Genetic Diversity, Population Structure and Parentage Analysis in Brazilian Grapevine Hybrids after Half a Century of Genetic Breeding

Geovani Luciano de Oliveira[a], Guilherme Francio Niederauer[a], Fernanda Ancelmo de Oliveira[a], Cinthia Souza Rodrigues[b], José Luiz Hernandes[b], Anete Pereira de Souza[a,c], Mara Fernandes Moura[b*]

[a] Molecular Biology and Genetic Engineering Center (CBMEG), State University of Campinas (UNICAMP), Av. Cândido Rondon, n° 400, Campinas, SP, Brazil

[b] Advanced Fruit Research Center, Agronomic Institute (IAC), Luiz Pereira dos Santos Avenue, n° 1.500, Jundiaí, SP, Brazil

[c] Department of Plant Biology, Biology Institute, State University of Campinas (UNICAMP), R. Monteiro Lobato, n° 255, Campinas, SP, Brazil

*****Corresponding author.

E-mail address: geoluciano92@gmail.com (G. L. Oliveira), gniederauer@protonmail.com (G. F. Niederauer), f.ancelmo.o@gmail.com (F. A. Oliveira), cinthia.rodrigues@iac.sp.gov.br (C. S. Rodrigues), jlhernandes@iac.sp.gov.br (J. L. Hernandes), anete@unicamp.br (A. P. Souza), mara.moura@sp.gov.br (M. F. Moura).

ABSTRACT

In the 1940s, the Agronomic Institute (IAC) started a grapevine breeding program to develop new cultivars adapted to the tropical and subtropical regions of Brazil. More than 2,000 crosses were carried out over 50 years, using 850 varieties as parents. However, among the thousands of hybrids developed by the program, only 130 are still maintained in the IAC grapevine germplasm. Little is known about their genetic makeup and usefulness for current breeding programs. In this study, we obtained genotypes of 130 Brazilian grape hybrids at 17 polymorphic microsatellite markers to evaluate the genetic diversity and population structure of the hybrids and verified their disclosed pedigrees. The results showed that the hybrid collection is highly diverse, with an expected heterozygosity (H_E) of 0.81 and an observed heterozygosity (Ho) of 0.79. A strong structure in three subgroups based mainly on the usage and combination of parental groups was revealed by STRUCTURE software and confirmed by discriminant analysis of principal components (DAPC). Through molecular profiling analysis, fifteen synonyms, one homonym and one duplicate were identified. Parentage analysis confirmed 22 full parentages, as well as 34 half-kinships. In addition, 14 pedigrees were invalidated, and eight mislabeling events were identified. No compatible parent was identified for 32.30% of the IAC hybrids, highlighting the severe genetic erosion that occurred in the IAC germplasm. The molecular characterization of the breeding hybrid bank collection contributes to our understanding the genetic basis of the varieties, guiding the efficient utilization of available genetic diversity. Together, our results could be applied to other breeding programs and assist in the selection of parents, management of the breeding collection, and conservation of grapevine genetic resources.

Keywords: SSR markers, genetic resources, plant breeding, pedigree analysis, *Vitis* spp., grape

1. Introduction

Brazil has diverse types of viticulture associated with different climatic conditions, soils and grapevine management strategies (Pereira et al., 2020). The great socioeconomic importance of viticulture in Brazil and the considerable environmental variation of production zones located in temperate, subtropical, and tropical regions, required the development of a genetic breeding program with the aim of developing varieties adapted to the various growing conditions throughout the nation (Ferri and Pommer, 1995).

The Agronomic Institute of Campinas (IAC) grape breeding program was initiated in 1943 to obtain varieties of wine grapes, table grapes, and rootstocks (Neto and Almeida, 1955). In the beginning, a collection of European and American cultivars and French hybrids of the Seibel, Seyve Villard, and Couderc series was established. This material was evaluated, and the varieties that exhibited the best characteristics in terms of production, vine vigor, taste qualities, and resistance to biotic and abiotic factors were selected to be used as parents in the first set of crosses (De Santos Neto, 1971). To expand the genetic base, fourteen wild *Vitis* species were used for their resistance to major pests and diseases, such as *Vitis rupestris*, *V. riparia*, *V. cinerea*, *V. caribaea*, *V. lincecumii*, and *V. labrusca*. Based on the results of the first crosses in the IAC breeding program, the hybrids with outstanding characteristics were used as parents (Pommer, 1993).

Over 50 years, since the beginning of the program, approximately 2,400 crosses have been performed using 850 different parental genotypes (Ferri and Pommer, 1995), leading to the release of varieties of wine grapes, table grapes, and rootstocks by the IAC. Among the thousands of hybrids developed by the program, only 130 are still maintained in the IAC grapevine germplasm; most of them were lost due to a variety factors, such as resource

limitations. The IAC hybrids are characterized by their complex pedigrees derived from crosses among three or more species, with a combination of alleles from different species of *Vitis*. Molecular characterization of these grapevine resources can help identify the genotypes that should be preserved and partially prevent or delay genetic erosion (Snoussi et al., 2004). Knowledge about the genetic diversity of germplasm resources is not only important for species protection but also necessary for the development and utilization of germplasm resources for crop improvement (Lassois et al., 2016).

The use of molecular markers has become an efficient method for genetic characterization and the determination of genetic relationships between germplasm accessions since the markers are not influenced by the environment and can be used in the early stages of plant development (Roychowdhury et al., 2014). Among the molecular markers identified in recent decades, microsatellites or simple sequence repeat (SSR) markers are highly polymorphic, abundant, reliably reproducible, relatively inexpensive to genotype, and transferable among several species of the genus *Vitis*, advantages that make them suitable and efficient for genetic analyses of grapevines (Cipriani et al., 2010; Lamboy, 1998; This et al., 2004). In addition, SSRs provide unique fingerprints for cultivar identification. They are inherited by Mendelian codominant segregation, confirming their suitability for genetic resource characterization, genome mapping, assisted selection, and parentage analysis (Karastan et al., 2018; Khadivi et al., 2019; Mihaljević et al., 2020; Saifert et al., 2018; Vezzulli et al., 2019).

