Metataxonomic comparison between internal transcribed spacer and 26S ribosomal large subunit (LSU) rDNA gene

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ABSTRACT

Next-generation sequencing has been used to strengthen knowledge about taxonomic diversity and ecology of fungi within food ecosystems. However, primer amplification and identification bias could edge our understanding into the fungal ecology. The aim of this study is to compare the performance of two primer pairs over two nuclear ribosomal RNA (rRNA) regions of the fungal kingdom, namely the ITS2 and 26S regions. Fermented cocoa beans were employed as biological material and the fungal ecology during fermentation was studied using amplicon-based sequencing tools, making use of a manually curated 26S database constructed in this study, and validated with SILVA's database. To explore potential biases introduced by PCR amplification of fungal communities, a mock community of known composition was prepared and tested. The relative abundances observed for ITS2 suggest that species with longer amplification fragments are underestimated and concurrently species that render shorter amplification fragments are overestimated. However, this correlation between amplicon length and estimation is not valid for all the species analysed. Variability in the amplification lengths contributed to the preferential amplification phenomenon. DNA extracted from twenty fermented cocoa bean samples were used to assess the performance of the two target regions. Overall, the metataxonomic data set recovered similar taxonomic composition and provided consistent results in OTU richness among biological samples. However, 26S region provided higher alpha diversity index and greater fungal rRNA taxonomic depth and robustness results compared with ITS2. Based on the results of this study we suggest the use of the 26S region for targeting fungi. Furthermore, this study showed the efficacy of the manually curated reference database optimized for annotation of mycobiota by using the 26S as a gene target.

1. Introduction

Fungi are eukaryotic microorganisms which belong to one of the most diverse kingdoms on Earth (Blackwell, 2011). They play an important role in the safety, quality, and stability of all foodstuff to some degree, whether they are required during processing or whether they have a negative impact during shelf life. Therefore, tracking fungal communities of food systems has been a concern in food research. To date, most recent studies on the microbial diversity of fermented food such as vegetable, seafood, beverages, cheese, olives and spontaneously fermented American cool ship ale fermentations have employed amplicon sequencing approaches (Bokulich et al., 2012; Cocolin et al., 2013; Ercolini et al., 2012; Li et al., 2011; Roh et al., 2010).

Illumina sequencing platform has been currently providing a sensitive description of the microbial dynamics within food ecosystems. Some of the advantages of using this technology is that it yields greater

sequencing coverage and increased sample throughput at lower cost *per* sample compared to other platforms (Caporaso et al., 2011; Quail et al., 2012). The sensitivity of this approach relies on the high coverage and accurate taxonomic resolution of short amplicon length (Quail et al., 2012). Recent advances in the microbial diversity using next-generation sequencing technologies (NGS) have underlined the importance of the reliability of PCR primers targeting a specific genetic marker (Bokulich and Mills, 2013). In spite of the importance of the amplification of shorter fragments amplified by PCR in NGS, recent studies described a more reliable community of fungi using shorter Internal Transcribed Spacer amplicons (ITS) of the nuclear ribosomal RNA (rRNA) (Bokulich and Mills, 2013; Ihrmark et al., 2012).

The ITS region is considered the universal barcode for identification of fungi and includes the ITS1 and ITS2 regions, separated by the 5.8S gene. These two regions (ITS1-2) are characterized by high evolutionary rates and are edged by highly conserved regions with suitable

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target sites for universal primers (Begerow et al., 2010). However, the complete ITS region located between the 18S and 28S genes in the nuclear ribosomal RNA is considered too long for 454 sequencing or other NGS (Bellemain and Carlsen, 2010). Therefore, various primers are used to amplify parts of the ITS region. In this study, we selected the primer ITS3tagmix1 that targets a site in the ITS2 and the degenerate reverse primer ITS4ngs which targets an ITS-flanking site in the ribosomal large subunit (LSU) encoding regions (White et al., 1990) based on their ability to amplify fungal species through *in silico* analysis (Bellemain and Carlsen, 2010; Tedersoo et al., 2015). Nevertheless, the nuclear rRNA large subunit (LSU/28S/26S) and small subunit (SSU/ 18S) genes have also been often used to address fungal diversity (Bonanomi et al., 2016; David et al., 2014; De Filippis et al., 2017a, 2017b; Garofalo et al., 2015; Stellato et al., 2015; Wang et al., 2015). To bring an overall perspective, most yeasts have been identified from sequence divergence in the D1/D2 domain of LSU rRNA (Kurtzman and Robnett, 1997). Despite the great resolution to recognized yeast species through 26S rRNA sequencing reactions, little is known about the potential uses and bias that can be introduced when using this target region in NGS. In this context, it is necessary for ecological studies to compare different targeting regions to describe the most accurate and reliable ecological populations in a food system. Given the nature of current challenges, the selection of a suitable genetic marker for the identification of fungi will help researchers to clear current issues insight into the selection of primer sets.

