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Populations of *Saccharomyces cerevisiae* in Vineyards: Biodiversity and Persistence Associated with Terroir

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Abstract: The origin terroir provides distinctive characteristics for wines, in relation to soil, climate, oenological practices, etc. Hence, the characterization of each wine region by multiple aspects would allow differentiation of its wines. Several approaches at different scales have studied terroir microbiological fingerprints: from global microbiome analysis up to intraspecific *Saccharomyces* biodiversity. Mature grapes are the primary source of yeasts, and *S. cerevisiae* is a key wine fermentative species. Malbec is the emblematic Argentinean variety and is mainly cultivated in the "Zona Alta del Rio Mendoza" (ZARM). In this work, the diversity of *S. cerevisiae* grape populations was studied at three vintages in two Malbec vineyards of the ZARM, to evaluate their annual diversity and behavior in different vintages. Rarefaction of classical ecological indices was applied for a statistically adequate biodiversity analysis. A total of 654 *S. cerevisiae* isolates were differentiated by Interdelta-PCR. Each yeast grape population showed a unique composition of *S. cerevisiae* strains; however, a narrow genetic relationship was found in each vineyard. A slight increase in the initial diversity and a stabilization in the diversity of *S. cerevisiae* populations were confirmed. These results add to the discussion about the contribution of yeasts to the terroir microbiological concept, and its limitations and stability over the time.

Keywords: *Saccharomyces cerevisiae*; Malbec grapes; biodiversity; rarefaction; terroir

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1. Introduction

The term terroir refers to an area that develops the collective knowledge of the interactions between biotic and abiotic environmental factors and applied vinicultural practices, providing distinctive characteristics for the products originating from this area [1]. Thus, terroir embraces wines with a sense of belonging to their geographical origin, in relation to the specific soil, topography, climate, landscape characteristics, and biodiversity features.

Since yeasts are part of the natural microbial communities on grapes [2], in the interests of preserving the biodiversity and regional influence on wine character, numerous studies have attempted to characterize yeast populations of grapes from different wine-growing regions worldwide, focusing specially on *Saccharomyces cerevisiae*, the key yeast specie for wine alcoholic fermentation [3]. These reports range from studies of the distribution, persistence, and diversity of *S. cerevisiae* strains in winery spontaneous fermentations [4–6], in vineyards [7–10], and even in the analysis of metapopulations to determine the biogeographic distribution of *S. cerevisiae* [11,12].

Biodiversity is a state or attribute defined as the variety within and among life forms of an ecosystem, at all organizational levels, whether occurring naturally or modified by humans [13]. In its broadest sense, it includes taxonomic (e.g., species or strains), functional, genetic, phylogenetic, and chemical diversity, etc., that can be measured at any scale, ranging from genes, to habitats, up to the entire biosphere [14].

Biodiversity specifically consists of three components: richness, which represents the number of different operational taxonomic units (OTUs) in the ecosystem studied; evenness, which describes the equitability in the distribution of individuals among the different OTU types; and disparity, which characterizes the level of similarity between the OTUs of the ecosystem studied [15]. Every adequate study of biodiversity must involve the use of diversity indices, as these mathematical functions allow the quantitative measurement of diversity by collapsing all the biological information into one number [14,16]. Alpha-diversity indices encompass richness and evenness in their equation, but they do not account for *disparity*, and hence this component of diversity is often neglected [15–17]. The oldest, simplest, and therefore most typically α-diversity indices used in classical studies of ecology are the Shannon (H′) and its evenness (J) [14,15,17].

Whilst the use of diversity indices enables the evaluation between different spatial regions, temporal periods, and OTU types (i.e., species, strains, etc.), they do not allow statistical comparisons [14]. Several studies reveal some inconsistencies/discrepancies regarding the use, differences, and meaning of terms such as richness and biodiversity, extending to the analysis of diversity indices and the worth of using these measures in the comparison and contrast of biological populations [15–17].

Rarefaction is a statistical method commonly applied in microbial studies to scale down samples to the same number of individuals [14,18]. In this procedure, the sample with the largest number of individuals is randomly subsampled without replacement and with multiple runs to generate a smoothed, or randomized, average accumulation curve with confidence intervals, so that diversity measures can be statistically compared across samples with an unequal number of individuals [14–16,18].

Although there are no theoretical guidelines, empirical examples suggest that samples of at least 20–50 individuals per sample (and ideally many more) are necessary for meaningful comparisons of abundance-based rarefaction curves. Thus, rarefaction curves require comparable sampling methods, random spatial arrangement of individuals, random and independent sampling of individuals, and larger samples or/with multiple samples drawn from a single community for precise estimation of diversity [14,17,18].

Malbec is the emblematic grape variety in the wine production of Argentina, and it is mainly cultivated in the "Zona Alta del Río Mendoza" (ZARM) region [19,20]. Previous studies have explored the microbial ecology involved in wine fermentations of the ZARM region [5,21–25]. However, the biodiversity of *S. cerevisiae* grape populations in different vineyards and vintages has not been addressed yet.

Therefore, in the present study, the grape populations of *S. cerevisiae* from two cv. Malbec vineyards of the ZARM region were characterized in three vintages to evaluate its annual diversity and evolution over the vintages, by applying the rarefaction method of the classical diversity indices. Rarefaction of the Shannon index (H′) allowed an accurate estimation of *S. cerevisiae* α-diversity, reducing the bias associated to enrichment and sampling procedures. Our results showed that vineyard ecosystem and vintage influence the composition of *S. cerevisiae* grape populations in each harvest. However, the biodiversity of *S. cerevisiae* in vineyards was preserved over time, and, interestingly, the analyses of genetic diversity showed the presence of closely related *S. cerevisiae* populations for each vineyard. Finally, this article also aims to examine diversity data from previous reports of *S. cerevisiae* populations, in order to provide arguments and useful recommendations so that future diversity studies would be more comparable and helpful in the discussion about *S. cerevisiae* contribution to the terroir microbiological concept.

2. Materials and Methods

2.1. Vineyard Selection

Two cv. Malbec vineyards were selected for this study. Vineyard ID belongs to the EEA Mendoza INTA (33°00′17.1″ S 68°51′12.8″ W, Luján de Cuyo, Mendoza, Argentina), and it was selected for its high polymorphic populations of *S. cerevisiae* [24]. It is located at 920 m above sea level, and it was planted in 2001 with a design of 2.5 m between rows and 1.5 m between plants. In this vineyard, only basic phytosanitary treatments are carried out (sulfur and copper are applied as fungicides), no coverages or fertilizers are used, and irrigation is carried out by flooding.

