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High-throughput sequencing of amplicons for monitoring yeast biodiversity in must and during alcoholic fermentation

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Abstract We compared pyrosequencing technology with the PCR-ITS-RFLP analysis of yeast isolates and denaturing gradient gel electrophoresis (DGGE). These methods gave divergent findings for the yeast population. DGGE was unsuitable for the quantification of biodiversity and its use for species detection was limited by the initial abundance of each species. The isolates identified by PCR-ITS-RFLP were not fully representative of the true population. For population dynamics, high-throughput sequencing technology yielded results differing in some respects from those obtained with other approaches. This study demonstrates that 454 pyrosequencing of amplicons is more relevant than other methods for studying the yeast community on grapes and during alcoholic fermentation. Indeed, this high-throughput sequencing method detected larger numbers of species on grapes and identified species present during alcoholic fermentation that were undetectable with the other techniques.

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Introduction

Numerous yeast genera and species are present on grapes, but the three principal species are *Hanseniaspora uvarum* (anamorph: *Kloeckera apiculata*), *Metschnikowia pulcherrima* (anamorph: *Candida pulcherrima*), and *Candida zemplinina*, previously called *Candida stellata* [1]. Most of the yeasts present on grapes are also found in musts. More than 20 yeast genera have been identified in musts and wine [33], and there is also considerable within-species biodiversity [8, 37].

These yeasts, often referred to as non-*Saccharomyces* (NS) yeasts, were long considered useless or irrelevant for winemaking [31]. However, interest has recently increased in the role of NS yeasts in wine fermentation [18, 20, 34, 41]: spontaneously fermented wines have a higher risk of spoilage, but are generally regarded as being more complex, with a better mouth-feel (texture), and integration of flavors than inoculated wines [17, 38]. However, spontaneous fermentations are ecologically complex, and the equilibrium between species depends on many factors. The presence of yeast species on grapes depends on the climatic conditions, the grape variety [28], disease pressure, the extent of damage to the grapes, and vineyard practices [3]. In particular, the use of antifungal agents in vineyards has been shown to affect the diversity of yeasts on grapes. For example, fungicide application promotes an increase in the size of *Metschnikowia* populations [32] and a decrease in the size of *Saccharomyces* populations.

Yeast biodiversity may be studied by culture-dependent or culture-independent approaches. One of the most frequently used approaches for the identification of isolates

from culture is analysis of the 5.8S ITS rDNA region [14]. Another strategy involves direct analysis of the microbial population present in the sample collected, by denaturing gradient gel electrophoresis (DGGE) [19, 33]. Direct methods have several advantages over culture-based approaches, including their rapidity and their ability to identify non-culturable microbes. However, detection by DGGE is difficult for species present at population densities below 10^3 CFU/ml or two orders of magnitude lower than those of the other species present [26, 30]. Recently developed high-throughput sequencing technologies, such as the 454 pyrosequencing of amplicons, can be used to characterize the microbial diversity of environmental ecosystems more precisely [35]. Amplicon pyrosequencing is an automated high-throughput sequencing technique involving the synthesis of single-stranded deoxyribonucleic acids and detection of the light generated by pyrophosphate release in a luciferase-coupled reaction [23]. This technique can be used for the rapid and accurate sequencing of nucleotide sequences from all species present in the sample, making it possible to study population structure. For example, the high-throughput sequencing of amplicons has recently been used to study the diversity and dynamics of the bacterial populations associated with an Irish kefir grain and the corresponding fermented product [13, 22]. Short-amplicon sequencing techniques have recently been used to monitor seasonal changes in winery-resident microbiota and to determine the bacterial diversity of botrytized wines [4–6].

We did not aim to assess the reproducibility of the various validated techniques in this study. Instead, the main aim was to compare the results obtained between the different techniques, applied to the same sample. We analyzed grapes from three different production systems to ensure that the samples analyzed were heterogeneous.

Materials and methods

Sampling

We used Chardonnay grapes harvested aseptically in 2011 from a single plot divided into three equal parts in Burgundy. Each part of the plot had been managed, since 2006, by one of three different farming methods, referred to as “conventional”, “organic”, and “ecophyto”. The conventional part of the vineyard was treated with chemicals, the “ecophyto” part was treated with the same products as the conventional part, but at half the dose. The “organic” part of the vineyard was not treated with any chemical product other than copper and sulfur. Details of the management procedures used are provided in Table 1. Equal amounts (3 kg) of grapes were collected from each part of the vineyard, in aseptic conditions. The harvested grapes were placed in sterile bags. Immediately after their collection, the grapes were pressed by hand-squeezing, in the plastic bags, and 250-ml aliquots of the resulting must were poured into sterile 500-ml fermenters and left to ferment at 22 °C. Samples were collected immediately before alcoholic fermentation (T1) in the middle of fermentation (T2), and two-thirds of the way through alcoholic fermentation (T3). Must composition at T0, medium composition (after partial alcoholic fermentation), and details of the cell population are provided in Table 2. At each time point, we isolated 50 colonies from YPD-agar plates or lysine-agar plates, for each of the three production method samples, and identified them by PCR-ITS-RFLP. In parallel, we extracted DNA directly from the medium at each time point for species identification by DGGE or amplicon sequencing.

Analysis of enological parameters and cell population

FTIR analysis using Bacchus (Cetim, France) was used to determine several wine parameters, including percent ethanol, pH titratable acidity (TA), volatile acidity, glucose, and fructose. Cell population was assessed by a spread plating procedure on YPD agar.