The main focus of this study is to address sequencing target regions and primer biases on one of the dominating taxonomic groups of fungi in the Dikarya, Ascomycota, which represents 53% of the described species of true Fungi (Koljalg et al., 2013). This phylum is important in the food industry and serves as a source for biomass production, but also includes known human and plant pathogens (Bekatorou et al., 2006; Berbee, 2001). We assessed two different targeting sites for amplicon-based Illumina NGS studies. The performance of the 26S primer set, delivering high coverage and accurate taxonomic assignment of short (~400 bp) fungal amplicon *versus* the performance with the ITS2 region was tested. This research intends to bring new insights in the field of taxonomic assignment, validation and resolution of uncertainties on using amplicon-sequencing approaches for fungi identification by using mock and fermented samples. Attention was paid for monitoring fungi in mock communities and biological samples, where taxonomic assurance of the technique, and mapping and monitoring fungi dynamics are investigated for food applications.

2. Materials and methods

2.1. Primer selection and in silico analysis

Primer pairs targeting the ITS2 region (Tedersoo et al., 2015), and the D1 domain of 26S rRNA gene (Cocolin et al., 2000), were selected and reported in Table 1. For the amplification of the D1 domain of the 26S, we modified the LS2-MF primer sequence position from reverse to forward, corresponding to nucleotide position 266 of *Saccharomyces cerevisiae* 26S gene as described by Cocolin et al. (2000) and a reverse primer NL4 (Jespersen et al., 2005). The Illumina overhang adapter sequences were added to locus-specific sequences. The D1 region from the 26S gene was amplified *in silico* to compare primer specificity and

Table 2
Fungal strains used for sequencing analysis and respective amplicon length.

Fungal species	Size bp	Size bp
	(26S)	(ITS2)
Alternaria alternata	454	414
Aspergillus flavus	455	430
Aspergillus fumigatus	459	439
Candida sake	467	393
Fusarium oxysporum	462	405
Fusarium verticillioides	458	415
Galactomyces geotrichum	502	324
Hanseniaspora opuntiae	435	484
Hanseniaspora osmophila	462	520
Kluyveromyces marxianus	427	521
Penicillium brevicompactum	456	428
Penicillium glabrum	458	426
Pichia kudriavzevii	472	431
Pichia membranifaciens	466	398
Plectosphaerella cucumerina	457	441
Saccharomyces cerevisiae	460	496
Saccharomycodes ludwigii	452	470
Schizosaccharomyces pombe	488	562
Starmerella bacillaris	389	361
Torulaspora delbrueckii	461	518

taxonomic coverage of both LS2-MF and NL4 by using Primer Prospector (Walters et al., 2011) against the constructed 26S databases and SILVA's database.

2.2. Mock community preparation, DNA extraction, and PCR amplification

Strains of yeast and filamentous fungi listed in Table 2 (DISAFA collection, Torino) were used and cultured on Malt Extract Agar (Oxoid, Milan, Italy) plus 25 mg l^{-1} streptomycin (Sigma, Milan, Italy) incubated at 28 °C for 72 h for yeast and 7 to 10 days for fungi. DNA extraction from yeast was carried out from a loopful of grown culture while 250 mg of mycelium was scraped from the plate for filamentous fungi. DNA extraction was carried out as described by Cocolin et al. (2000). DNA from each strain was quantified by using the Qubit dsDNA assay kit (Thermo Fisher Scientific, Milan, Italy) and standardized at 5 ng/μl. A pool (Mock-DNA) containing each of the standardized strain DNA was then obtained and subject to amplification of the ITS2 and the 26S regions. PCR was carried out for the two target regions using a PCR mixture prepared with 12.5 μl of the 2× Kapa HiFi HotStart ReadyMix Taq (Roche, Milan, Italy), 5 μl of each primer (1 μM), 2.5 μl of DNA as a template. Each PCR were subject to the following amplification conditions: thirty cycles of 30 s of denaturation (95 °C), 30 s of primer annealing (55 °C), and 30 s of primer elongation (72 °C), followed by a final elongation step (72 °C) of 10 min.

The amplification of each fungal strain was carried out by using the same couple of primers, each amplicon was then purified using the Agencourt AMPure XP beads (Beckman Coulter Genomics) and quantified using Qubit dsDNA assay kit. Based on the amplicon size of DNA assessed by using a Biorad experion workstation (Biorad, Milan, Italy), amplicons concentration was determined. Amplicons were diluted at 20 mM and aliquots of 10 μl were pooled together to construct a Mock-

Table 1
Primers used for Illumina MiSeq sequencing.