Prior to the use of molecular markers, the putative parentage of new grape cultivars was recovered from breeders' notes, which could be incomplete or inaccurate (Raimondi et al., 2017). Several studies have used microsatellite markers to clarify the parentage relationships between grape cultivars, allowing for more accurate retrieval of breeding information by

confirming or invalidating declared pedigrees and identifying new genetic relationships (Aliquó et al., 2017; Migliaro et al., 2019; Mihaljević et al., 2020).

Little is known about the genetic makeup of IAC grape hybrids and their usefulness for current breeding programs. Success in grapevine breeding depends on the understanding and use of the available gene pool of varieties and breeding clones (De Oliveira et al., 2020). Currently, there is great interest in understanding the genetic basis of complex traits and in discovering new germplasm traits to leverage them for efficient tropical grapevine breeding. IAC grape hybrids are thought to have high genetic value as a source of diversity. This valuable genetic resource can play an important role in the development of new varieties with favorable traits, such as adaptability to climate change, disease resistance, or an original flavor.

The goal of this study was to investigate, at the molecular level, the grapevine hybrids developed over 50 years of breeding by the IAC Grapevine Breeding Program to assess their genetic diversity and population structure. Another aim was to clarify pedigree information to enable better categorization and understanding of the remaining interbreeds for use in future cross-breeding programs and the development of genetic conservation strategies.

2. Material and Methods

2.1. Plant material and DNA extraction

A total of 130 accessions of grapevine hybrids were analyzed in this study (Supplementary Table 1). The accessions were developed by the IAC Grapevine Breeding Program and belong to the Grapevine Germplasm Bank of the IAC located in Jundiaí, São Paulo (SP), Brazil. Each accession consisted of three clonally propagated plants, sustained in an

espalier system and pruned in August every year, leaving one or two buds per branch. For sampling, young leaves of a single plant were collected from each accession.

Total genomic DNA was extracted from the young leaves homogenized in a TissueLyser (Qiagen, Valencia, CA, USA) following the cetyltrimethylammonium bromide (CTAB) method previously described by Doyle (1991). The DNA concentration was quantified by using a NanoDrop 8000 (Thermo Scientific), and the quality was checked using 1% agarose gel electrophoresis.

2.2. Microsatellite analysis

A set of 17 microsatellites was selected to genotype the IAC hybrids in the study, including the international set of seven SSR loci recommended by the International Organization of Vine and Wine (OIV) for universal grapevine identification (OIV, 2001): VVS2 (Thomas and Scott, 1993), VVMD5, VVMD7 (Bowers et al., 1996), VVMD25, VVMD27 (Bowers et al., 1999), VrZAG62, and VrZAG79 (Sefc et al., 1999). Ten additional markers previously developed to assess grapevine diversity were also included: VVIn74, VVIr09, VVIp25b, VVIn56, VVIn52, VVIq57, VVIp31, VVIp77, VVIv36, and VVIr21 (Merdinoglu et al., 2005). Additional information about the loci is available in Supplementary Table 3.

PCR was performed using forward primers labeled with fluorescent dyes (6-FAM, PET, VIC, or NED), and amplification was conducted as described by De Oliveira et al. (2020). Capillary electrophoresis was conducted in an ABI 3500 (Applied Biosystems, Foster City, CA, USA). Allele calling was performed with Geneious software v. 8.1.9 (Kearse et al., 2012) using the internal GeneScan-600 (LIZ) Size Standard Kit (Applied Biosystems, Foster City, CA, USA).

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137 *2.3. Genetic diversity and population structure analysis*

138 Descriptive statistics based on the genotyping data were generated using GenAlEx v. 6.5
139 (Peakall and Smouse, 2012) to indicate the number of alleles per locus (Na), effective number of
140 alleles (Ne), observed heterozygosity (HO), expected heterozygosity (HE), and fixation index
141 (F). The null allele frequency (r) and the polymorphism information content (PIC) were
142 estimated using CERVUS 3.0.7 (Kalinowski et al., 2007). Discriminating power (Dj) values
143 were estimated to compare the efficiencies of microsatellite markers in varietal identification and
144 differentiation (Tessier et al., 1999).

145 A model-based Bayesian analysis implemented in the software package STRUCTURE v.
146 2.3.4 (Pritchard et al., 2000) was used to determine the approximate number of genetic clusters
147 (K) within the full dataset and to assign individuals to the most appropriate cluster. All
148 simulations were performed using an admixture model, with 100,000 replicates for burn-in and
149 1,000,000 replicates for Markov chain Monte Carlo (MCMC) processes in ten independent runs.
150 The number of clusters (K) tested ranged from 1 to 10.

151 A DAPC analysis as implemented in the R package adegenet was also performed, using a
152 nonparametric approach, free from Hardy–Weinberg constraints (Jombart et al., 2010). The
153 *find.clusters* function was used to detect the number of clusters in the germplasm, running
154 successive K-means clustering with increasing numbers of clusters (K). We used 20 as the
155 maximum number of clusters. The optimal number of clusters was estimated using the Bayesian
156 information criterion (BIC). The DAPC results were presented as multidimensional scaling plots.

157

158 *2.4. Parentage and identity analysis*

A search for compatible trios (parents and offspring) and duos (parent-offspring) combinations among the SSR profiles was carried out using a likelihood-based method implemented in CERVUS v.3.0.7 software (Kalinowski et al., 2007). The analysis was performed with molecular data from the IAC grapevine genetic database, including 280 additional genotypes (De Oliveira et al., 2020). Most of these accessions were European and American cultivars that were used as parents over the years by the IAC Grapevine Breeding Program.

The likelihood of each detected trio and duo was determined based on the natural logarithm of the overall likelihood ratio (LOD) score. The CERVUS program calculates allelic frequencies using a simulation approach and determines the confidence in parentage assignments by calculating critical values of the LOD score. One hundred thousand offspring were simulated with a proportion of 0.01 sampled parents, including the possibility of self-fertilization and the existence of relatives among potential parents. The maximum number of mismatching loci for trios and duos was set to 1, and the parentage relationship was considered significant when the trio or pair confidence was represented by a probability greater than 95%. Last, the results of the analysis were compared with the IAC historical records to verify declared parents.