Yeast isolation

Aliquots of each sample were serially diluted, and 100 µl of each dilution was plated on two different media: a non-selective YPD agar (0.5 % w/v yeast extract, 1 % w/v, peptone, 2 % w/v glucose and 2 % w/v agar) and lysine medium agar (OXOID, France). All media were supplemented with chloramphenicol (200 ppm) to inhibit bacterial development. The plates were incubated at 28 °C. Cultures on lysine medium were used to estimate the NS population, as *Saccharomyces* cannot grow on media containing lysine as the sole carbon source [16]. After 24–48 h, 50 colonies were randomly selected from cultures on each medium, cultured in YPD and stored at −80 °C in YPD broth supplemented with glycerol (25 % final concentration).

Identification of yeast isolates

Genomic DNA was prepared from yeast cultures on YPD-agar, after 2 days of incubation, with Whatman FTA filters [7]. Briefly, 5 µl of an aqueous suspension of yeast (20 µl) was applied directly onto a Whatman FTA microcard and allowed to dry for about 1 h at room temperature. Small discs (2 mm in diameter) were then removed from the dried FTA filters with a micropunch and washed by incubation for 5 min with 100 µl of Whatman FTA wash reagent, and then for 1 min with 100 µl of TE buffer (10 mM Tris–HCl, pH 7.5, 0.1 mM EDTA). The washed filter discs were dried by incubation for 5 min at 65 °C, and the PCR mix was then added directly.

Table 1 Details of the management procedures for the production systems studied

Farming system	Organic		Ecophyto		Conventional	
	Treatments	No. of repeats	Treatments	No. of repeats	Treatments	No. of repeats
Management procedure	Sulfur (80 g/l, Thiovit jet Micro Billes, Syngenta) and with copper sulfate	7	Sulfur (Thiovit jet Micro Billes, Syngenta)	1	Spiroxamine (Prosper, Bayer)	1
	Copper sulfate (20 g/l, Bordeaux mixture RSR Disperss, Cerexagri)	4	and with same chemicals as the conventional plot but at half the dose	0.5	Tebuconazole (Corail, Bayer)	1
			Tebuconazole (Corail, Bayer)	1	Dithianon + Dimethomorph (Arco DTI, BASF)	1
			Kresoxim-methyl (Stroby DF, BASF)	1	Kresoxim-methyl (Stroby DF, BASF)	1
			Metrafenone (Vivando, BASF)	1	Fosethyl-Aluminum + Folpel + Cymoxanil (Valiant Flash, Bayer)	1
			Tetraconazole (Greman, Phyteurop)	1	Metrafenone (Vivando, BASF)	1
					Cymoxanil + Folpel (Sarman F, Phyteurop)	1
					Tetraconazole (Greman, Phyteurop)	1
					Meptyldinocap (Karathane 3D, DOW)	1
Dates	26 April, 2011 and 24 June, 2011		26 April, 2011 and 15 June, 2011		28 April, 2011 and 5 July, 2011	

The 5.8S-ITS region was amplified by PCR with the primers ITS1 5′-TCCGTAGGTGAACCTGCGG-3′ and ITS4 5′-TCCTCCGCTTATTGATATGC-3′. PCR was performed in 50 μl of 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 μM of each primer, 0.025 U of *Taq* polymerase (Promega Corp., Madison, WI, USA) and 50 ng of yeast DNA. A MyCycler thermal cycler (Bio-Rad, Hercules, CA, USA) was used with a program described elsewhere [14]. PCR products were digested with the restriction enzymes *Hae*III, *Hin*fI, *Cfo*I, and *Dde*I (Fermentas, France). The PCR products and their restriction fragments were subjected to electrophoresis for 1 h at 110 V in 2 and 3 % agarose gels, respectively, which were then stained with ethidium bromide (14 mg/ml) for visualization of the DNA bands under UV light. Fragment sizes were estimated by comparison with DNA size markers (GeneRuler 100 bp Plus DNA Ladder, Thermo Fisher Scientific, Inc. Waltham, MA, USA), with Quantity One 4.6.5 software from Bio-Rad.

Total DNA extraction

We collected 5-ml aliquots of must or fermented must midway and two-thirds of the way through the alcoholic fermentation and centrifuged them for 5 min at 4 °C and 3,000 × *g*. The resulting pellet was suspended in 5 ml of milliQ water and filtered through glass wool, to separate the cells from must debris. The filtered suspension was centrifuged again (5 min at 4 °C and 3,000 × *g*). DNA was extracted as described by White et al. [40]. The DNA concentrations of the samples were then standardized (50 ng/μl) on the basis of optical density at 260 nm, by adding DEPC-treated water, as appropriate, and the samples were then stored at −20 °C.

DGGE analysis

The D1 domain of the fungal 26S rRNA gene was amplified with the primers NL1-GC (5′-GCCATATCAATAAGCGGAGGAAAG-3′) and LS2 (5′-ATTCCCAAACAACTCGACTC-3′), as reported in a previous study [10]. The NL1-GC primer had a 39-bp GC-clamp sequence at its 5′ end to prevent the complete denaturation of amplicons. PCR was performed in a reaction volume of 50 μl, with 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 μM of each primer, 0.025 U of *Taq* polymerase (Promega Corp., Madison, WI, USA) and 10–100 ng of yeast DNA. Reactions were run for 30 cycles of denaturation at 95 °C for 60 s, annealing at 52 °C for 45 s and extension at 72 °C for 60 s. An initial 5-min denaturation at 95 °C and a final 7-min extension at 72 °C were used. The products (250 bp) were analyzed by electrophoresis in 2 % agarose gels containing 0.5 μg/ml ethidium bromide, with visualization under UV light.