Primer	Features	Primer sequence	Target region	Amplicon length	Reference
ITS3tagmix1	Fwd	5′-CTAGACTCGTCACCGATGAAGAACGCAG-3′	ITS2	385	Tedersoo et al., 2015
ITS4ngs	Rev	5′-TTCCTSCGCTTATTGATATGC-3′	ITS2		Tedersoo et al., 2015
LS2-MF	Fwd	5′-GAGTCGAGTTGTTTGGGAAT-3′	LSU D1	369	This study
NL4	Rev	5′-GGTCCGTGTTTCAAGACGG-3′	LSU D1		Jespersen et al., 2005

Amp. In total, two independent Mock-DNA and Mock-Amp were obtained by two independent DNA extraction, quantification and pooling procedure.

2.3. DNA extraction and PCR amplification of fermented cocoa beans

A total of twenty fermented cocoa beans samples were collected and DNA extracted as following original study (Mota-Gutierrez et al., 2018). Samples were collected during a fermentation period of 0, 48, 96 and 120 h. Detailed information of samples is reported in Mota-Gutierrez et al., 2018 and in supplementary table S1. Briefly, total DNA was extracted from the pellet of cocoa matrix by using the MasterPure Complete DNA & RNA Purification kit (Illumina Inc., San Diego, CA) following the manufacturer's instructions. DNA was quantified by using the Qubit dsDNA assay kit (Thermo Fisher Scientific), standardized at 5 ng/μl and subject to amplification of the two target regions using primers and procedure as described above.

2.4. Library preparation and sequencing

Sequencing was performed for the two target regions and for the three target samples (Mock-DNA, Mock-Amp and cocoa samples). After the first purification step following the Illumina sample preparation procedure, the library was combined with the sequencing adapters and dual indices using the Nextera XT Index Kit (Illumina, San Diego, USA), obtaining the multiplexed paired-end libraries. Individual libraries concentration in nM were calculated based on the size of amplicons by using a Biorad Experion workstation (Biorad) and diluted to 4 nM, denaturated with 0.2 N NaOH and spiked with 20% (v/v) of PhiX. The combination of pool library and PhiX were diluted to 12 pM and paired-end sequencing was performed on the MiSeq platform, using MiSeq Reagent Kit V3 (2 × 250 bp) (Illumina, San Diego, USA), following the standard Illumina sequencing protocol.

2.5. Constructed 26S rRNA sequence database

The construction database of fungal rRNA gene sequence of the 26S gene was used to select primers, which amplify the D1 region of a broad fungal taxa. The sequences were downloaded from the Nucleotide database of the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/nucleotide/; accessed March 07, 2018). The database was constructed using the large subunit rRNA gene sequences, 23.381 sequences were downloaded using diverse taxonomic ID and the query word "26S rRNA". The final constructed database consisted of 4 phyla, 27 classes, 172 families, and 442 fungal strains. Incomplete sequences or sequences with absent taxonomies were removed. Duplicate sequences and sequences that clustered together at 99% of similarity were discarded by using Prinseq and USEARCH respectively (Schmieder and Edwards, 2011). A taxonomy file, matching exactly seven taxonomic levels (root, subphylum, class, order, family, genus and species) was generated from the corresponding taxonomy strings to be compatible with implementation in the NGS analysis pipeline QIIME. Both files were manually curated for accuracy and consistency. Sequences obtained from the constructed 26S database from biological and mock samples were compared using SILVA's database. All sequences identified by D1 domain of 26S rRNA sequence analysis from biological samples and mock communities were compared with our constructed database.

2.6. Bioinformatics

Paired-end reads (2 × 250 bp) were first merged using the FLASH software (Magoč and Salzberg, 2011), with default parameters. Joint reads were further quality filtered (Phred < Q20) using the QIIME 1.9.0 software (Caporaso et al., 2011). Chimeras were then removed with the adopted USEARCH version 8.1 software. Lastly, OTUs were picked at 99% of similarity by means of UCLUST clustering methods (Edgar, 2010) and representative sequences of each cluster were used to assign taxonomy. For the 26S data, each cluster was used to assign taxonomy using the Constructed 26S rRNA gene database and SILVA, while for the ITS dataset the UNITE rRNA ITS database version 2012, by means of the RDP Classifier. Sequences were double-checked using the BlastN search tool (http://www.ncbi.nlm.nih.gov/blast/) to confirm the taxonomy assignment. Cocoa samples datasets (ITS2 and 26S) were rarefied at 10,018 reads after raw read quality filtering, and OTU tables were filtered for OTUs occurring at 1% of the relative abundance in at least 2 samples. While for mock community reads from the two target regions were rarefied at 17,313 reads.