On the contrary, vineyard S was selected since its *S. cerevisiae* populations showed low polymorphism [24]. The distance between both vineyards is 19 km. Vineyard S is located on the property of a commercial winery (International Route 7 km 6.5, Luján de Cuyo, Mendoza, Argentina) at 1050 m above sea level. The Malbec vineyard plot has a size of 5.03 ha, and it is located near the winery building. It was implanted in the year 2000, with a design of 145 rows arranged from North to South, with 2.5 m between rows and 1.33 m between plants.

In vineyard S, irrigation is carried out by drip, and the agricultural practices involve four applications of fungicides between flowering and harvest. The active compounds applied are copper oxychloride in combination with sulfur, strobirulins, and triazoles. Additionally, during November, nitrogen fertilizer is applied, and mulching of grape and seeds pomace is placed in two rows of the vineyard during harvest. These products represent a winery surplus which probably contain high populations of *S. cerevisiae* commercial strains [9,24]. They were used as soil coverage to moderate the temperature, improve the water content of the soil, and suppress diseases and pests [26].

Finally, it is important to point out that optimal sanitary conditions were recorded in both vineyards.

2.2. Sampling, Yeast Isolation, and Identification

A systematic sampling plan with random start was designed. Ten different sites statistically distributed in each vineyard were selected, and the vines in those places were marked to collect samples. The sampling sites were assigned equidistant on the surface described of both vineyards (30 rows for vineyard ID and 145 rows in vineyard S). They were identified by number (from 1 to 10); hence, consecutive numbers corresponded to neighboring sites, and the distance between sampling sites increased as its identification number increased.

From every sampling site, 2 kg of mature healthy Malbec grapes were picked under aseptic conditions, placed into sterile plastic bags, and maintained at 4 °C until laboratory processing. Overall, 60 grapes samples were collected during three harvests in 2004, 2010, and 2011 (coded hereafter as H04, H10, and H11) in both vineyards. The three vintages exhibited typical meteorological conditions for the ZARM region (climatic group IH + 2 IF + 1 IS + 1), characterized by a hot climate, cold nights, and moderate drought with annual rainfall oscillating around 200 mm [20].

Grapes were aseptically crushed, and the musts were incubated at 25 °C to favor *S. cerevisiae* development by spontaneous fermentation at laboratory scale. The process was daily monitored by measuring weight loss until 75% of the initial sugars were consumed [24]. Decimal dilutions were made on 0.1% (*w/v*) peptone water and spread on a WL Nutrient Agar medium (Oxoid, Basingtoke, UK), with chloramphenicol (50 µg/mL) to inhibit bacterial growth. Petri dishes were incubated at 28 °C for 48 h, and yeast count was performed. Considering the colony morphology (texture, surface, margin, elevation, and color) of *S. cerevisiae* previously described [27], 10 to 20 colonies from plates containing 10–300 colonies [28] were purified by streak plating and sub-culture on YPD media (Agar 2 g/L, Yeast Extract 5 g/L, Peptone 5 g/L, and Dextrose 40 g/L). Likewise, all isolates were plated on Lysine Media (Oxoid, Basingtoke, UK) to confirm *Saccharomyces* genera assignment, since *Saccharomyces* yeasts are unable to grow with L-lysine as the sole nitrogen source [29].

2.3. *S. cerevisiae* Molecular Characterization and Data Analysis

Total DNA extraction of *S. cerevisiae* isolates was performed according to Hoffman and Winston [30], and they were differentiated at strain level by Interdelta-PCR [31] following the detailed protocol previously described [25].

To normalize the molecular profiles and construct a presence/absence matrix, the Interdelta-PCR data were processed with PyElph software [32]. Software PAST 3.21 [33] and FIG TREE version 1.4.4 [34] were used for clustering molecular profiles by UPGMA (Unweighted Pair Group Method using Arithmetic averages) based on the Dice coefficient with a cut-off of 80% similarity for strain identity. To verify the presence of commercial yeast strains between the *S. cerevisiae* grapes isolates, a set of 44 Interdelta-PCR patterns previously obtained from the commercial wine yeasts most extensively used in the ZARM region was also included [25].

2.4. Genetic and Ecological Diversity Analysis of *S. cerevisiae* Grape Populations

The binary matrix built with the Interdelta-PCR data was also used for the genetic diversity analysis using Popgene 1.32 software [35]. Each amplification band was considered as a different locus, and three estimators were calculated:

a. Average genetic diversity (h): intra-population genetic diversity calculated as average diversity per locus.
b. Coefficient of interpopulation genetic diversity (Gst): equivalent to Wright's F_{st} [36], it quantifies the level of differentiation among populations considering the total genetic diversity of the populations compared (H_t) and the genetic diversity of each population (H_s).
c. Nei's coefficient of genetic identity among populations [37]: it considers all isolates from every population. Another dendrogram was constructed by UPGMA from these identity coefficients.

Additionally, the ratio between the number of molecular patterns and the number of *S. cereviasie* isolates expressed as a percentage was calculated as an approximate estimative biodiversity [8] or Polymorphism index [38].

The ecological diversity of the *S. cerevisiae* grape populations was evaluated using Estimate*S* software [39] for the calculation of the diversity indices most widely used in ecology [14,16]: richness (S), the Shannon (H') index, and evenness (J). In our study, S is the total number of Interdelta-PCR patterns in a grape population. *H* was calculated as

$$H = -\sum_{i}^{S} p_i \ln p_i \quad (1)$$

where p_i is the proportion of a specific molecular pattern in the data set. It is estimated as n_i/n, where n_i is the number of *S. cerevisiae* isolates for a specific Interdelta-PCR profile and n is the total number of unique Interdelta-PCR patterns [40,41]. Finally, evenness (J) was determined as

$$J = \frac{H}{\ln(S)} \quad (2)$$

where "S" is the richness and "H" is the Shannon index. Its value ranges between 0 (meaning low equitability or high dominance by a few OTUs) and 1 (representing total equitability in the OTU abundance representation detected in the sampling) [16,41].

Since the three vintages showed a different number of *S. cerevisiae* isolates, a mathematical correction was critical to statistically compare biodiversity between harvests. This procedure is called rarefaction, and it was also performed with Estimate*S* software 9.1 [39]. Rarefaction standardizes the diversity indexes by sampling effort, so that the habitat with the largest number of individuals is randomly subsampled without replacement and with multiple runs to generate an average diversity index that can be compared to the index of another habitat based on the same number of individuals [15,16].

3. Results

3.1. Vineyard S. cerevisiae Populations

Grape samples from ten isolation sites statistically distributed in two Malbec vineyards of the ZARM region (ID and S) were independently analyzed at three harvests (H04, H10, and H11) to evaluate the annual diversity of *S. cerevisiae* populations in different areas of the same vineyard and its evolution over the vintages. Fermentation was carried out as an enrichment procedure to favor the development of *S. cerevisiae* for subsequent plating and isolation.