Table 2 Main enological characteristics of musts and wines obtained from the grapes produced by the three production systems

Farming system:	Organic		Ecophyto		Conventional	
Sampling time:	Must	T3	Must	T3	Must	T3
Glucose (g l^{-1})	98	46	98	53	102	71
Fructose (g l^{-1})	99	28	100	39	103	0
Total sugar (g l^{-1})	197	74	198	92	205	71
Ethanol (%)	0	6.10	0	7.50	0	6.50
Acetic acid (g l^{-1})	0	1.1	0	0.3	0	2.3
Malic acid (g l^{-1})	1	0.6	1.04	0.7	1.18	0.8
pH	3.35	3.05	3.57	3.17	3.44	3.21
Cell population (CFU/ml)	10^6	6×10^9	5×10^5	10^8	10^7	10^9

Vertical polyacrylamide gels (acrylamide-bis acrylamide 19:1, Bio-Rad, Hercules, CA, USA), with a denaturing gel of 35–50 % polyacrylamide, were used for DGGE. The 100 % chemical denaturing solution consisted of 7 M urea (Sigma–Aldrich) and 20 % (v/v) formamide (Sigma–Aldrich) in 2 mM Na_2 EDTA.H_2O in Tris–acetate, pH8.5 (1 × TAE). We mixed 20-μl samples of diluted PCR amplicons (1/10) with 10 μl of (100 %) glycerol before loading on the gel.

A DCode apparatus (Bio-Rad) was used for DGGE in 1 × TAE, at 60 °C for 6 h, with a constant voltage of 130 V. Gels were stained with 1 × GelRed (Biotium, Hayward, CA, USA) in 1 × TAE and the bands were visualized and photographed under UV transillumination. Bands were excised from the gels and the DNA was eluted overnight in 40 μl of 10 mM Tris pH8, 1 mM EDTA (TE) at 4 °C. The DNA was re-amplified with the same pair of primers, without the GC-clamp, and sequenced with a cycle extension DNA sequencer (Beckmann Coulter Cogenics, Essex, United Kingdom). The BLASTN algorithm was applied to the GenBank database for sequence identification (http://www.ncbi.nlm.nih.gov/BLAST/).

Primers, 18S rRNA gene amplification, and 454 pyrosequencing conditions

A 350-base (on average) 18S rRNA gene fragment was amplified from each DNA sample (5 ng) with the universal primers FR1 (5′-ANCCATTCAATCGGTANT-3′) and FF390 (5′-CGATAACGAACGAGACCT-3′) [9], under the following PCR conditions: 94 °C for 3 min, followed by 35 cycles of 1 min at 94 °C, 1 min at 52 °C and 1 min at 72 °C, and a final extension phase for 5 min at 72 °C. PCR products were purified with a MinElute PCR Purification kit (Qiagen NV, Venlo, Netherlands) and quantified with the PicoGreen (Molecular Probes, Eugene, Oregon, USA) staining kit, according to the manufacturer's instructions. A second PCR, carried out under the same conditions, but with only nine cycles, was performed with the purified PCR products (5 ng), with 10-bp multiplex identifiers added to the 5′ end of the primers for the specific identification of each sample and the prevention of PCR biases [2]. PCR products were purified with the MinElute gel extraction kit (Qiagen NV) and quantified as previously described. Equal amounts of each sample were pooled to give a total of 500 ng and 454-adaptors were added, as recommended by the manufacturer. The pyrosequencing step was carried out on a GS Junior apparatus (Roche 454 Sequencing System), by the GenoSol platform (INRA, Dijon, France, http://www2.dijon.inra.fr/plateforme_genosol/). The raw datasets are freely available from the EBI database system (in the Short-Read Archive), under project accession no. PRJEB4220.

Sequence treatment and bioinformatic analysis of 18S rRNA gene sequences

Raw reads were processed with the GnSPipe pipeline first described by Terrat et al. [39] and recently optimized by the GenoSol platform (INRA, Dijon, France). All reads were first filtered and deleted if (a) the exact primer sequence was not found at the start of the sequence, (b) the sequences contained any ambiguity (N_s), (c) or the sequence was <250 bases long. The reads retained for the analysis were reverse-complemented as required, because the sequencing was not oriented. Rigorous dereplication (i.e., clustering of strictly identical sequences) was then carried out with a PERL program. The dereplicated reads were then aligned, with Infernal software [27], and clustered into operational taxonomic units (OTU) with a PERL program clustering rare reads with abundant reads without taking differences in homopolymer lengths into account. All single-singletons (reads detected only once and not clustered, that might be artifacts, such as PCR chimeras and large sequencing errors) were then checked on the basis of the quality of their taxonomic assignments [21, 29]. Thus, single-singletons were compared with the contents of the Silva 111 reference database, with a megaBLAST approach (the

threshold value was set to 85 % similarity at the phylum level). Finally, for efficient comparison of the datasets and to avoid biased community comparisons, the retained reads were homogenized by random selection restricted to the smallest dataset. All the retained high-quality reads were then (1) taxonomically assigned, according to the Silva 111 reference database, through a megaBLAST approach (threshold set to 85 % similarity for all taxonomic levels); (2) aligned and clustered into OTUs with the PERL program, as described above.

Results and discussion

The main goal of this study was to compare the results obtained with culture-dependent and culture-independent techniques. We ensured that the samples tested were heterogeneous, by collecting grapes from three different production systems for analysis with these techniques.

Biodiversity of the yeasts on grapes

In total, we isolated 1,200 colonies, 1,121 of which we were able to identify: the isolates were shown to belong to ten different species by the culture-dependent PCR-ITS-RFLP method [14] (Table 4). The most frequently identified species were *H. uvarum/K. apiculata* (66.5 % of all isolates) and *C. zemplinina* (23.55 % of all isolates). The other non-*Saccharomyces* species present were *Kluyveromyces thermotolerans, Cryptoccocus magnus, Sporobolomyces roseus, Aureobasidium pullulans, Bulleromyces albus, M. pulcherrima*, and *Rhodoturula nothofagi*. These species are the most frequently described in published studies, but have been reported to be present at very different frequencies [10, 20]. *Saccharomyces cerevisiae* was not isolated from any of the harvested grapes. This result is consistent with the reported extreme rarity of *S. cerevisiae* on grapes [24].