2.7. Statistical analyses

Statistics and plotting were carried out in the R environment (www.r-project.org). Alpha diversity indices were calculated using the diversity function of the vegan package (Dixon, 2007). OTUs table were used to find differences between target regions by Anosim statistical test in R environment. A two-sided permutation test with 999 permutations was performed to compare the OTUs distribution and alpha diversity between the two datasets. Pairwise Kruskal-Wallis Wilcoxon test or one way- ANOVA coupled with the Duncan honestly significant difference (HSD) test were used as appropriate to determine significant differences in alpha diversity or OTU abundance from mock communities and biological samples. Statistical analysis was acquired through the function *aov* through the *stats* package and principal component analysis were plotted using the function *dudi.pca* through the *made4* package using R version 3.3.2.

2.8. Accession numbers

The ITS2 and 26S rRNA gene sequences are available at the Sequence Read Archive of the National Center for Biotechnology Information (NCBI), under the SRA accession number SRP126081 (fermented cocoa samples ITS2) and SRP150401 (fermented cocoa samples 26S and mock sequences data).

3. Results

3.1. In silico performance of 26S primers

We performed an *in-silico* analysis of the 26S primer set against our constructed database and SILVA using Primer Prospector. LS2-MF primer showed the lowest weighted score (Fig. S1A) indicating higher coverage across the database sequences and lower number of mismatches if compared with NL4 (Fig. S1B). Comparing the taxonomic coverage of LS2-MF and NL4 against *Zygomycota*, *Glomeromycota*, *Ascomycota* and *Basidiomycota* sequence, LS2-MF showed the best performance with a coverage higher than 80% for all the phyla except for *Glomeromycota* (Fig. S2A), while NL4 account for the 20% of the coverage against our constructed database (Fig. S2B). Regarding the performance of the primer sets against the SILVA database, the score of the primers was higher compared with our database (data not shown).

3.2. Performance of primers by mock community analysis

A mock community containing twenty fungal species (Table 2) was prepared to validate the performance of the two target regions. The possible effect of the bias introduced by PCR (Mock-DNA) and that of sequencing (Mock-Amp) was then evaluated. Amplicon length of the single species showed little variation when the 26S gene was amplified (461 ± 30 bp) while for ITS2 we observed greater dispersion in size (445 ± 55 bp) (Table 2). Significant difference in mycobiota composition (Anosim statistical test, $P < 0.05$) by using the two target regions or mock communities (DNA or AMP) was observed by Principal
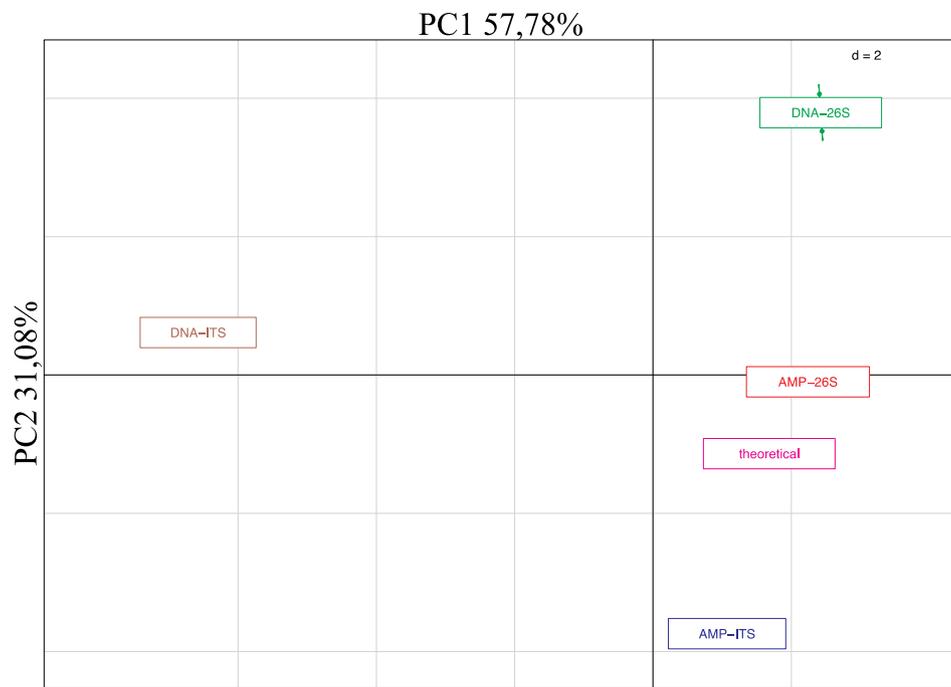
Fig. 1. Principal component analysis (PCA) based on mock mycobiota composition.