Yeast count was performed after incubation at 28 °C for 48 h. In H04, total yeast counts in both vineyards ranged from 2.18×10^7 to 2.61×10^7 CFU. mL^{-1}, although no *S. cerevisiae* yeasts were isolated from three sampling sites in vineyard S (Table 1). In H10, *S. cerevisiae* growth was confirmed in all samples, except for site 8 in vineyard S. Vineyard ID showed higher populations in the order of $4.02 \times 10^7 \pm 2.1$ CFU. mL^{-1}, whereas in vineyard S the average count was $3.71 \times 10^7 \pm 2.2$ log CFU. mL^{-1}, corresponding in all cases to *Saccharomyces*-type yeasts. In H11, *S. cerevisiae* growth was verified after enrichment in all sampling sites of both vineyards, with populations varying from 3.80×10^6 to 6.53×10^7 for vineyard S and from 1.40×10^6 to 5.09×10^7 CFU/mL for vineyard ID.

Table 1. *S. cerevisiae* isolates, molecular patterns, and biodiversity by sampling site according to vineyard and harvest.

V	H	VIS	NI	MP					B (%)	
ID	H04	1	11	**1** (45.45%)	2 (36.36%)	3 (18.18%)			27.27	31.32
		2	12	4 (8.33%) 9 (8.33%)	5 (8.33%) 10 (8.33%)	6 (8.33%) 11 (16.67%)	7 (8.33%) 12 (8.33%)	8 (16.67%) 13 (8.33%)	83.33	
		3	8	**1** (25%)	14 (12.5%)	15 (12.5%)	16 (25%)	**25** (25%)	62.50	
		4	9	**1** (22.22%) 22 (11.11%)	17 (11.11%)	18 (11.11%)	19 (11.11%)	**20** (33.33%)	66.67	
		5	7	**1** (71.43%)	**25** (28.57%)				28.57	
		6	8	**20** (12.5%)	21 (25%)	23 (12.5%)	24 (12.5%)	**25** (37.5%)	62.50	
		7	8	**26** (100%)					12.50	
		8	7	**26** (100%)					14.29	
		9	7	**24** (42.86%)	**25** (57.14%)				28.57	
		10	6	**20** (66.67%)	**25** (33.33%)				66.67	
	H10	1	13	27 (69%)	28 (7.7%)	29 (15.4%)	**30 (7.7%)**		30.77	11.29
		2	20	**30** (100%)					5.00	
		3	14	31 (92.9%)	32 (7.1%)				14.29	
		4	10	33 (90%)	34 (10%)				20.00	
		5	11	35 (45.5%)	36 (54.5%)				18.18	
		6	10	37 (100%)					10.00	
		7	12	38 (100%)					8.33	
		8	11	**30** (100%)					9.09	
		9	13	39 (100%)					7.69	
		10	10	40 (100%)					10.00	
	H11	1	15	44 (33.3%)	45 (66.7%)				13.33	8.44
		2	17	**43** (88.2%)	46 (5.9%)	47 (5.9%)			17.65	
		3	15	41 (6.7%)	48 (6.7%)	**49** (20%)	50 (66.7%)		26.67	
		4	15	**43** (80%)	51 (20%)				6.67	
		5	15	**42** (6.7%)	52 (66.7%)	53 (20%)	54 (6.7%)		26.67	
		6	15	55 (81.3%)	56 (18.7%)				13.33	
		7	15	57 (80%)	58 (20%)				13.33	
		8	15	**49** (100%)					6.67	
		9	15	**43** (100%)					6.67	
		10	15	**42** (100%)					6.67	
	Subtotal		**359**			**58**			**16.15**	

Table 1. *Cont.*

V	H	VIS	NI	MP					B (%)	
S	H04	1	6	1 (50%)	**2** (8.35%)	**3** (33.33%)	**6** (8.35%)		66.67	14.29
		2	6	**2** (100%)					16.67	
		3	6	**2** (100%)					16.67	
		4	13	**3** (61.54%)	**4** (23.07%)	5 (7.69%)	**6** (7.69%)		30.77	
		5	[a]							
		6	7	**3** (85.71%)	7 (14.29%)				28.57	
		7	6	**4** (16.67%)	8 (50%)	**9** (33.34%)			50.00	
		8	5	**3** (60%)	**9** (20%)	10 (20%)			60.00	
		9								
		10								
	H10	1	14	**11** (92.9%)	**12** (7.1%)				14.29	7.22
		2	14	**11** (85.7%)	**12** (14.3%)				14.29	
		3	14	**11** (78.6%)	**13** (21.4%)				14.29	
		4	14	**11** (64.3%)	**13** (35.7%)				14.29	
		5	9	**11** (77.8%)	**13** (22.2%)				22.22	
		6	9	**11** (100%)					11.11	
		7	11	**11** (81.8%)	14 (18.2%)				18.18	
		8								
		9	6	**13** (66.7%)	15 (16.7%)	16 (16.7%)			16.67	
		10	6	**17** (100%)						
	H11	1	15	**26** (100%)					6.67	7.38
		2	15	**24** (100%)					6.67	
		3	15	**24** (100%)					6.67	
		4	15	**21** (100%)					6.67	
		5	15	**21** (100%)					6.67	
		6	15	**21** (100%)					6.67	
		7	14	**23** (100%)					7.14	
		8	15	18 (100%)					6.67	
		9	15	**11** (20%)	19 (26.7%)	20 (20%)	22 (13.3%)	**23** (20%)	33.33	
		10	15	25 (100%)					6.67	
	Subtotal		295			26			9.15	
	Total		654			84				12.84

V: vineyard, ID: EEA Mendoza INTA Vineyard; S: winery vineyard; H: harvest, H04: 2004th; H10: 2010th; H11: 2011th; VIS: vineyard's isolation site; NI: number of *S. cerevisiae* isolates; MP: molecular Interdelta-PCR patterns and incidence in percentages. Consecutive Arabic numbers were assigned by vineyard to indicate different patterns. Molecular patterns repeatedly isolated are in bold, and patterns coincident with *S. cerevisiae* commercial strains are shaded (pattern 1 from ID and pattern 11 from S: EC1118, Lallemand; pattern 25 from ID and pattern 26 from S: D254, Lallemand; and pattern 3 from S: Zymaflore F10, Laffort). B (%): estimative biodiversity calculated as the ratio between the number of molecular patterns and the number of *S. cerevisiae* isolates expressed as a percentage [8,38]. [a]—Indicates no isolation of *S. cerevisiae*.