To identify possible synonyms (different names for the same genotype), homonyms (common name for different genotypes) and duplicates, an individual identity analysis was also carried out using the CERVUS software. The minimum number of matching loci was set to 10, and 1 fuzzy match was allowed.

3. RESULTS

3.1. Genetic diversity

One hundred thirty IAC grape hybrids were analyzed at 17 SSR loci, and a total of 202 alleles were detected (Table 1). The number of alleles per SSR locus (Na) ranged from 4 (VVIq57) to 15 (VVMD25), with an average of 11.88. The number of effective alleles per locus (Ne) varied from 2.12 (VVIq57) to 9.93 (VVIp31), with a mean value of 6.11.

Locus	**Na**	**Ne**	**H_O**	**H_E**	**PIC**	**D*j***	***r***
VVIn74	12	5.72	0.76	0.83	0.80	0.83	0.04
VVIr09	13	5.30	0.81	0.81	0.79	0.82	-0.01
VVIp25b	11	3.29	0.57	0.70	0.65	0.70	0.10
VVIn56	6	2.57	0.62	0.61	0.54	0.62	-0.01
VVIn52	12	7.89	0.69	0.87	0.86	0.88	0.12
VViq57	4	2.12	0.57	0.53	0.47	0.53	-0.04
VVip31	14	9.93	0.88	0.90	0.89	0.91	0.01
VVip77	14	7.87	0.73	0.87	0.86	0.88	0.09
VVIv36	10	4.50	0.79	0.78	0.75	0.78	-0.01
VVIr21	14	5.49	0.81	0.82	0.80	0.82	0.00
VVS2	14	7.08	0.85	0.86	0.84	0.87	0.01
VVMD5	12	7.80	0.85	0.87	0.86	0.88	0.01
VVMD7	14	9.22	0.92	0.89	0.88	0.90	-0.02
VVMD25	15	5.03	0.85	0.80	0.78	0.81	-0.04
VVMD27	13	6.44	0.85	0.84	0.83	0.85	-0.01
VrZAG62	12	7.11	0.93	0.86	0.85	0.87	-0.04
VrZAG79	12	6.54	0.88	0.85	0.83	0.86	-0.02
Total	202	103.90					
Mean	11.88	6.11	0.79	0.81	0.78	0.81	
SE[a]	0.71	0.53	0.03	0.02	0.03	0.02	

Table 1. Genetic parameters of the 17 microsatellite loci obtained from 130 grapevine accessions. Na, number of alleles; Ne, number of effective alleles; H_O, observed heterozygosity;

189 H_E, expected heterozygosity; PIC, polymorphic information content; D*j*, discrimination power; *r*,
190 estimated frequency of null alleles.
191 [a]Standard error of mean values.

192 The mean observed heterozygosity (H_O) and mean expected heterozygosity (H_E) were
193 very similar (0.79 and 0.81, respectively). A significantly high (>0.20) probability of null alleles
194 (r) was not detected in any of the analyzed loci. The PIC had a mean value of 0.78, and the
195 discrimination power (Dj) was greater than 0.80 for 13 of the 17 loci, with a mean value of 0.81.
196 When the PIC and D*j* of each locus were analyzed together, 11 loci exhibited high values for
197 both indexes (>0.80).

198 Of the 202 SSR alleles found, 49% displayed a frequency greater than 5% and were
199 classified as common alleles, 37.6% had frequencies between 1% and 5% and were considered
200 less-common alleles, and 13.4% had a frequency less than 1% and were rare alleles, suggesting
201 that this collection, despite originating from the same breeding program, includes great
202 biodiversity.
203
204 *3.2. Population structure analysis*

205 The STRUCTURE analysis indicated relatedness among the 130 accessions, with the
206 highest ΔK value for K = 3, suggesting that three genetic clusters were sufficient to interpret our
207 data (Supplementary Fig. 1). Based on a membership probability threshold of 0.70, 28 hybrids
208 were assigned to cluster SV, 45 hybrids were assigned to cluster MG, and 44 hybrids were
209 assigned to cluster TV. Thirteen hybrids were not assigned to defined clusters and were assigned
210 to the admixed group (Fig. 1).

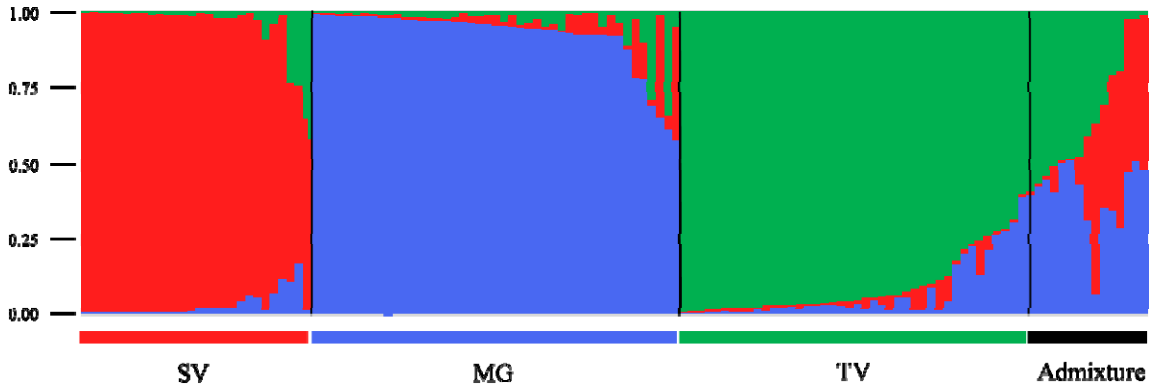

Fig. 1. Bar graph of the estimated membership coefficient (q) for each of the 130 individuals. Each genotype is represented by a single vertical line, which is partitioned into colored segments in proportion to the estimated membership in each cluster. Cluster SV: genetic predominance of wine hybrids obtained by crossing Seibel hybrids with wine grape cultivars of *V. vinifera*; cluster MG: genetic predominance of table hybrids obtained by crossing fine table muscat grapes with other varieties (mainly interspecific hybrids); cluster TV: genetic predominance of hybrids originated from crosses that used wild *Vitis* (tropical vines) as one of the base parents; Admixture: interspecific hybrids with a membership of $q < 0.70$.