Table 3

Relative abundance (%) of the fungal species identified in mock communities amplified using two different target regions. The expected concentration is referred to as theoretical.

OTU	Theoretical	26S				Theoretical	ITS2			
		DNA		AMP			DNA		AMP	
Alternaria alternata	5[ab]	3.62 ±	0.34[b]	5.71 ±	0.47[a]	5[a]	7.67 ±	0.94[b]	6.63 ±	0.09[ab]
Aspergillus flavus	5[ab]	5.78 ±	0.46[a]	4.03 ±	0.07[b]	5[a]	9.39 ±	0.17[b]	7.19 ±	0.26[a]
Aspergillus fumigatus	5[a]	4.82 ±	1.34[a]	3.71 ±	0.09[a]	5[a]	8.41 ±	0.20[b]	6.14 ±	0.01[c]
Candida sake	5[a]	0.24 ±	0.20[b]	0.06 ±	0.02[b]	5[a]	4.57 ±	0.38[a]	6.19 ±	0.03[b]
Fusarium oxysporum	5[ab]	3.99 ±	0.25[b]	5.08 ±	0.22[a]	5[a]	8.11 ±	0.00[b]	6.33 ±	0.04[a]
Fusarium verticillioides	5[a]	6.27 ±	0.04[c]	4.63 ±	0.02[b]	5[a]	10.44 ±	0.12[c]	6.60 ±	0.27[b]
Galactomyces geotrichum	5[a]	13.39 ±	0.61[c]	6.94 ±	0.11[b]	5[b]	0.00 ±	0.00[a]	0.00 ±	0.00[a]
Hanseniaspora opuntiae	5[b]	2.94 ±	0.16[a]	3.46 ±	0.33[a]	5[c]	0.70 ±	0.07[a]	3.67 ±	0.01[b]
Hanseniaspora osmophila	5[a]	10.63 ±	0.56[b]	4.73 ±	0.13[a]	5[b]	1.63 ±	0.34[a]	4.78 ±	0.08[b]
Kluyveromyces marxianus	5[b]	3.68 ±	0.08[a]	3.33 ±	0.16[a]	5[a]	8.55 ±	0.33[b]	6.71 ±	0.08[b]
Penicillium brevicompactum	5[a]	6.00 ±	0.92[a]	3.55 ±	0.29[a]	5[a]	14.39 ±	0.09[c]	9.04 ±	0.08[b]
Penicillium glabrum	5[a]	4.86 ±	0.19[a]	4.88 ±	0.22[a]	5[b]	7.08 ±	0.11[a]	5.69 ±	0.07[b]
Pichia kudriavzevii	5[a]	7.24 ±	1.89[a]	10.76 ±	0.45[a]	5[b]	0.12 ±	0.14[a]	2.11 ±	0.16[b]
Pichia membranifaciens	5[a]	6.63 ±	1.83[a]	4.82 ±	0.28[a]	5[b]	2.58 ±	0.39[a]	3.57 ±	0.13[b]
Plectosphaerella cucumerina	5[b]	1.11 ±	0.13[a]	4.33 ±	0.02[b]	5[b]	3.51 ±	0.62[a]	4.45 ±	0.26[b]
Saccharomyces cerevisiae	5[b]	1.86 ±	0.09[a]	4.20 ±	0.49[b]	5[a]	4.74 ±	0.03[a]	5.44 ±	0.10[b]
Saccharomycodes ludwigii	5[b]	3.51 ±	0.00[a]	4.49 ±	0.00[b]	5[b]	0.68 ±	0.03[a]	4.66 ±	0.03[b]
Schizosaccharomyces pombe	5[b]	5.00 ±	0.11[b]	3.71 ±	0.19[a]	5[b]	0.01 ±	0.00[a]	0.00 ±	0.00[c]
Starmerella bacillaris	5[a]	4.21 ±	1.68[a]	5.91 ±	0.11[a]	5[b]	0.16 ±	0.07[a]	0.19 ±	0.10[b]
Torulaspora delbrueckii	5[c]	2.46 ±	0.02[a]	3.90 ±	0.17[b]	5[a]	5.38 ±	0.11[a]	5.30 ±	0.52[a]

Component Analysis (Fig. 1).