A total of 654 *S. cerevisiae* isolates were purified from the 56 grape samples of vineyards ID and S in the three vintages. Among them, 84 *S. cerevisiae* molecular patterns were differentiated by Interdelta-PCR (Table 1). Vineyard ID presented 359 *S. cerevisiae* isolates which were typed into 58 molecular patterns, showing a higher number of isolates and *S. cerevisiae* strains than vineyard S, which gathered 295 *S. cerevisiae* isolates and 26 different molecular patterns (Table 1). This result suggests greater richness in vineyard ID in contrast to vineyard S.

In both vineyards, a non-homogeneous distribution of *S. cerevisiae* strains was verified. In general, sampling sites harbored between 1 and 10 different molecular patterns (mainly 2 in vineyard ID, and 1 in vineyard S); however, *S. cerevisiae* could not be isolated from vineyard S in four cases (Figure 1, Table 1). It is also interesting to point out that 23% of the sampling sites of the ID vineyard presented more than 4 different molecular patterns (between 5 and 10, Figure 1). Most molecular patterns found in the sampling sites were different, but in some cases neighboring sites shared the same molecular pattern. For example,

in H10, vineyard ID shared pattern 30 between sites 1 and 2, whereas vineyard S presented patterns 11 and 12 in sites 1 and 2 and patterns 11 and 13 in sites 3, 4, and 5 (Table 1).

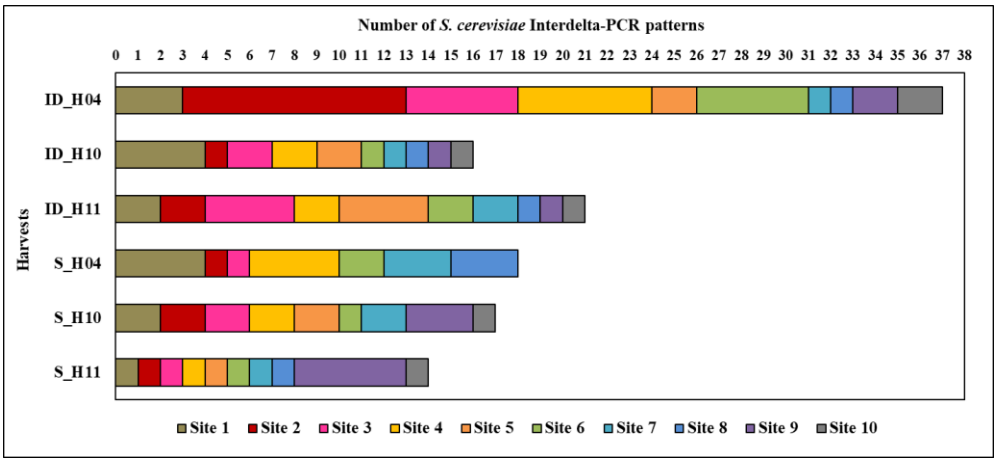

Figure 1. Number of *Saccharomyces cerevisiae* Interdelta-PCR patterns by vineyard, harvest, and sampling site. ID: EEA Mendoza INTA Vineyard; S: winery vineyard; H: harvest; H04: 2004th harvest; H10: 2010th harvest; H11: 2011th harvest.

Each vineyard was characterized by a unique composition of *S. cerevisiae* grape populations in every harvest season, showing an almost complete change in the strains presented in the population every year. Some 26 different molecular patterns were obtained from vineyard ID in H04, and 14 and 18 unique molecular patterns were obtained in H10 and H11, respectively. In contrast, 10 different Interdelta-PCR patterns were found in vineyard S in H04, 7 molecular patterns were recovered from H10, and finally 11 in H11. Patterns 1, 20, 24, 25, 26, 30, 42, 43, and 49 were found in only one vintage, but across several sampling sites of vineyard ID (Table 1). In vineyard S, the same behavior was observed in patterns 2, 3, 4, 6, 9, 12, 13, 17, 21, 23, and 24, whereas pattern 11 was the only Interdelta-PCR profile observed in two vintages (Table 1). Overall, vineyards ID and S exhibited different *S. cerevisiae* populations, and there were almost no coincident strains between them. However, two Interdelta-PCR patterns were found simultaneously in both vineyards, and they corresponded to commercial *S. cerevisiae* strains extensively used in the ZARM region: patterns 1 and 25 from vineyard ID with patterns 11 and 26 from vineyard S, respectively (Table 1).

The estimative biodiversity [8,38] is shown in Table 1 per vineyard, sampling site, and harvest. Values ranged from 8.44 to 31.32% in vineyard ID, while vineyard S showed lower values (from 7.22 to 14.29%). In both cases, H04 exhibited the highest ratio between the number of molecular patterns and the number of *S. cerevisiae* isolates obtained (Table 1).

Some 84 different molecular patterns were processed with PyElph software [32], and a presence–absence matrix was constructed to evaluate the molecular relationship between the harvest's populations of *S. cerevisiae* from vineyards ID and S. Similarity based on the Dice coefficient was calculated, and UPGMA clustering was obtained. The dendrogram constructed using a cut-off of 80% similarity for strain identity showed that molecular patterns did not group according to the vintage or vineyard isolation site. Nevertheless, most of the clusters exhibited patterns belonging to the same vineyard, thus allowing a certain discrimination by location (Figure S1).

3.2. Analysis of S. cerevisiae Population Genetic Diversity

The six grape *S. cerevisiae* populations of the ZARM region (ID_H04, ID_H10, ID_H11, S_H04, S_H10, and S_H11) were analyzed using software Popgene 1.32 [35] to study their genetic variability and population structure. Estimators of genetic diversity calculated are summarized in Table 2. A total of 38 weight bands (considered as different loci) corresponding to amplified Interdelta-PCR bands from 2500 to 100 bp were analyzed. The

percentage of the polymorphic loci varied from 68.42 to 92.11%, showing that more than 26 bands were polymorphic in the studied populations (Table 2).

Table 2. Estimators of genetic diversity for the grape populations of *S. cerevisiae* in vineyards ID and S across H04, H10, and H11.

Populations Studied	L	P (%)	h
ID_H04	34	89.47	0.2475 (0.1745)
ID_H10	33	86.84	0.2668 (0.1727)
ID_H11	34	89.47	0.2473 (0.1699)
S_H04	28	73.68	0.2493 (0.1883)
S_H10	26	68.42	0.0967 (0.1081)
S_H11	35	92.11	0.3133 (0.1818)

L = number of polymorphic loci, P (%) = percentage of polymorphic loci, and h = average intra-population genetic diversity and standard deviation (in brackets) [37].