The clustering level (K = 3) was mainly based on the use and combination of parental gene pools. Cluster SV was formed primarily by wine hybrids originating from crosses between Seibel hybrids and wine grape cultivars of *V. vinifera*. Cluster MG was composed of table hybrids originating from crosses with Muscat grapes. In the TV cluster, there was no clear discrimination based on human use, and hybrids for wine, table and rootstock were found in this group. However, all these hybrids were developed from crosses with tropical vines (wild *Vitis*). The hybrids in the admixed group exhibited a more complex origin, and some of them had associations with the three clusters simultaneously.

Additionally, DAPC was performed with no prior information about the groupings of the evaluated accessions. Inspection of the BIC values revealed that the division of the accessions into three clusters was the most likely scheme to explain the variance in this set of genotypes (Supplementary Fig. 2). In the preliminary step of data transformation, the maintenance of 90 principal components (PCs) allowed DAPC to explain 99% of the total genetic variation. The DAPC scatter plot, based on the first and second discriminant functions, showed the distribution of the three groups (Fig. 2) with great genetic differentiation between them and low variance within the groups.

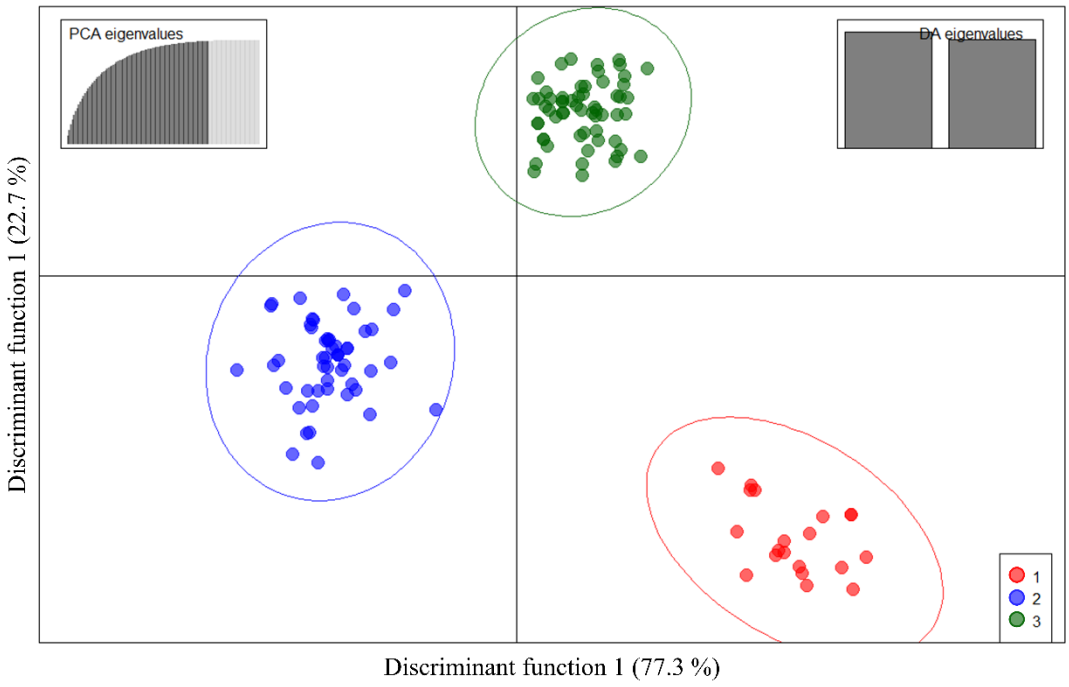

Fig. 2. DAPC scatterplots based on the K-means algorithm used to identify the proper number of clusters. Dots represent individuals, and the clusters are presented in different colors. The individuals were allocated into three clusters: 1 (red), wine hybrids obtained by crossing Seibel hybrids with *V. vinifera* wine cultivars; 2 (blue), table hybrids obtained through crosses with fine muscat grapes; 3 (green), hybrids obtained through crosses with wild *Vitis*.

The allocation of individuals into clusters according to the DAPC showed several similarities to that resulting from STRUCTURE, and both analyses showed the same pattern of clustering. Essentially, Clusters 1 (red), 2 (blue), and 3 (green) of the DAPC reflected the subgroups SV, MG, and TV detected by STRUCTURE, respectively. By the DAPC grouping, 20 hybrids were allocated to Cluster 1, 53 to Cluster 2, and 57 to Cluster 3. Despite the similarities between the analyses, the DAPC analysis proved to be more discriminating. Individuals in the STRUCTURE admixed group were distributed among the DAPC clusters, and no cases of overlap between clusters were observed, indicating a more delineated genetic structure.

3.2.1 Parentage analysis

To improve the search for possible first-order kinship relationships, the genetic profiles of 280 accessions stored in the IAC grapevine genetic database were added to parentage analyses, completing a total of approximately 410 genotypes.

The critical LOD values determined by simulation for strict confidence (95%) of parentage were 20.00 and 6.16 for trios (offspring and two inferred parents) and duos (parent-offspring), respectively. Offspring resulting from self-pollination were not detected. A total of 31 compatible trios were identified with a high confidence level using a maximum of one mismatched locus as the threshold, with LOD values ranging from 23.15 to 46.49 (Table 2). The complete pedigrees of 22 IAC hybrids reported in the IAC records were validated, while for four trios identified, one parent was validated and the other was not (Supplementary Table 1). For seven IAC hybrids, both declared parents were invalidated, and for two of them, no other possible parent (consistent with the offspring's SSR profiles) was found in the IAC grapevine genetic database.