The PCR-DGGE profiles obtained for the DNA extracted directly from the must before and during alcoholic fermentation are shown in Fig. 1. Most of the yeast species identified by the culture-dependent PCR ITS-RFLP method were also identified by DGGE. No minor yeast species (*K. thermotolerans, C. magnus, R. nothofagi*) were identified. However, this is unsurprising, as DGGE cannot detect minor species, particularly if their population densities are below 10^3–10^4 CFU/g of grapes or CFU/ml of must, or when their abundance is two orders of magnitude lower than those of other species, as reported in previous studies [26, 30]. The efficiency of PCR with the NL1/LS2 primer set has already been reported to be poor for *M. pulcherrima* [26]. Finally, a high-throughput sequencing approach was used to assess grape yeast biodiversity.

In total, 57,048 raw reads longer than 250 bp were generated by 454 pyrosequencing analysis. Reads that did not fulfill the quality criteria were removed (see "Materials and methods"); 32,527 high-quality sequence reads were retained for subsequent analyses. Between 1,582 and 9,404 high-quality sequence reads were obtained per sample (Table 3). Normalization was carried out to obtain the same number of reads for each sample for the quantitative estimation of diversity. We then clustered high-quality reads according to their similarity, giving 87–387 OTUs per sample (Table 3).

Figure 2 shows rarefaction curves for the OTUs obtained, based on 97 % sequence identity, for each sample. These curves show that saturation was not reached for samples after pressing (T1) (Fig. 2, Panel A), even when re-sampling analyses were performed, with normalization as a function of sample size (Fig. 2, Panel B). The rarefaction curves for samples CT1, ET1, and OT1 followed similar patterns, but the ET1 sample was richer in OTUs than the other two samples, consistent with previous results for OTU richness and index calculations (Table 3). Moreover, rarefaction curves for samples midway (T2) and two-thirds of the way (T3) through alcoholic fermentation suggested that fungal diversity was well represented, because the curves flattened over time and the number of sequences analyzed increased

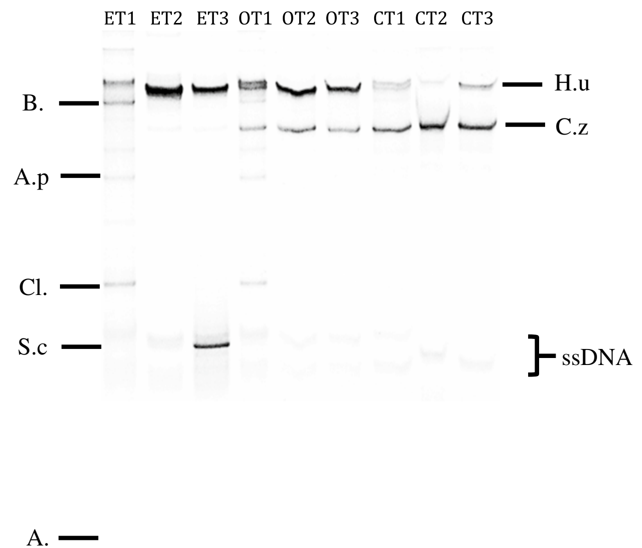


Fig. 1 PCR-DGGE profiles of amplified 26S rRNA from samples collected from the ecophyto (*E*), organic (*O*), and conventional (*C*) vineyard plots before alcoholic fermentation (*T1*), midway (*T2*), and two-thirds of the way through (*T3*) the fermentation. Identification was based on a BLASTn comparison of the sequences obtained from the PCR-DGGE bands with GenBank: *Hanseniaspora uvarum (H.u); Candida zemplinina (C.z), Botryotinia fuckeliana (B.) Aureobasidium pullulans (A.p). Cladosporium sp. (Cl.). Saccharomyces cerevisiae (S.c) Alternaria sp (A.).* The bands common to all isolates (labeled ssDNA) are single-stranded DNA artifacts

Table 3 Summary of 454 pyrosequencing data, estimated OTU richness, sample coverage, and indices (Chao1, ACE, and Shannon) for fungal 18S rDNA libraries from Chardonnay grape samples

Farming system	Organic			Ecophyto			Conventional		
Sampling time	T1	T2	T3	T1	T2	T3	T1	T2	T3
Raw reads	5,660	4,446	2,817	5,512	6,745	13,456	6,370	4,952	7,090
High-quality reads kept after filtering steps	3,295	2,390	1,582	3,091	3,217	9,404	3,308	2,836	3,404
High-quality reads kept after the homogenization step	1,582	1,582	1,582	1,582	1,582	1,582	1,582	1,582	1,582
Number of OTUs (3 % distance level)—genus level	330	124	102	387	146	87	285	90	95
Shannon diversity index	3.85	1.72	1.59	4.08	1.31	1.31	3.65	1.90	1.89
Chao1 richness index	1,023	359	287	1,273	408	301	913	405	326
ACE richness index	1,130	416	250	1,450	489	351	1,017	370	298
ESC	0.27	0.60	0.65	0.26	0.61	0.67	0.36	0.60	0.58

ESC: $C_x = 1 - (N_x/n)$, where N_x is the number of unique high-quality reads and n is the total number of high-quality reads