The average genetic diversity (h) was used as an estimate of intra-population genetic diversity, since it is a function associated with the observed variants' frequency (Mercado et al., 2011). Overall, *h* values ranged from 0.2473 ± 0.17 (ID_H04) to 0.3133 ± 0.18 (S_H11), indicating an acceptable intermediate biodiversity in most of the fermentative grape-associated *S. cerevisiae* populations analyzed (Table 2). Notwithstanding this, population S_H10 showed a coefficient value considerably lower, thus reflecting a lower diversity in its *S. cerevisiae* strains since they shared a significant number of bands (Table 2). This finding is line with the low number of *S. cerevisiae* Interdelta-PCR patterns observed among the 97 isolates found in S_H10 (Table 1).

The total genetic diversity of the populations (H_t) was moderated, as it was the average 0.2684 ± 0.0151, and most of the genetic diversity was found within the populations (H_s = 0.1963 ± 0.0081). The degree of genetic differentiation among the *S. cerevisiae* populations was analyzed by the coefficient of inter-population diversity (G_{st}), equivalent to Wright's F_{ST} [36]. The distinction between low (F_{ST} = 0–0.05), moderate (F_{ST} = 0.05–0.15), high (F_{ST} = 0.15–0.25), and very high (F_{ST} > 0.25) genetic differentiation has been suggested by Wright [36]. In our study, G_{st} had a value of 0.2687, hence indicating a high differentiation level among the six fermentative grape-associated *S. cerevisiae* populations analyzed.

In general, identity between the six *S. cerevisiae* populations was high, with an average value of 0.89 (Table S1). The only exception was the S_H10 population, which showed a lower identity compared to the other populations. This finding was also evidenced in the dendrogram obtained by Nei's genetic identity among the six *S. cerevisiae* grape populations analyzed (Figure 2). As the Cophenetic Correlation Coefficient (CP) was 0.991 for this dendrogram, the clustering gave an accurate picture of the genetic relationships among the fermentative grape-associated *S. cerevisiae* populations. Figure 2 evidenced a close relationship kept between the three *S. cerevisiae* grape populations of vineyard ID (ID_H04, ID_H10, and ID_H11), whilst S_H04 and S_H11 appeared as a second group, and population S_H10 emerged as a highly separate cluster. Altogether, these results suggest that biodiversity and distribution of *S. cerevisiae* strains depend on certain population substructure characteristics for each vineyard.

3.3. Analysis of S. cerevisiae Population Ecological Diversity

To evaluate the annual diversity of *S. cerevisiae* populations in different areas of the same vineyard and its evolution over the vintages, classical ecological indices were calculated. Estimate*S* software [39] was employed for the estimation and rarefaction of richness (S), evenness (J), and the Shannon index (H′), as each harvest presented a different number of isolates [15,16]. Results are summarized in Table 3 and Figure 3, respectively.

Table 3. Rarefaction of richness (*S*), evenness (*J*), and Shannon diversity indices (H') of the six grape-associated *S. cerevisiae* populations analyzed from two Malbec vineyards (ID and S) in the ZARM region during three harvests (H04, H10, and H11).

ID_H04				ID_H10				ID_H11				S_H04				S_H10				S_H11			
NI	***S***	***H′***	***J***	**NI**	***S***	***H′***	***J***	**NI**	***S***	***H′***	***J***	**NI**	***S***	***H′***	***J***	**NI**	***S***	***H′***	***J***	**NI**	***S***	***H′***	***J***
8.3	3.9 (0.8)	1.1 (0.7)	0.8	12.4	1.6 (0.4)	0.2 (0.3)	0.5	15.4	2.2 (0.5)	0.5 (0.3)	0.6	7.0	2.6 (0.7)	0.7 (0.5)	0.7	10.8	1.9 (0.8)	0.4 (0.3)	0.7	14.9	1.4 (0.4)	0.2 (0.5)	0.5
16.6	7.1 (1.4)	1.6 (0.5)	0.8	24.8	3.3 (0.8)	0.9 (0.2)	0.8	30.8	4.4 (1.0)	1.1 (0.3)	0.8	14.0	4.6 (0.9)	1.2 (0.4)	0.8	21.6	3.0 (1.1)	0.7 (0.3)	0.6	29.8	2.7 (0.8)	0.8 (0.4)	0.8
24.9	9.5 (1.9)	1.9 (0.4)	0.8	37.2	5.0 (1.2)	1.3 (0.2)	0.8	46.2	6.5 (1.4)	1.5 (0.3)	0.8	21.0	6.0 (1.1)	1.4 (0.3)	0.8	32.3	3.7 (1.2)	0.8 (0.3)	0.6	44.7	3.9 (1.1)	1.2 (0.4)	0.8
33.2	11.7 (2.3)	2.1 (0.4)	0.8	49.6	6.4 (1.5)	1.6 (0.2)	0.8	61.6	8.3 (1.7)	1.7 (0.3)	0.8	28.0	7.3 (1.2)	1.5 (0.2)	0.8	43.1	4.5 (1.4)	0.9 (0.2)	0.6	59.6	4.8 (1.4)	1.3 (0.3)	0.9
41.5	14.3 (2.7)	2.2 (0.3)	0.8	62.0	7.6 (1.9)	1.7 (0.2)	0.9	77.0	10.0 (2.1)	1.9 (0.2)	0.8									74.5	5.9 (1.7)	1.5 (0.3)	0.9
49.8	17.1 (3.1)	2.4 (0.3)	0.8	74.4	8.9 (2.2)	1.9 (0.2)	0.9	92.4	11.6 (2.4)	2.0 (0.2)	0.8	35.0	8.4 (1.3)	1.6 (0.2)	0.8	53.9	4.9 (1.5)	0.9 (0.2)	0.5	89.4	7.1 (2.0)	1.7 (0.2)	0.9
58.1	19 (3.5)	2.4 (0.3)	0.8	86.8	10.2 (2.5)	2.0 (0.2)	0.9	107.8	13.5 (2.7)	2.1 (0.2)	0.8	42.0	9.3 (1.4)	1.7 (0.1)	0.7	64.7	5.6 (1.6)	0.9 (0.2)	0.5	104.3	7.9 (2.2)	1.8 (0.2)	0.9
66.4	21.5 (3.8)	2.5 (0.2)	0.8	99.2	11.5 (2.8)	2.1 (0.1)	0.9	123.2	15.0 (3.0)	2.2 (0.2)	0.8	**49**	**10** (1.5)	**1.7**	**0.7**	75.4	6.1 (1.7)	0.9 (0.1)	0.5	119.2	9.0 (2.4)	1.9 (0.2)	0.9
74.7	23.4 (4.2)	2.6 (0.2)	0.8	111.6	12.7 (3.1)	2.2 (0.1)	0.9	138.6	16.7 (3.3)	2.3 (0.1)	0.8					86.2	6.6 (1.8)	1.0 (0.1)	0.5	134.1	9.9 (2.7)	2.0 (0.1)	0.9
83	**26** (4.5)	**2.7**	**0.8**	**124**	**14** (3.4)	**2.3**	**0.9**	**154**	**18** (3.6)	**2.4**	**0.8**					**97**	**7** (1.9)	**1.0**	**0.5**	**149**	**11** (2.9)	**2.1**	**0.9**

Estimate*S* software [39] was used for the calculation and rarefaction of the diversity indices. Values are expressed as mean and standard deviation (in brackets). Numbers in bold represent the global diversity found in a specific vintage. NI: number of *S. cerevisiae* isolates.