Offspring ID Number	Offspring Name	First candidate parent	Second candidate parent	Trio loci compared	Trio loci mismatch	Trio LOD score*
1	IAC 16-02	Ravat 34	Muscat Hamburg	16	0	25.35
2	IAC 21-14 (Madalena)	Ravat 34	Moscato Giallo	15	1	23.15
3	IAC 23-08	Ravat 34	Muscat Hamburg	16	1	27.88
5	IAC 74-01 (Iara)	Seibel 10096	Syrah	15	0	26.88
6	IAC 74-02	Seibel 10096	Syrah	17	0	27.08
8	IAC 116-31 (Rainha)	Seibel 7053	Pinot Noir	17	0	37.46
9	IAC 137-04	Ravat 34	Semillon	17	0	33.95
13	IAC 192-54	Seibel 8712	Muscat Hamburg	17	0	32.95
21	IAC 313 (Tropical)	Golia	*Vitis cinerea*	16	1	46.49
24	IAC 339-03	Muscat Hamburg	*Vitis cinerea*	16	0	38.79
25	IAC 341-02	Moscatel Rosado	*Vitis cinerea*	17	0	42.93
29	IAC 388 (Santa Tereza)	Italia	IAC 82-01	17	0	30.86
33	IAC 405-06	Moscatel Rosado	*Vitis cinerea*	17	0	43.79
37	IAC 460-01	Highland	Sultanina	14	0	27.55
40	IAC 496-15	Seibel 7053	Gewurztraminer	16	0	34.51
80	IAC 871-05 (Geni)	IAC 501-06 (Soraya)	IAC 544-14	17	0	38.41
81	IAC 871-13 (A Dona)	IAC 501-06 (Soraya)	IAC 544-14	17	0	38.41
82	IAC 871-18	IAC 501-06 (Soraya)	IAC 544-14	16	0	39.75
82	IAC 871-41 (Patricia)	IAC 501-06 (Soraya)	IAC 544-14	17	0	36.09
99	IAC 966-01	Seibel 7053	Pinot Noir	17	1	31.37
114	IAC 1742	Muscat Hamburg	Niagara Branca	16	1	26.45
119	IAC Juliana	IAC 21-14 (Madalena)	Italia	17	0	30.26

121	SR 496-09	Seibel 7053	Gewurztraminer	16	0	36.50
122	SR 496-15 (Dr. Júlio)	Seibel 7053	Gewurztraminer	12	0	25.42
123	SR 496-25	Seibel 7053	Gewurztraminer	17	1	31.94
124	SR 5010-08	Seibel 7053	Seibel 10096	16	1	27.24
126	SR 501-17 (IAC Ribas)	Seibel 7053	Syrah	17	0	41.10
127	SR 5012-34 (Dona Emília)	Seibel 7053	Cabernet Sauvignon	16	1	26.68
128	SR 501-33	Seibel 7053	Syrah	17	1	33.77
129	SR 507-38	Seibel 7053	Semillon	16	1	25.11
130	SR 507-08	Seibel 7053	Cabernet Sauvignon	17	1	29.89

Table 2. Putative full parentages of 31 IAC grapevine hybrids inferred based on the maximum likelihood approach.

* A maximum of only one locus mismatch was allowed, and the parentage relationship was considered significant when the trio confidence probability was greater than 95% (LOD ≥ 20).

A total of 39 compatible duos were also identified, and all of them were recognized as cases of putative direct (first-degree) relationships (Table 3). The partial pedigrees of 34 IAC hybrids reported in the IAC records were validated, while for eight IAC hybrids, the identified parent did not correspond to any of the declared parents. Moreover, no reliable trios or duos within the IAC grapevine genetic database were found for the other 42 genotypes.

Offspring ID Number	**Offspring Name**	**Candidate parent**	**Pair loci compared**	**Pair loci mismatch**	**Pair LOD score***
10	IAC 138-22 (Máximo)	Ravat 34	15	1	8.26
11	IAC 141-51	Sémillon	17	0	14.05
12	IAC 158-12	Muscat Hamburg	12	0	8.77
14	IAC 202-28	Muscat Hamburg	16	0	10.96
15	IAC 202-43	Muscat Hamburg	17	0	11.26
20	IAC 274-21	Ravat 34	15	1	7.36
23	IAC 338-04	*Vitis cinerea*	9	0	13.01
30	IAC 393-04	Muscat Hamburg	15	0	11.03
31	IAC 393-05	Muscat Hamburg	17	1	11.71
32	IAC 403-01	Sultanina	15	0	17.82
34	IAC 408-01	*Vitis cinerea*	14	0	21.93
36	IAC 457-11 (Iracema)	Sultanina	17	0	13.05
39	IAC 486-03	Italia	16	0	12.51
52	IAC 574-01	IAC 74-1 (Iara)	14	0	9.72
53	IAC 583-03	Ruby Cabernet	12	0	11.03
54	IAC 584-53	Sauvignon Gris	16	0	16.81
55	IAC 589-02	Sémillon	17	0	11.70
56	IAC 592-01	Ruby Cabernet	13	0	12.16
61	IAC 720-01	Carignane	13	1	8.51
63	IAC 733-39	IAC 544-14	12	0	11.98
69	IAC 768-02	IAC 457-11 (Iracema)	17	1	6.18
70	IAC 772-41	IAC 514-6 (Maria)	14	0	11.51
72	IAC 778-04	IAC 544-14	16	0	11.23
73	IAC 804-13	IAC 583-03	15	1	8.33

75	IAC 822-21	IAC 405-06	17	0	16.25
76	IAC 842-04 (Eugenio)	IAC 501-06 (Soraya)	12	0	9.98
77	IAC 860-05	IAC 514-6 (Maria)	11	0	8.06
86	IAC 901-01	IAC 457-11(Iracema)	16	1	6.21
88	IAC 903-47	IAC 457-11(Iracema)	17	1	7.90
89	IAC 904-11	IAC 514-6 (Maria)	12	0	9.67
90	IAC 904-30	IAC 544-14	16	0	15.22
92	IAC 904-47	IAC 514-6 (Maria)	16	0	11.61
95	IAC 915-02	IAC 457-11 (Iracema)	17	1	6.42
97	IAC 960-11	IAC 138-22 (Máximo)	16	1	9.39
101	IAC 1025-17	IAC 904-30	15	0	15.55
102	IAC 1117-06	IAC 768-02	15	1	6.48
108	IAC 1410-08 (Ezequiel)	IAC 501-06 (Soraya)	17	0	18.20
113	IAC 1726-03 (Roberta)	IAC 871-18	16	0	18.48
120	Jd 930 (Moscatel de Jundiai)	Seyve Villard 5276	16	1	11.19

Table 3. Possible direct (first-degree) relationships of 38 IAC grapevine hybrids based on the maximum likelihood approach.