ESC estimated sample coverage, *OTU* operational taxonomic unit

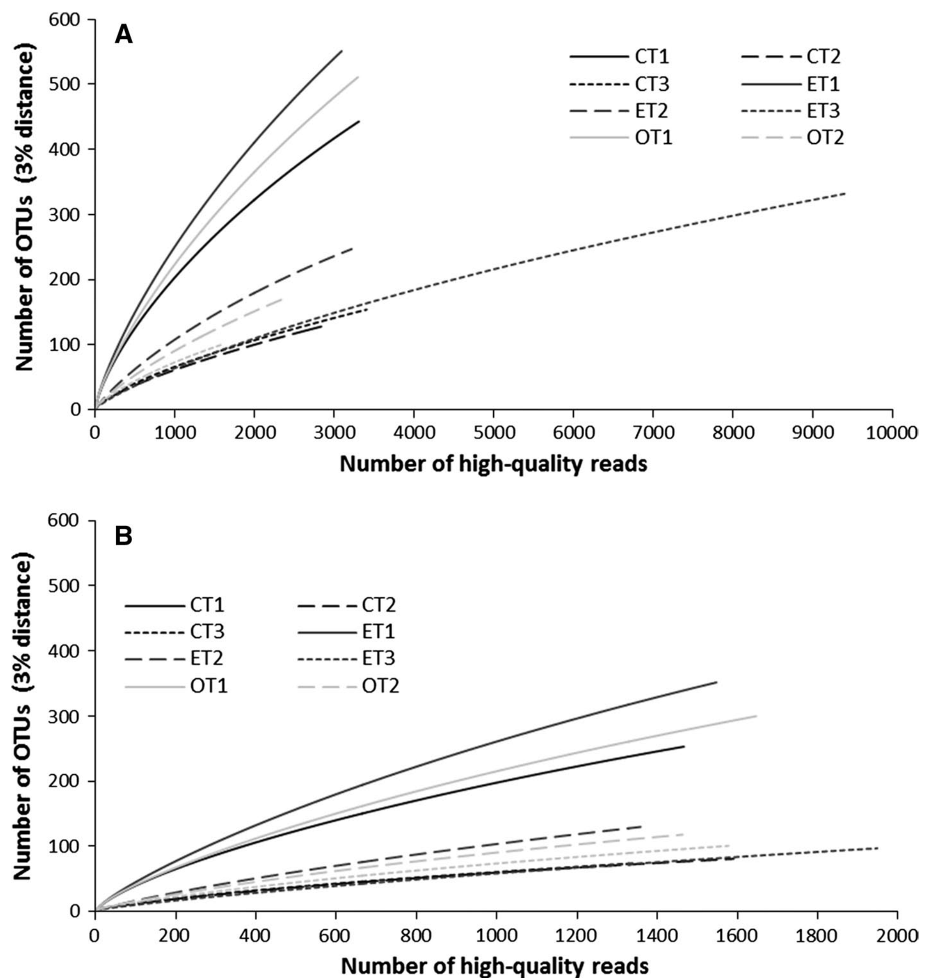


Fig. 2 Rarefaction curves for total high-quality 18S rDNA reads from Chardonnay grape samples (organic *O*, ecophyto *E*, and conventional *C*) at a 3 % distance level (**a**) and rarefaction curves normalized with respect to high-quality 18S rDNA library sizes at a 3 % distance level (**b**)

(Fig. 2). Thus, the 454 pyrosequencing approach clearly revealed higher levels of fungal diversity than reported in previous studies, particularly for mold species.

Even though biodiversity was assessed on only one grape sample per production system, our results confirm previous findings. Indeed, different species profiles were found for the different production systems used (Table 4). *H. uvarum* accounted for 85 % of the yeasts present on grapes produced by the organic method, but only between 43 and 62 % of those produced by the two other methods.

Table 4 Comparison of the various molecular techniques used to characterize the fungal diversity of three different grape production systems (organic, ecophyto, and conventional)