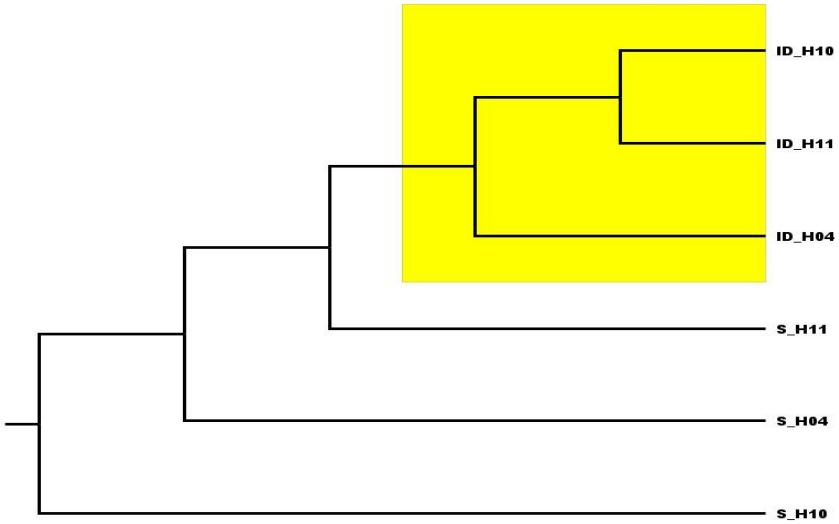

Figure 2. Dendrogram constructed with UPGMA method and Nei's genetic identity [37] of the six *S. cerevisiae* grape populations of the ZARM region analyzed. Cophenetic Correlation Coefficient (CP) = 0.9910. Populations from ID vineyard are highlighted.

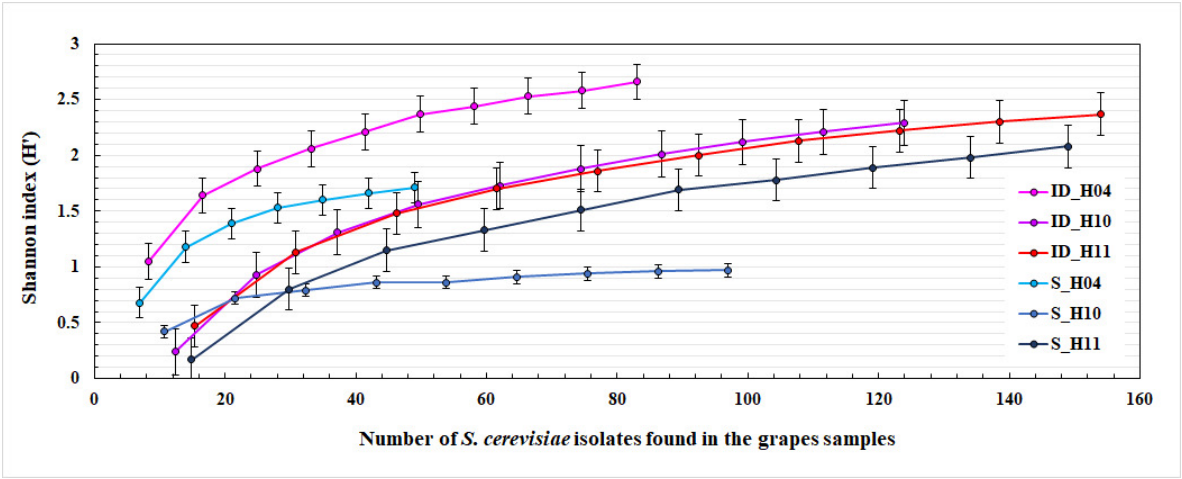

Figure 3. Rarefaction curves of Shannon diversity indices (H') of the number of *S. cerevisiae* isolates obtained from the six vineyard *S. cerevisiae* populations of the ZARM region analyzed. Estimate*S* software [39] constructed the different curves using a sub-sampling technique, which allows the (balanced) comparison of Shannon indices based on the same number of isolates.

The Shannon index (H') measures the diversity within a population and considers both richness and evenness, i.e., it considers both the number of diverse strains and the number of isolates showing the same genetic profile [25,40,41]. Overall, the Shannon diversity indices for the harvests were typical (bold values in the last row in Table 3), as they were all included within the range of 1.5 to 3.5 [38,39]. The exception was S_H10, which showed a very low value of diversity (Table 3).

Evenness (J) quantifies the equitability in the distribution of individuals among the different OTU types detected in the sampling. Values of J ranged between 0.8 and 0.9, suggesting that most of the harvests showed a good equitability in the abundance of *S. cerevisiae* Interdelta-PCR patterns obtained. These results are in agreement with the previous observations regarding the difference determined in most Interdelta-PCR patterns found in the vineyard sampling sites, as well as the non-homogeneous distribution of *S. cerevisiae* strains in these sites.

The Shannon diversity indices' *(H′)* rarefaction curves of the number of *S. cerevisiae* isolates obtained from the vineyard-associated populations of the ZARM region are shown in Figure 3. This technique was applied as an important step towards the statistically significant evaluation of diversity. Thus, to test if rarefaction curves are statistically different, error bars must be compared around the points in discussion [14,17,18]. Rarefaction curves confirmed that S_H10 exhibited a significant lower diversity than the other harvests evaluated from both vineyards (error bars of the final point in the curve, Figure 3).

Nonetheless, it is important to point out that the diversity of *S. cerevisiae* populations in vineyard S stabilized at last, since the error bars of the S_H11 curve overlapped with the S_H04 curve, the diversity of the first vintage evaluated. Moreover, S_H11 showed a slightly increase on the initial diversity of S_H04. Thus, these results suggested that this vineyard ecosystem could overcome the disturbance that caused a diversity drop in H10.