* A maximum of only one locus mismatch was allowed, and the parentage relationship was considered significant when the pair confidence probability was greater than 95% (LOD ≥ 6.16).

The two most common varieties that emerged as a parent in 17 proposed trios and five duos were ‘Seibel 7053’ (syn. Chancellor) and ‘Muscat Hamburg’. The next most recurrent parent in seven crosses (four trios and three duos) was the hybrid IAC 544-14, which had an unverifiable pedigree, as its declared parents were IAC hybrids that are now extinct.

3.2.2 Genotype identity

Among the 130 IAC hybrids analyzed, 15 synonyms were identified, with hybrids having the same molecular profile but identified with different names (Supplementary Table 4). In addition, one case of duplication and one case of homonymy were detected. The two hybrids

287 labeled IAC 746-03 showed the same molecular profile, while the molecular profiles of the two
288 hybrids labeled IAC 514-6 were different.

289 Through pedigree validation, eight synonyms were identified as possible mislabeling, and
290 their correct identification is proposed in Supplementary Table 4. It is possible that the other
291 seven synonyms identified in this study were also due to mislabeling; however, it was not
292 possible to propose a correct identification in these cases either because no parent was identified
293 in the parentage analysis or because both hybrids of the synonym group had the same pedigree.

294

295 **4. DISCUSSION**

296

297 *4.1. Genetic diversity*

298 The results of this study revealed high levels of heterozygosity among the evaluated
299 genotypes, with a high percentage of less-common and rare alleles (51%). Since heterozygosity
300 is an indicator of genetic variability in a population and is related to the polymorphic nature of
301 each locus, these results highlight the potential of this genetic material as a source of genetic
302 diversity. The wide abundance of parents used in the crosses and the different purposes of the
303 breeding program were probably the factors responsible for the high genetic diversity we
304 observed. Among the 850 genotypes used as parents by the IAC breeding program, there were
305 *Vitis vinifera* cultivars from different countries, wild species and intra- and interspecific hybrids.
306 Approximately 2,400 combinations were performed using these parents to obtain wine, table and
307 rootstock grape varieties adapted to conditions in Brazil (Ferri and Pommer, 1995).

308 We detected an H_E of 0.81 across the entire hybrid set in the 17 evaluated loci (Table 1).
309 This result is similar to those found in other grapevine collections characterized by an abundance

of interspecific hybrids (Migliaro et al., 2019; Schuck et al., 2009) but greater than that of collections composed only of *V. vinifera* accessions (Boz et al., 2011; De Lorenzis et al., 2014). Laucou et al. (2011) and Emanuelli et al. (2013) showed that the genetic diversity found in non-*vinifera* varieties was higher than that in the *V. vinifera* sector, indicating that taxonomically broader genotypes contribute to an increase in genetic diversity, as expected by the heterogeneity of IAC hybrids, since most have wild *Vitis* in their genealogy.

The high number of alleles obtained by the 17 SSR primer set positively impacted the PIC and discrimination power (D*j*). No locus was identified with a high frequency of null alleles (> 0.20). According to the classification of Botstein et al. (1980), all the loci in the study can be considered highly informative (PIC > 0.50), except for VVIq57 (0.47). This locus also presented the lowest Dj value, certainly due to its reduced number of alleles (4), which limits the power to distinguish genotypes. All 16 other SSR loci analyzed proved to be adequate for grape cultivar discrimination and it can be considered an efficient set for genetic diversity studies.

4.2. Cluster analysis and genetic structure

The genetic structure was impacted by the different objectives and strategies adopted by the IAC breeding program, such as the development of grape varieties for wine, table and rootstock adapted to the climatic conditions in Brazil through crosses between *V. viniferas* cultivars, complex hybrids and wild *Vitis* species known as tropical vines. Population structure analysis using STRUCTURE software revealed the presence of three primary clusters in our set of hybrids (Fig. 1), two of which were strongly based on human usage and the other had no clear distinction regarding use but had a strong influence of tropical vines.

Most of the hybrids developed for use as wine grapes were concentrated in the SV cluster. Based on the analysis of the genealogy of the hybrids of this cluster, there was a clear direction in the use of Seibel series hybrids crossed with wine grape cultivars of *V. vinifera*. Seibel series hybrids were widely used in the state of São Paulo from the 1930s through the 1950s, and they exhibited good productivity, good affinity with the rootstocks used in the region, and satisfactory resistance to the main pests and diseases. However, they had some problems regarding the quality of the wine produced (Ribas, 1967). On the other hand, the *V. vinifera* cultivars known for producing high-quality wines had low adaptation to the climatic conditions in Brazil. The SV cluster reflects one of the strategies used in the breeding program to develop cultivars capable of producing high-quality wines in the tropical and subtropical conditions in Brazil. Basically, all hybrids in this cluster were obtained from crosses of the Seibel series with *V. vinifera* cultivars, except SR 5010-08 and SR 5010-21, which were obtained by crossing two Seibel hybrids.