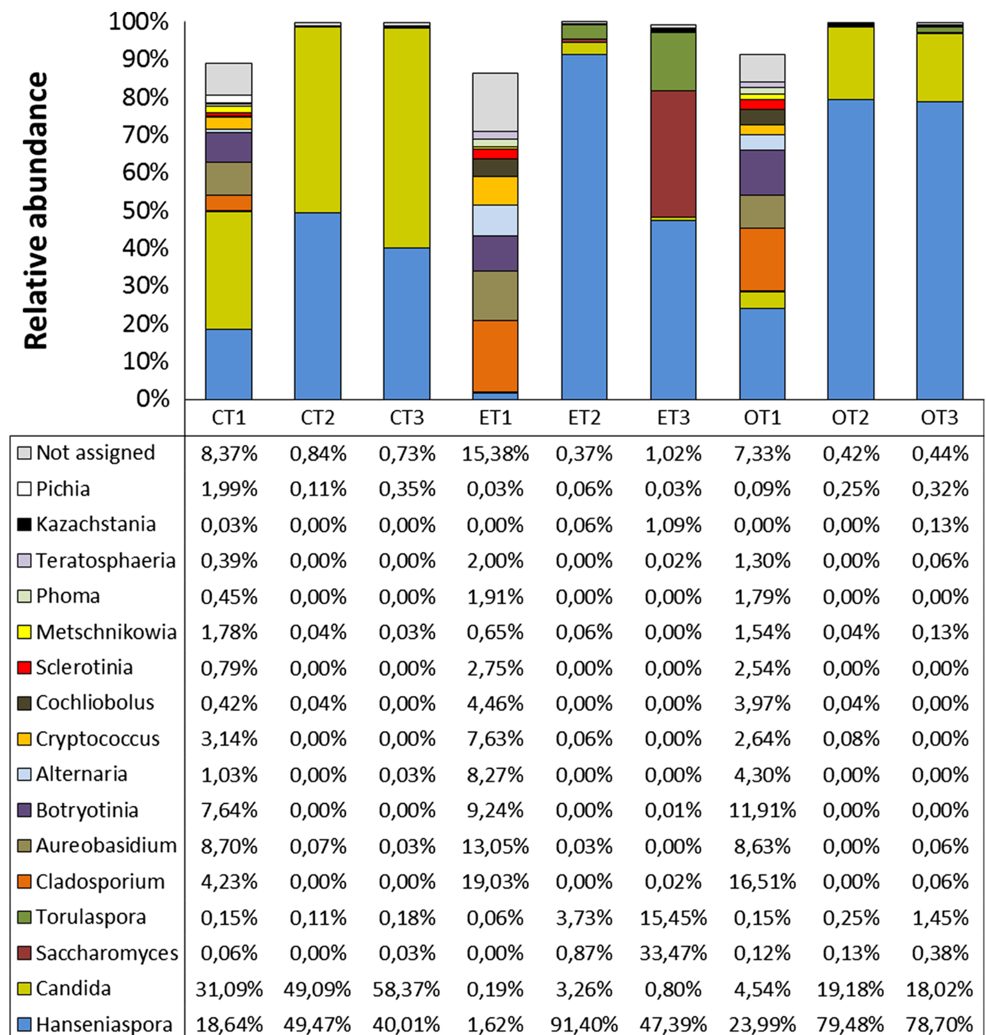
Farming system	Organic									Ecophyto									Conventional								
Sampling time	T1			T2			T3			T1			T2			T3			T1			T2			T3		
Techniques	PYRO (%)	PCR ITS (%)	DGGE	PYRO (%)	PCR ITS (%)	DGGE	PYRO (%)	PCR ITS (%)	DGGE	PYRO (%)	PCR ITS (%)	DGGE	PYRO (%)	PCR ITS (%)	DGGE	PYRO (%)	PCR ITS (%)	DGGE	PYRO (%)	PCR ITS (%)	DGGE	PYRO (%)	PCR ITS (%)	DGGE	PYRO (%)	PCR ITS (%)	DGGE
Hanseniaspora uvarum	56.7	85.00	+	80.3	51.00	++	79.4	73.50	++	6.4	62.00	+	92.0	96.00	+++	48.8	88.00	++	29.3	43.00	+	50.2	5.00	+	40.7		+
Candida zemplinina	10.6	13.00	+	19.4	8.50	+	18.2	26.50	+	0.8		+	3.3			0.8			49.0	52.00	++	49.8	57.50	++	59.3	100.00	++
Saccharomyces cerevisiae							0.4						0.9		+	34.5	12.00	++									
Aureobasidium pullulans	20.3		+							52.6	7.40	+							14		+						
Metschnikowia pulcherrima	3.5	1.70					0.1			2.4	26.00								3	4.70							
Sporobolomyces roseus	2.1				18.50					7.2	3.70			4.00									20.00				
Bulleromyces albus					21.00																		17.50				
Torulaspora delbrueckii	0.5			0.3			1.5						3.7			15.9											
Cryptococcus magnus	6.1				1.40					30.5									5								

PYRO 454 pyrosequencing of 18S rRNA genes, *PCR ITS* PCR-5.8S-ITS –RFLP, *DGGE* PCR- DGGE of 26S rRNA genes

Such differences in biodiversity between production systems have been reported before [12, 25]. However, we will not discuss the effects of production systems on biodiversity in any greater detail here because (1) this was not the aim of this study; (2) this aspect has already been studied, (3) the various studies reported to date have reported conflicting results, and (4) as recently shown [36], such studies would require very large sample sizes, taking into account the high level of diversity within individual plots. However, the analysis of heterogeneous samples by next-generation sequencing revealed greater differences in yeast biodiversity than could be picked up with the other two techniques. More than 16 species were identified by amplicon sequencing, whereas PCR-ITS-RFLP and DGGE identified only five and seven different species, respectively (Figs. 1, 3, 4). Moreover, amplicon sequencing revealed greater differences in the nature of the species present than the other two techniques, as described above.

Changes in biodiversity during alcoholic fermentation

Although results should be taken with cautious since no duplicate have been performed, all three methods showed a decrease in biodiversity during alcoholic fermentation, consistent with previous reports [28]. Indeed, as fermentation progresses, the biodiversity of the yeast community decreases as shown by the richness indices (Table 3), with the emergence of a dominant species [15, 33]. However, differences were observed in the results obtained with the different techniques (Table 4). For example, for all three sampling times, the culture-dependent method showed *C. zemplinina* to be the dominant species in conventional production system samples. High-throughput sequencing results indicated that, for this production system, *H. uvarum* accounted for 49.47 and 40.01 % of the total yeast population at T2 and T3, respectively (Table 4). DGGE also confirmed the presence of *Hanseniaspora* at T2 and T3. This suggests that culture-dependent identification approaches may lead to incorrect interpretation.