In both samples (Mock-DNA and Mock-Amp), the target region ITS2 showed similar abundances with respect to the theoretical value for two fungal species, namely *Torulaspora delbrueckii* and *Plectosphaerella cucumerina* (Table 3). Similarly, with the 26S target gene, for six species, abundances retrieved from both Mock-DNA and Mock-Amp samples were comparable to the theoretical values (*Aspergillus fumigatus*, *Pichia membranifaciens*, *Pichia kudriavzevii*, *Penicillium glabrum*, *Penicillium brevicompactum* and *Starmerella bacillaris*). Furthermore, for the 26S region, the species *Alternaria alternata*, *Aspergillus flavus* and *Fusarium oxysporum* rendered different abundances in the Mock-DNA and the Mock-Amp but in both samples the values were comparable to the theoretical.

For 18 out of the 20 fungal species tested, the ITS2 region resulted in underestimation or overestimation with respect to the theoretical value in the Mock-DNA, Mock-Amp or both ($P < 0.05$). Four species were significantly overestimated (*A. fumigatus* (439 bp), *F. verticillioides* (415 bp), *K. marxianus* (521 bp) and *P. brevicompactum* (428 bp)) while other 4 were significantly underestimated (*Galactomyces geotrichum* (324 bp), *Hanseniaspora opuntiae* (484 bp), *Schizosaccharomyces pombe* (562 bp) and *Starmerella bacillaris* (361 bp)), in both samples. Interestingly, *G. geotrichum* and *S. pombe* were not detected in any of the

two samples. Nine more species resulted to be significantly different from the theoretical value (either higher or lower) in the Mock-DNA or Mock-Amp sample only (Table 3).

When the 26S region was targeted, 11 out of the 20 species were either underestimated or overestimated in the Mock-DNA, Mock-Amp or both. More specifically, 4 species were underestimated in both types of samples (*C. sake* (467 bp), *H. opuntiae* (435 bp), *K. marxianus* (427 bp), *T. delbrueckii* (461 bp)) and only one (*G. geotrichum* (502 bp)) was overestimated. Five more species were significantly different from the theoretical value (either higher or lower) in the Mock-DNA or Mock-Amp sample only (Tables 2 and 3). It also should be pointed out that we did not observe a clear correlation between amplicon size and over or underestimation.

In the Mock-Amp samples, correct relative quantification was obtained for 13 out of the 20 species targeting the 26S region and for 10 out of 20 species with the ITS2 region. In the Mock-DNA samples, correct relative quantification was obtained for 10 out of 20 species in the 26S region and for 4 out of 20 species targeting the ITS2 (Table 3).

Remarkably, *G. geotrichum* and *S. pombe* were only detected when the 26S region was targeted while *H. opuntiae* was the only species that was consistently underestimated, independently from the target region or sample. Overall, 26S sequencing data aligned better to theoretical abundance values for the fungal species tested than did the ITS2 sequencing data.

3.3. Mycobiota in biological samples

Sequencing of 20 fermented cocoa beans samples collected during a previous experiment, after amplification with the primers ITS2 and 26S showed a mean sequence length of 412 and 390 bp respectively and an estimated sample coverage of 97.73 and 95.87%, respectively (see

Table S1). The 26S target region revealed greater OTU richness compared to the ITS2 region ($P < 0.05$) as shown in Fig. 2. Overall, 20 and 37 fungal OTUs were identified during the fermentations using the primer set ITS2 and 26S, respectively. In addition, we observed differences in length distribution across the two target genes. Histogram of reads length of 26S showed that the higher reads proportion were around 380 bp while for ITS2 we observed a varied distribution of the reads length around 370 bp, 400 bp, 420 bp, and 450 bp (see Fig. S3). Eleven OTUs, namely *Candida jaroonii*, *Candida tallmaniae*, *Fusarium*, *Hanseniaspora*, *Hanseniaspora opuntiae*, *Hanseniaspora uvarum*, *K. marxianus*, *S. cerevisiae*, *Saccharomycopsis crataegensis*, *Torulaspora delbrueckii* and *Pichia pijperi* were detected by both targeting regions (Fig. 3). The relative abundance of several fungal species was significantly different according to the type of amplicon used ($P < 0.05$, Fig. 4), in which significantly higher relative abundance was found for *S. cerevisiae*, *P. pijperi*, and *H. uvarum* ($P < 0.05$) using ITS2 target gene, while *Hanseniaspora* showed higher abundance when using the 26S ($P < 0.05$, Fig. 4).