On the other hand, rarefaction curves also allowed us to confirm that there were no differences in the annual diversity and its evolution over the vintages in the case of vineyard ID, as the error bars of the three rarefaction curves overlapped (Figure 3). This fact highlights the real importance of standardizing diversity by sampling effort, as one could think that ID_H04 would be the most diverse harvest just because it presented the greatest number of Interdelta-PCR patterns and the highest values of the Shannon index (Table 3) and estimative biodiversity (B%) [8,38] (Table 1).

4. Discussion

In the present study, 84 different Interdelta-PCR patterns were found among 654 *S. cerevisiae* isolates obtained from 56 spontaneous fermentations performed with Malbec grapes from two vineyards (ID and S) located in the ZARM region, during three vintages (H04, H10, and H11). Taking into consideration previous knowledge about the low proportion of *S. cerevisiae* in healthy and mature grapes [2,29], all samples were subjected to a selective enrichment by spontaneous fermentation, since the conditions imposed by grapes must favor *S. cerevisiae* isolation [5,8,9,24]. Selective enrichment is crucial not only to favor the isolation of *S. cerevisiae*, but also to avoid underestimation of the recovery of vineyard strains more susceptible to the stressful conditions of alcoholic fermentation: high concentrations of sugar increasing ethanol content, low pH, microbial competition, variable nutrient availability, suboptimal oxygen levels, and the impact on the cellular redox status [8,42]. *S. cerevisiae* yeast count obtained after enrichment reflected a non-homogeneous distribution of yeast populations in the ten sampling sites distributed along the same vineyard, which is behavior already reported [24].

Spontaneous fermentations can be considered as complex multifactorial process, where strain diversity is one variable for a rapid onset [43]. Indeed, fermentation conditions stimulate the development of *S. cerevisiae* diversity in the population within the fermentation as well. Whether one strain dominates throughout a fermentation or there is a progressive change in dominant genotypes depends on the selective potential imposed by enrichment conditions, the relative abundance, and the genomic and phenotypic composition of the initial population of *S. cerevisiae* [44]. In this sense, experimental design acquires a vital importance, as the vineyard sampling, the scale of fermentation (laboratory or winery), the moment and the number of samples obtained in the fermentation process, and the detection limit of the direct-plating method directly affect the yeast isolation and the diversity results obtained.

Since OIV is the international organization which reports technical and scientific aspects of viticulture and winemaking, our culture approach is defined based on its official compendium for microbiological analysis [28]. Therefore, our detection limit varied between 6.66 and 10% (20 *S. cerevisiae* strains picked from 300 isolates, or 1 strain in 10 isolates). Previous similar studies on *S. cerevisiae* biodiversity have generally applied detection limits of between 2.5 and 4 % [8,45,46], all regarded as acceptable compromises for a good estimation of *S. cerevisiae* population composition, even when rare strains might not have been detected.

In regards to vineyard sampling design, even when 10 sampling sites statistically distributed were evaluated in both vineyards, our results showed that *S. cerevisiae* diversity and distribution depended on the vineyard isolation site. Similar conclusions were also found in the first study of *S. cerevisiae* diversity from the ZARM region, which evaluated eight vineyards [24]. Likewise, another study applied for the first time the Theory of Sampling to obtain grapes from adjacent and well-established commercial vineyards within the same terroir [47]. These authors concluded that microbial diversity was unevenly distributed within individual vineyards, reinforcing the importance of sampling multiple locations in the vineyard to precisely assess the biodiversity of the ecosystem.

Despite this evidence, we found that numerous field studies on *S. cerevisiae* diversity generally took a small random number of grape samples or analyzed very few sites within vineyards [48–52], and consequently could be reporting patterns that are not representative of the entire ecosystem. This probably occurs due to the relatively large spatial areas that need to be sampled in diversity studies, and the financial and logistical limits related to the number of samples that can be analyzed [47].

Nonetheless, numerous studies on the ecology, population dynamics, and biodiversity of *S. cerevisiae* strains in both vineyards and wineries worldwide have described the dynamic nature of *S. cerevisiae* populations [4–7,10,24,25,38,41,45]. In fact, many of these reports have also applied Interdelta-PCR for the intraspecific differentiation of *S. cerevisiae* strains. This simple, robust, and highly discriminative technique allows the discrimination of *S. cerevisiae* isolates whilst at the same time no amplicons are generated for other *Saccharomyces* and non-*Saccharomyces* yeast species [4,10,24,25,31,38].

The Interdelta-PCR data were normalized, i.e., the combinations of band patterns from each *S. cerevisiae* isolate were compared to define *S. cerevisiae* strains, and they were also used to build a binary matrix of presence/absence [32]. First, the similarity of the molecular patterns was evaluated with a dendrogram built by the UPGMA method and Dice coefficient using a cut-off of 80% for strain identity. No specific clusters could be ascribed to the vineyard isolation site or vintage; however, various clusters exhibited Interdelta-PCR patterns from the same vineyard, thus allowing certain discrimination by vineyard location. In contrast, another three-year survey on yeast populations of must from wineries in two Italian vine-growing areas territories found that *S. cerevisiae* strains were casually distributed without any evident relationship between Interdelta-PCR profile and territory, year, or matrix of isolation [10].

Moreover, the molecular data binary matrix was used to analyze the genetic diversity of the six vintage-associated *S. cerevisiae* populations (combination of vineyards and harvests). The pattern and degree of populational divergence among these populations was estimated by the coefficient of inter-population diversity (G_{st}) over all loci, equivalent to Wright's F_{ST} [36]. In our study, the value of G_{st} was 0.2687, therefore indicating a high divergence among the *S. cerevisiae* populations analyzed. The dendrogram built with Nei's genetic identity also supported this finding, as population S_H10 emerged separately from the cluster of vineyard S and the vineyard ID group. Altogether, our results suggest that biodiversity and distribution of *S. cerevisiae* strains depend on certain population substructure characteristics for each vineyard. A previous report, studying the genetic structure of fermentative vineyard-associated *S. cerevisiae* populations from Vinho Verde (Portugal), found that values of genetic differentiation correlated with the size of the vineyards [9]. These authors hypothesized that *S. cerevisiae* strains may become more distinctive in a larger vineyard that constitutes a bigger "evolutionary playground". Accordingly, vineyard S presents a wider area than vineyard ID, which may have affected its vintage-associated *S. cerevisiae* populations.

Conversely, another report found fewer divergent groups of *S. cerevisiae* strains in vineyards with the same grape varieties in close proximity [43]. The lack of correlation between genetic and geographic distances has been explained as a consequence of a lower number of strains from the population analyzed [40], and also due to scarce genetic flux

between vineyards' *S. cerevisiae* populations [24] because wild yeasts generally have cycles of sexual reproduction by "self-mating", alternating with clonal reproduction [53].