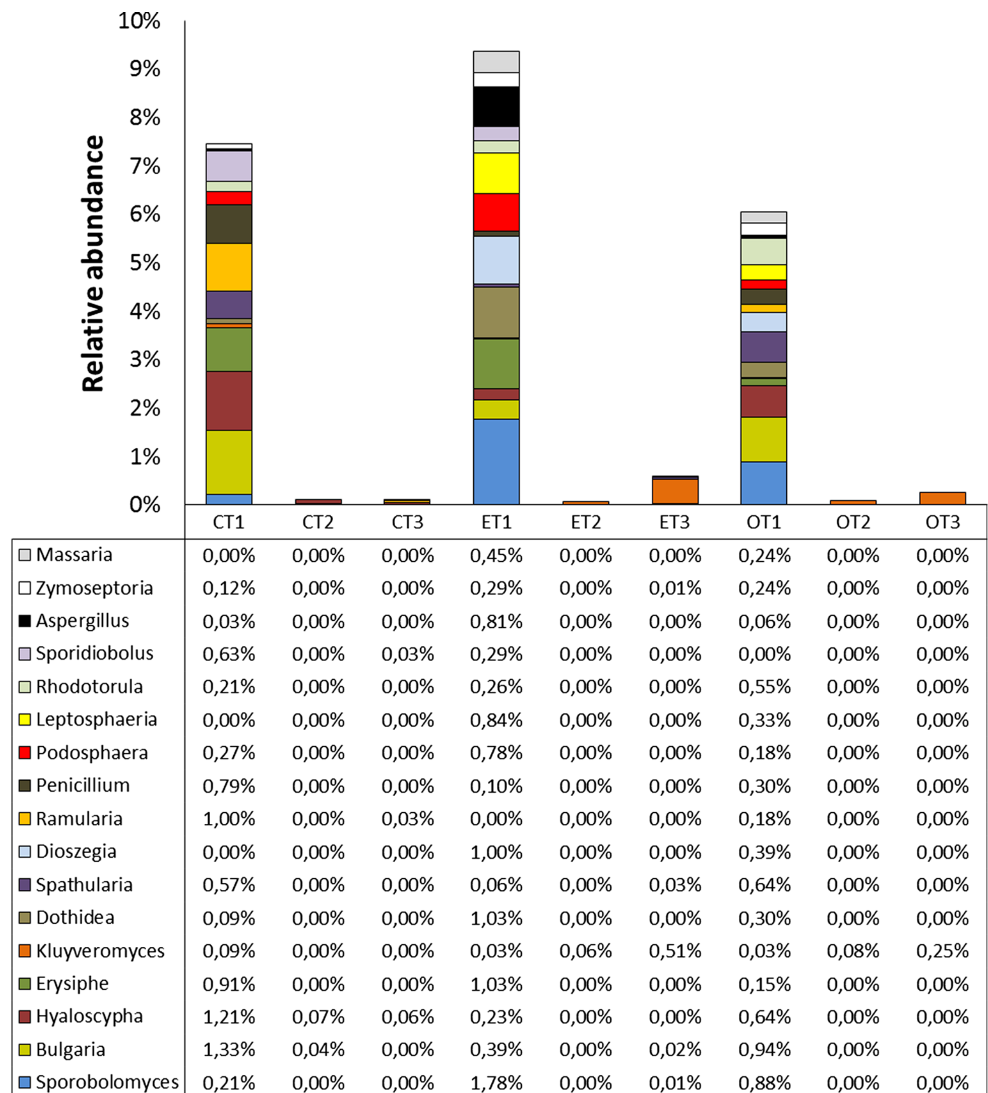
	CT1	CT2	CT3	ET1	ET2	ET3	OT1	OT2	OT3
Not assigned	8,37%	0,84%	0,73%	15,38%	0,37%	1,02%	7,33%	0,42%	0,44%
Pichia	1,99%	0,11%	0,35%	0,03%	0,06%	0,03%	0,09%	0,25%	0,32%
Kazachstania	0,03%	0,00%	0,00%	0,00%	0,06%	1,09%	0,00%	0,00%	0,13%
Teratosphaeria	0,39%	0,00%	0,00%	2,00%	0,00%	0,02%	1,30%	0,00%	0,06%
Phoma	0,45%	0,00%	0,00%	1,91%	0,00%	0,00%	1,79%	0,00%	0,00%
Metschnikowia	1,78%	0,04%	0,03%	0,65%	0,06%	0,00%	1,54%	0,04%	0,13%
Sclerotinia	0,79%	0,00%	0,00%	2,75%	0,00%	0,00%	2,54%	0,00%	0,00%
Cochliobolus	0,42%	0,04%	0,00%	4,46%	0,00%	0,00%	3,97%	0,04%	0,00%
Cryptococcus	3,14%	0,00%	0,00%	7,63%	0,06%	0,00%	2,64%	0,08%	0,00%
Alternaria	1,03%	0,00%	0,03%	8,27%	0,00%	0,00%	4,30%	0,00%	0,00%
Botryotinia	7,64%	0,00%	0,00%	9,24%	0,00%	0,01%	11,91%	0,00%	0,00%
Aureobasidium	8,70%	0,07%	0,03%	13,05%	0,03%	0,00%	8,63%	0,00%	0,06%
Cladosporium	4,23%	0,00%	0,00%	19,03%	0,00%	0,02%	16,51%	0,00%	0,06%
Torulaspora	0,15%	0,11%	0,18%	0,06%	3,73%	15,45%	0,15%	0,25%	1,45%
Saccharomyces	0,06%	0,00%	0,03%	0,00%	0,87%	33,47%	0,12%	0,13%	0,38%
Candida	31,09%	49,09%	58,37%	0,19%	3,26%	0,80%	4,54%	19,18%	18,02%
Hanseniaspora	18,64%	49,47%	40,01%	1,62%	91,40%	47,39%	23,99%	79,48%	78,70%

Fig. 3 Relative abundances, based on the taxonomic assignation of high-quality 18S rDNA reads of fungi from Chardonnay grape samples (organic *O*, ecophyto *E*, and conventional *C*), for the 16 major genera detected before alcoholic fermentation (*T1*), and midway (*T2*), and two-thirds of the way through (*T3*) alcoholic fermentation

For the organic production system, we identified five species at T2, and only two at T3 (*H. uvarum* and *C. zemplinina*) by PCR-ITS-RFLP. The sequencing of amplified 18S rRNA gene fragments resulted in the identification of three species at T2 and five at T3. The two major species were identified by all three techniques, but pyrosequencing also identified minority species occurring at T3, such as *S. cerevisiae*, *Torulaspora delbrueckii*, and *M. pulcherrima,* which were not detected by either PCR-ITS-RFLP or DGGE.