3.4. Performance of the new constructed 26S database against SILVA

To validate the new 26S database, biological samples and mock communities identified by D1 domain of 26S rRNA sequence analysis were compared with SILVA's database (Quast et al., 2013). Significant difference in mycobiota composition of the two databases was observed in mock communities (Anosim statistical test, $P < 0.05$). In detail, the constructed 26S database assigned successfully the twenty fungal species, while SILVA's database assigned only ten (*A. alternata*, *H. osmophila*, *K. marxianus*, *P. kudriavzevii*, *P. membranifaciens*, *S. ludwigii*, *S. pombe*, *S. bacillaris*, *T. delbrueckii* see Table S2). Interestingly, *S. cerevisiae* was not detected by using the SILVA's database from the mock
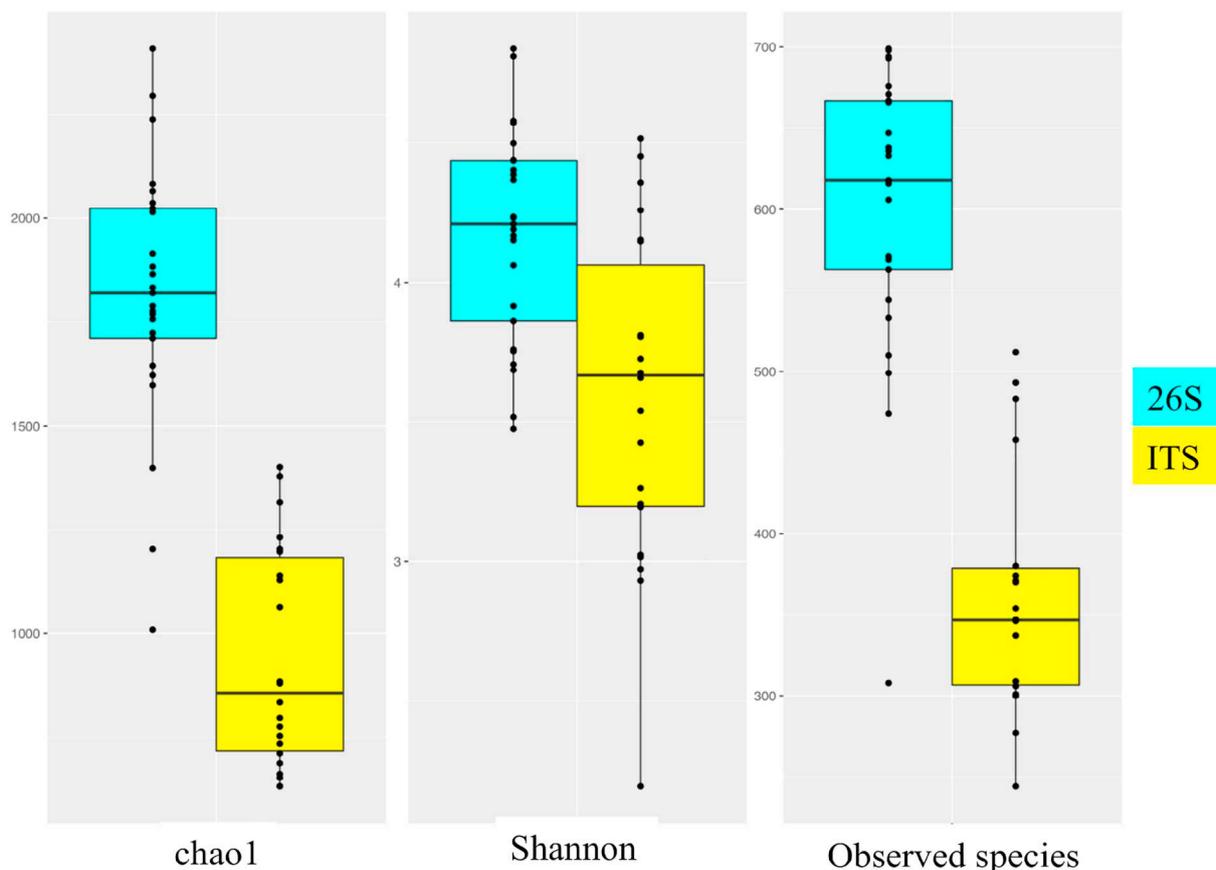