The MG cluster was formed by table grape hybrids with a predominance of genealogies based on crosses with Muscat grapes. In the 1950s, there was a high market demand for muscat-flavored table grapes in Brazil, for which high prices were paid. Most of the Muscat grapes used in the country had adaptability problems, such as cluster rot, berry splitting, and susceptibility to fungal diseases, mainly downy mildew and powdery mildew. Given this scenario, one of the focuses of the breeding program was to obtain new varieties resistant to the main fungal diseases, having satisfactory development in the conditions in Brazil, with fruits of high palatability, high sugar content, low acidity, and muscatel flavor (Neto and Almeida, 1955). Most of the hybrids in the MG cluster were the result of this approach, arising mainly from crosses with ‘Moscatel Branco’ (Moscato Giallo), ‘Moscatel Rosado’, ‘Muscat Hamburg’, and ‘Italia’. The ‘Italia’

355 cultivar, an offspring of the 'Muscat Hamburg', was widely used as a parent in the IAC breeding
356 program, probably because it is one of the most cultivated table grapes in São Paulo due to its
357 characteristics traditionally appreciated by consumers and farmers (Ferri and Pommer, 1995).

358 Unlike previous clusters, there was no clear discrimination based on usage in the TV
359 cluster, and hybrids for wine, table, and rootstock were found in this group. However, all hybrids
360 have the presence of wild *Vitis* in their genealogy in common. The use of tropical vines was
361 intense in the IAC breeding program to promote climate adaptability and disease resistance.
362 These vines have small-sized fruits with a low percentage of pulp and their chemical
363 composition is without a satisfactory balance, not meeting the requirements for table or wine.
364 However, their characteristics related to vigor, resistance, productivity, and adaptation to regions
365 with high humidity and temperature during the summer led these species have an important role
366 in the search for the expansion of the genetic base of the new Brazilian varieties (Ferri and
367 Pommer, 1995).

368 A small number of the hybrids remained admixed, with evidence of a greater genetic
369 complexity of these genotypes. The intra- and interspecific crossings carried out during breeding
370 cycles in search of novelties and hybrid vigor promoted the miscegenation of grapevine cultivars,
371 resulting in hybrids with a heterogeneous genetic composition (De Oliveira et al., 2020). The
372 admixed group hybrids certainly carry alleles from different gene pools; they occupy an
373 intermediate position and belonging simultaneously to more than one cluster. The hybrids IAC
374 339-03, IAC 393-04, and IAC 192-54 are examples. IAC 339-03 and IAC 393-04 were the result
375 of crosses between the cultivar 'Muscat Hamburg' with the tropical vines *V. smalliana* and *V.*
376 *shuttleworthii* x *V. rufotomentosa,* respectively. The mixture of gene pools was detected by
377 STRUCTURE, which assigned a membership probability threshold of approximately 0.5 to the

MG and TV clusters, representing the genetic clusters of the two parental cultivars (Muscat grapes and tropical vines). A similar situation was observed for the hybrid IAC 192-54 developed from the cross between 'Muscat Hamburg' and 'Seibel 8712', assigned to the MG and SV clusters, respectively. This hybrid also presented an intermediate membership of 0.5 to the two groups. The other hybrids from the Admixture group exhibited a similar or even more complex origin than these examples, and some of them had associations with the three clusters simultaneously.

The clustering performed by DAPC resulted in the same clustering pattern found by STRUCTURE but with a greater distinction between genotypes. Since the DAPC minimizes within-group genetic variance and maximizes between-group genetic variance, individuals in the STRUCTURE admixed group were distributed among the DAPC clusters. The genotypes in this study are the result of human manipulation of cultivars (displacements, breeding, clonal propagation); therefore, deviations from Hardy-Weinberg equilibrium (HWE) are expected. This feature can lead to greater accuracy in the DAPC results since this method does not assume the absence of linkage disequilibrium or specific models of molecular evolution to identify genetic clusters (Jombart et al., 2010). Cluster 1 formed by DAPC exhibited a more precise separation of the hybrids; only wine hybrids remained in this cluster, and the few table hybrids present in the STRUCTURE SV cluster were assigned to Cluster 2 in DAPC analysis, where the hybrids of this class were concentrated.

Knowledge about the genetic structure of IAC hybrids will certainly help to minimize the use of closely related genotypes as parents in breeding programs, avoiding the risk of inbreeding depression and the reduction of genetic variation. Information regarding genetic diversity,

400 population structure, and molecular markers may facilitate the selection of desirable traits in
401 grapes and is important for ensuring the conservation of genetic resources.
402
403 *4.3. Parentage analysis and its use in genotype identity*
404 Among the IAC hybrids analyzed in this study, only 30 (23.07%) were actually released
405 as varieties. The others remained exclusively in the IAC grapevine germplasm without any
406 published genealogy information. In this study, we made available the genealogy information of
407 all 130 hybrids (Supplementary Table 1) recovered through research carried out in the breeder's
408 notes and institution's internal records. We also performed the parentage analysis of these
409 hybrids with molecular data for the first time and used the results to validate the parentage
410 declared in the historical records.
411 Nine trios and eight duos had their declared pedigrees invalided by parentage analysis. In
412 all IAC hybrids examined, 'Seibel 11342' was invalidated as a parent, with 'Ravat 34' being the
413 true parent. The correct identification of this cultivar in the IAC germplasm was suggested
414 previously in a recent study (De Oliveira et al., 2020), and was confirmed in this study as a
415 mislabeling that likely occurred beginning with the first crosses in 1944, indicating that Seibel
416 11342 was not introduced in the IAC grapevine breeding program. The use of 'Ravat 34' instead
417 of 'Seibel 11342' in a substantial number of crosses increased the inaccuracy of the breeder's
418 data, since important hybrids such as IAC 21-14 Madalena and IAC 138-22 Máximo were
419 released with incorrect genealogy information and were later used as parents in new crosses.
420 Some hybrids with invalidated pedigrees were identified as synonyms by identity
421 analysis (Supplementary Table 4). Since most of the IAC hybrids were never released and were
422 kept exclusively in the IAC germplasm, the synonyms found were probably "internal

synonyms", originating from cases of misnaming that occurred over the years. Misidentification in breeding programs is common, especially for ancient clonal species such as *Vitis* spp., and it can occur during material propagation, during the planting and duplicating of collections, or even during seedling selection (Raimondi et al., 2017). Through pedigree validation, we proposed the correct identification of eight synonyms. In these cases, the synonym presented the same genetic profile as a hybrid with a validated pedigree. For the other seven synonyms, correct identification was more complex, since some had extinct parents and others had the same parents. Further ampelographic and passport data are necessary for these synonyms to check for true synonym status (not yet known), to identify possible somatic mutations not detected with a small number of SSR markers (Cipriani et al., 2010; Liang et al., 2015) and to discard false synonymy resulting from grafting errors or erroneous former morphological identification (De Andrés et al., 2007; Lassois et al., 2016).