For the ecophyto production system, only two species were identified by PCR-ITS-RFLP at T2. One of these species, *S. roseus,* was not detected by DGGE or pyrosequencing. High-throughput sequencing revealed the presence of three minor species (*C. zemplinina*, *S. cerevisiae*, *T. delbrueckii*) two of which were undetectable with the two other techniques, the remaining species, *S. cerevisiae*, being detected by DGGE. At T3, *S. cerevisiae* was detected, but was present at low levels, with *H. uvarum* identified as the majority species by PCR-ITS-RFLP. Based on the sequencing results, *Saccharomyces* accounts for up to one-third of the total population, whereas *Torulaspora* accounts for 15 % of the total population. However, *Torulaspora* was not identified by the other two methods. For the conventional production system, no *H. uvarum* was detected by PCR-ITS-RFLP at T3, with *C. zemplinina* considered to account for 100 % of the population at this time point, in analyses carried out with this technique. By contrast, both DGGE and 454 pyrosequencing techniques revealed the presence of *H. uvarum* at this time point.

Grape samples from the three production systems considered differed in terms of yeast biodiversity, with potential effects on population dynamics during alcoholic fermentation, consistent with previous findings [11, 12, 25]. However, firm conclusions about the influence of production system on yeast biodiversity would require the analysis of a very large number of samples. Indeed, the trends observed



	CT1	CT2	CT3	ET1	ET2	ET3	OT1	OT2	OT3
Massaria	0,00%	0,00%	0,00%	0,45%	0,00%	0,00%	0,24%	0,00%	0,00%
Zymoseptoria	0,12%	0,00%	0,00%	0,29%	0,00%	0,01%	0,24%	0,00%	0,00%
Aspergillus	0,03%	0,00%	0,00%	0,81%	0,00%	0,00%	0,06%	0,00%	0,00%
Sporidiobolus	0,63%	0,00%	0,03%	0,29%	0,00%	0,00%	0,00%	0,00%	0,00%
Rhodotorula	0,21%	0,00%	0,00%	0,26%	0,00%	0,00%	0,55%	0,00%	0,00%
Leptosphaeria	0,00%	0,00%	0,00%	0,84%	0,00%	0,00%	0,33%	0,00%	0,00%
Podosphaera	0,27%	0,00%	0,00%	0,78%	0,00%	0,00%	0,18%	0,00%	0,00%
Penicillium	0,79%	0,00%	0,00%	0,10%	0,00%	0,00%	0,30%	0,00%	0,00%
Ramularia	1,00%	0,00%	0,03%	0,00%	0,00%	0,00%	0,18%	0,00%	0,00%
Dioszegia	0,00%	0,00%	0,00%	1,00%	0,00%	0,00%	0,39%	0,00%	0,00%
Spathularia	0,57%	0,00%	0,00%	0,06%	0,00%	0,03%	0,64%	0,00%	0,00%
Dothidea	0,09%	0,00%	0,00%	1,03%	0,00%	0,00%	0,30%	0,00%	0,00%
Kluyveromyces	0,09%	0,00%	0,00%	0,03%	0,06%	0,51%	0,03%	0,08%	0,25%
Erysiphe	0,91%	0,00%	0,00%	1,03%	0,00%	0,00%	0,15%	0,00%	0,00%
Hyaloscypha	1,21%	0,07%	0,06%	0,23%	0,00%	0,00%	0,64%	0,00%	0,00%
Bulgaria	1,33%	0,04%	0,00%	0,39%	0,00%	0,02%	0,94%	0,00%	0,00%
Sporobolomyces	0,21%	0,00%	0,00%	1,78%	0,00%	0,01%	0,88%	0,00%	0,00%

Fig. 4 Relative abundances, based on the taxonomic assignment of high-quality 18S rDNA reads for the fungi in Chardonnay grape samples (organic *O*, ecophyto *E*, and conventional *C*), for the first 17 minor genera detected before alcoholic fermentation (*T1*), and midway (*T2*), and two-thirds of the way through (*T3*) alcoholic fermentation

may reflect sample variation rather than differences due to the production system [36], and it would be very difficult to draw any firm conclusions on the effect of production system from our data. Nevertheless, it would be of considerable interest to test this hypothesis in future studies.

The three methods gave different results. This was not unexpected, as such differences have already been reported for comparisons between DGGE and isolation by culture [30], and between culture-dependent methods and ARISA [36].

Our results demonstrate that culture-dependent identification methods detect fewer species than other methods. Indeed, the number of species identified by the ITS-RFLP analysis of isolates was similar to the number of species identified by DGGE, but far lower than that identified by the high-throughput sequencing of amplicons (Figs. 3, 4). The metagenomic approach based on the 454 pyrosequencing of amplified 18S rRNA genes revealed much greater fungal diversity than previously described, particularly for mold species, although some of the species identified were present at very low densities (Figs. 3, 4). This detection of greater community diversity than documented by other methods indicates the superiority of metagenomic approaches.

The findings of this study suggest that culture-dependent methods are not the most appropriate methods for studies of yeast biodiversity. Indeed, yeasts differ considerably in their ability to grow on standard media and this may lead to the unintentional selection of certain species. PCR-DGGE is not suitable for use in biodiversity studies either because it is not quantitative. Nevertheless, the profile of yeasts determined with this technique was very similar to that obtained by the high-throughput sequencing technique. Our results suggest that DGGE cannot detect yeast species present at a frequency of <8 % of the total population. The results of this study also demonstrate that metagenomic sequencing is a very powerful method for studies of the biodiversity of yeasts in musts and during alcoholic fermentation. Indeed, high-throughput sequencing makes it possible to analyze large numbers of samples rapidly and may therefore be a good approach for further investigations of the recently reported variability between vineyards [36].

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