Overall, *S. cerevisiae* communities in each harvest season were characterized by the appearance of new strains, i.e., the isolated *S. cerevisiae* strains were unique for each vineyard and were also not re-isolated in consecutive years. Previous reports have described this behavior as "not perennial" [8,9,43].

Among the first works performed on *S. cerevisiae* diversity, Vezhinet et al. [54] found *S. cerevisiae* strains with permanence over the years and widely distributed in a Champagne vineyard area. Therefore, these authors associated the terroir concept to the notion of specific native strains representative of an oenological area which persist in it for a certain period [54]. Over the years, this perception has been largely debated: is terroir really linked to specific native *S. cerevisiae* strains? How long does it take to consider a strain as a native of a particular area? When assigning a strain membership to a territory, where should the area boundary be placed? [10,55].

Taking into consideration that most of the studies on *S. cerevisiae* diversity during unique or many vintages support the "not perennial" behavior of *S. cerevisiae* strains over the years, many of them have also not reported *S. cerevisiae* strains distributed widely in great areas [8,10,24], and additionally the terroir definition also includes biodiversity features. Would it be possible that yeast terroir is linked to sustained *S. cerevisiae* diversity in time and space, rather than the persistence of specific native strains in a particular area/winery over time?

Estimative biodiversity (B, %) was the first index used to characterize wine *S. cerevisiae* diversity [8]. Schuller et al. registered a total polymorphism of 18.33% in a three-year survey about *S. cerevisiae* diversity in three vineyards from Vinho Verde (Portugal) [8]. Later, the first study of *S. cerevisiae* diversity in eight vineyards from the ZARM region exhibited an estimative biodiversity that was surprisingly high (32.20%) [24]. After that, a biodiversity study of *S. cerevisiae* populations in spontaneous fermentations from Apulia (Italy) used this measure, but named it the polymorphism index; the study registered a higher total level of polymorphism (48.32%) during a single harvest analyzed [38]. As far as we are aware, de Celis et al. [4] had the highest level of polymorphic *S. cerevisiae* populations from wine spontaneous fermentations, accounting for 97.04% in four Spanish cellars. Nevertheless, other recent reports showed total levels of polymorphism of 10.18, 12.11, and 36.06% in *S. cerevisiae* populations from spontaneous fermentations in Italy, Portugal, and Spain, respectively [6,41,43].

Our results showed a similar low–medium value (12.84%) and a declining trend in both vineyards ID and S compared with the previous report of Mercado et al. [24].

Similarly, most of these studies have also established their results by the calculation of richness (S) and the Shannon index (H′), the most widely used indices in ecology [14,16]. The problem is that the three descriptive indices have biases, and by themselves they do not allow statistical comparisons of diversity [15,17,18].

A simple count of the number of OTUs in a sample is usually a biased underestimation of the real number of OTUs, simply because increasing the sampling effort (through counting more individuals, examining more sampling units, or sampling a larger area) will inevitably increase the number of OTUs observed. Furthermore, as richness (S) does not account for the OTU abundance distribution (i.e., evenness), it gives exceptionally rare OTUs equal weight as exceptionally common OTUs, an entirely unintuitive estimate of diversity [15]. For all this, even the simplest comparison of OTU richness between two samples is not straightforward unless the number of individuals is identical in the two samples (which rarely occurs) or the two samples represent the same degree of coverage (completeness) in sampling [17].

On the other hand, while the Shannon index (H′) is a much more balanced estimation of diversity because it accounts for richness and evenness, it also tends to heavily weight *richness*. Hence, it is very sensitive to sample size, as every new OTU increases the value of the index [14,16]. An estimator of the Shannon index (H′) frequently used in ecology is

based on the steepness of the rarefaction curve—recall that the rarefaction procedure reduces bias in estimating richness, and thus reduces the same bias in the Shannon estimator [15].

Sampling variation is a critical factor of biodiversity studies, and ignoring the sampling effects may obscure the influence of overall abundance or sampling intensity on OTU richness. In diversity analysis, it is equally important to adjust for sampling differences (e.g., by rarefaction), as measuring the variance (or standard error) of an estimator and providing a confidence interval that reflect sampling uncertainty [14,15,17]. As far as we are aware, this is the first report that has applied rarefaction for the calculation of α-diversity indices of *S. cerevisiae* vineyard strains discriminated by Interdelta-PCR, offering a reliable and precise method to the analysis of biodiversity. Rarefaction of classical ecological indexes confirmed a slightly increase in the initial diversity in vineyard S and a stabilization in the diversity of *S. cerevisiae* populations in the other vineyard, ID. Therefore, even when harvest environmental conditions have a strong impact on yeast population structure [10,38,41], if diversity indices of *S. cerevisiae* populations give similar assessments in time and space, the biodiversity is sustained, and this persistence becomes an important terroir characteristic as the viability of ecosystems relies on sustained biodiversity. Furthermore, intra-species diversity has been recognized as often playing a more significant role in ecosystem functioning and dynamics than species diversity [15]. These results offer useful recommendations so that future diversity studies of *S. cerevisiae* populations would be more comparable worldwide and contribute to the discussion about yeast contribution to the terroir microbiological concept.

5. Conclusions

The annual diversity of *S. cerevisiae* vineyard grape populations and its evolution over the vintages in two cv. Malbec vineyards of the ZARM region was evaluated. Overall, we could summarize that:

Genetic biodiversity analysis of *S. cerevisiae* populations revealed a high differentiation between vineyards, and most of the variation was found within the populations. These results suggest a certain population substructure characteristic for each vineyard that should be deepened to confirm *S. cerevisiae* biogeographic distribution in the ZARM region.

While the composition of *S. cerevisiae* strains in the vineyard's populations of the three harvests evaluated was completely different, the values of the diversity indices remained stable. Thus, the biodiversity over time in this ecosystem was preserved.

Rarefaction of classical ecological diversity indices represents a promising tool as a statistically reliable method for evaluating biodiversity. This technique is easy to run with freely available software; it performs better with repeated and larger samples, and hence it can be used to properly compare the diversity of different communities.

This work offers useful recommendations so that future diversity studies of *S. cerevisiae* populations could be more comparable between different harvests and wine-growing areas, so they could provide solid evidence to the discussion about the contribution or not of *S. cerevisiae* to the wine terroir concept.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9030292/s1, Figure S1: Dendrogram showing molecular relationships for 84 Interdelta-PCR patterns obtained during three harvests in two Cv. Malbec vineyards of the ZARM region. Numbers at each branch represent bootstrap values (over 100 bootstrap replicates). CP: 0.612. Patterns code corresponds to vineyard, harvest, and identification number; Table S1: Nei's genetic identity matrix of the six *S. cerevisiae* grape populations of the ZARM region.

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