In addition to these synonyms, one case of homonymy was also found. The hybrids IAC 514-6 (ID: 46) and IAC 514-6 (Maria) (ID: 47) shared the same name but not the same genetic profile. In the literature, this variety has been described as a seedless white table grape (Pommer, 1993; Pommer et al., 1995), and according to IAC phenotyping data (unpublished), only the IAC 514-6 (Maria) (ID: 47) genotype matches these descriptions. Both genotypes corresponded to white table grapes, but only IAC 514-6 (Maria) (ID: 47) was a seedless grape, and the other presented well-developed seeds. This evidence points to the hybrid IAC 514-6 (Maria) (ID: 47) as the correct variety. IAC 514-6 (ID: 46) was another genotype that could not be identified, likely another result of mislabeling.

In this study, no compatible parent was identified for 42 IAC hybrids (32.30%) within the IAC grapevine genetic database, and for another 39 (30%), only one compatible parent was

446 detected. The low number of reconstructed trios (both parents and offspring) points to the severe
447 genetic erosion of the IAC germplasm since the late 1980s. Most hybrids with unverifiable
448 parents were the result of crosses between genotypes developed by the IAC breeding program
449 that became extinct. At the beginning of the breeding program, a large volume of crosses was
450 carried out, and numerous hybrids were obtained. Many of these hybrids were not released as
451 cultivars but played an important role as intermediaries in the use of wild species, often being
452 used as parents (Ferri and Pommer, 1995). The importance and justification for the preservation
453 of this large volume of local genotypes was overlooked, since most of them were not
454 economically interesting at the time; the lack of financial support resulted in the loss of a large
455 part of the IAC genetic resources.

456 The proposed parentages were not confirmed for IAC 282 or IAC 1319, nor were other
457 possible genitors found in the IAC grapevine genetic database. This might be due to the mistaken
458 identity of both parents or, more likely, to a mistake in seedling labeling, or even in material
459 propagation from mother plants during field collection establishment or duplication.

460 Several grape cultivars have previously been reported to have an important role in the
461 establishment of local genetic networks, such as ‘Muscat Hamburg’, ‘Seibel 7053’, ‘Italia’, and
462 ‘Niagara Rosada’ in southeastern Brazil (Ferri and Pommer, 1995; Neto and Almeida, 1955;
463 Ribas, 1967). Data analysis showed the significant contribution of ‘Seibel 7053’ and ‘Muscat
464 Hamburg’ to the generation of IAC grapevine diversity; they were involved as progenitors in 12
465 and 10 identified pedigrees, respectively.

466 The species *Vitis cinerea* was validated as a parent in six pedigrees (Tables 2 and 3). This
467 species was introduced in the breeding program, along with other tropical vines, to introduce
468 characteristics of disease resistance and adaptability to tropical climates (Neto and Almeida,

1955). The genetic profiles of the other tropical vines used as parents by the breeding program, such as *Vitis gigas, V. shuttleworthii* x *V. rufotomentosa*, and *Vitis caribaea*, were not found in the IAC grapevine genetic database. These species were probably lost along with many other IAC hybrids due to severe genetic erosion that occurred in the germplasm.

Declared pedigrees are not necessarily a reliable tool, either because they are often too generic (such as *V. shuttleworthii* x *V. rufotomentosa*) or the declared parents do not match the true parents due to mislabeling issues (Migliaro et al., 2019). Therefore, genetic data analysis is essential to verify the consistency of declared parents, and it can help correct mislabeling and ensure true variety identification (Raimondi et al., 2017). Microsatellite markers are among the most commonly used molecular markers for genetic analysis in grapevines, since the alleles are inherited via Mendelian codominant segregation, confirming their suitability for investigating hereditability and cultivar parentage (Aliquó et al., 2017; De Lorenzis et al., 2014; Mihaljević et al., 2020; Sefc et al., 2009). In this study, the 17 SSRs used were valuable for drawing robust conclusions regarding first-degree relationships, supporting or questioning known information, suggesting new possible parentage, and identifying probable cases of misidentification.

5. CONCLUSIONS

Despite the serious genetic erosion that occurred in the IAC grapevine germplasm, this study revealed that there is still a high level of genetic diversity present in the set of conserved hybrids developed by the breeding program. However, this loss of genetic resources made it impossible to fully validate the pedigrees of most individuals, since many IAC hybrids used as key parents were no longer present in the collection.

The combination of the results obtained by the parentage and identity analyses allowed us to identify cases of genotype mislabeling, information that is extremely useful for curating the collection. Additional phenotypic and passport data checking is necessary to address pending identification questions. The overall diversity structure was shown to be rather strong and coincided with the usage of the varieties and the strategies adopted by the breeding program based on combinations of parental groups.

Many of the hybrids in this study were not properly recognized as cultivars and can be considered a source of genetic diversity with the potential for utilization; they could be used to obtain new varieties that may exhibit crucial features for developing sustainable viticulture in tropical and subtropical areas. All these data point to the importance and justification of preserving these genotypes in germplasm repositories.

CRediT authorship contribution statement

Geovani Luciano de Oliveira: Conceptualization, Data curation, Methodology, Formal analysis, Visualization, Investigation, Writing - original draft; Writing - review & editing. **Guilherme Francio Niederauer:** Methodology, Writing - original draft. **Fernanda Ancelmo de Oliveira:** Conceptualization, Visualization, Writing - review & editing. **Cinthia Souza Rodrigues:** Writing - review & editing, Investigation. **José Luiz Hernandes:** Methodology, Writing - review & editing**. Anete Pereira de Souza:** Funding acquisition, Project administration, Resources, Supervision. **Mara Fernandes Moura:** Conceptualization, Visualization, Writing - review & editing, Funding acquisition, Project administration, Resources, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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