Fig. 2. Boxplotsdescribe α-diversity measures (Chao1, Shannon index and number of observed species) of fermented cocoa bean samples. Individual points and brackets represent the richness estimate and the theoretical standard error range, respectively.
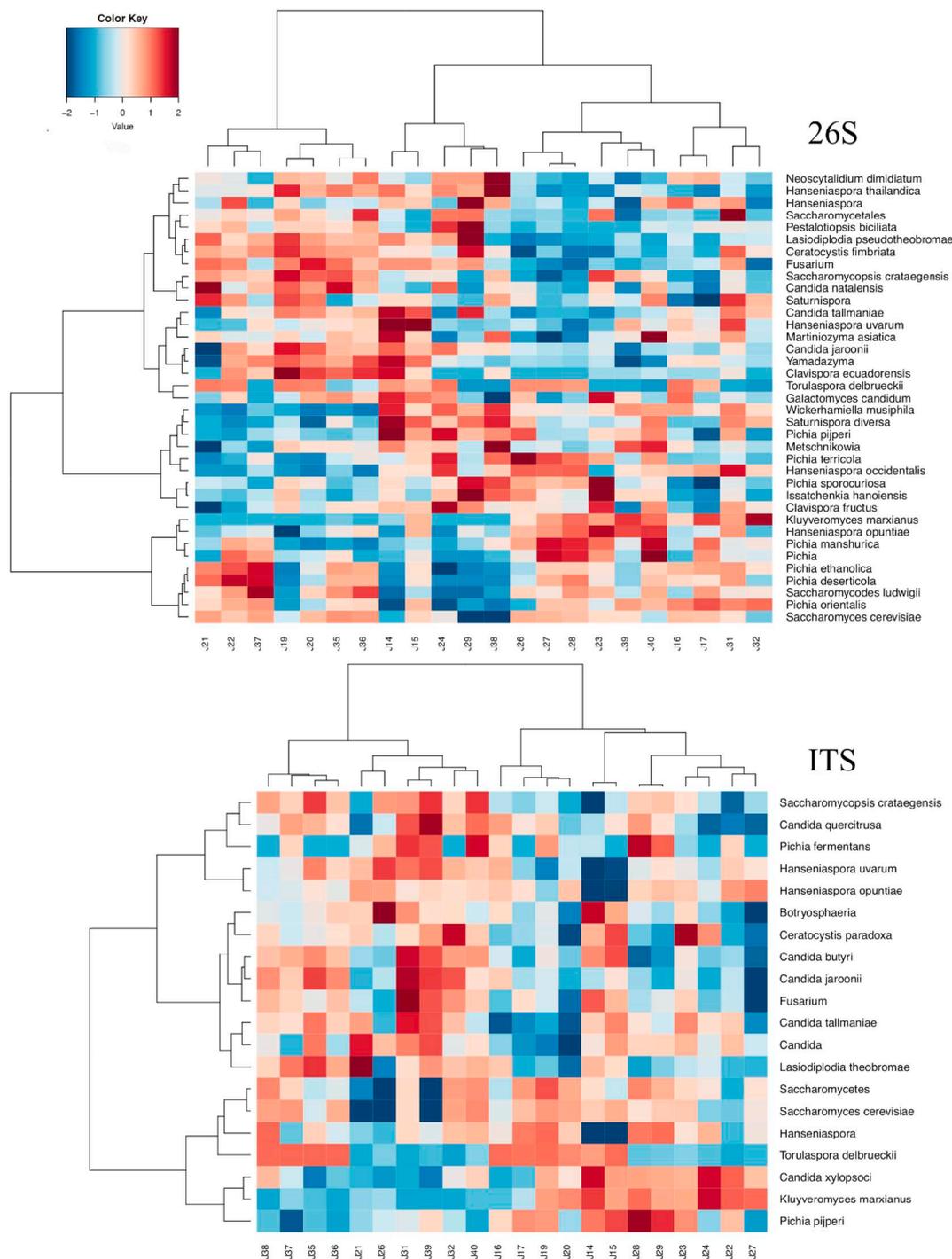
Fig. 3. Distribution of OTUs in fermented cocoa bean samples in the amplicon datasets divided into 26S (upper figure) and ITS2 (lower figure). Only OTUs with an incidence above 1% in at least 2 samples are shown.

communities. In contrast, no significant differences in the OTU distributions and the alpha diversity calculations were observed between the two datasets from biological samples. In depth, the constructed 26S database assigned 37 fungal species, described above, while SILVA's database assigned 35 (data not shown). However, we observed that *S. cerevisiae* and *H. uvarum* were not detected by using the SILVA's database.

4. Discussion

New tools and molecular techniques have been used to detect microbial ecology in the past decades. Recently, the interest in the use of amplicon sequencing to identify taxonomically relevant taxa in food has increased. However, this approach has potential biases as previously described (Bowers et al., 2015; Fouhy et al., 2016) where primer selection is considered one of the most important sources of biases (Bokulich et al., 2014; Bonanomi et al., 2016; David et al., 2014; De Filippis et al., 2017a; Ercolini, 2013; Garofalo et al., 2015; Stellato et al., 2015; Stielow et al., 2015; Wang et al., 2015). The 26S region (D1 domain) of the rRNA encoding gene and the ITS2 region have been proposed as good candidates for identifying fungal species when using NGS technologies due to the high taxonomic resolution (Tedersoo et al.,
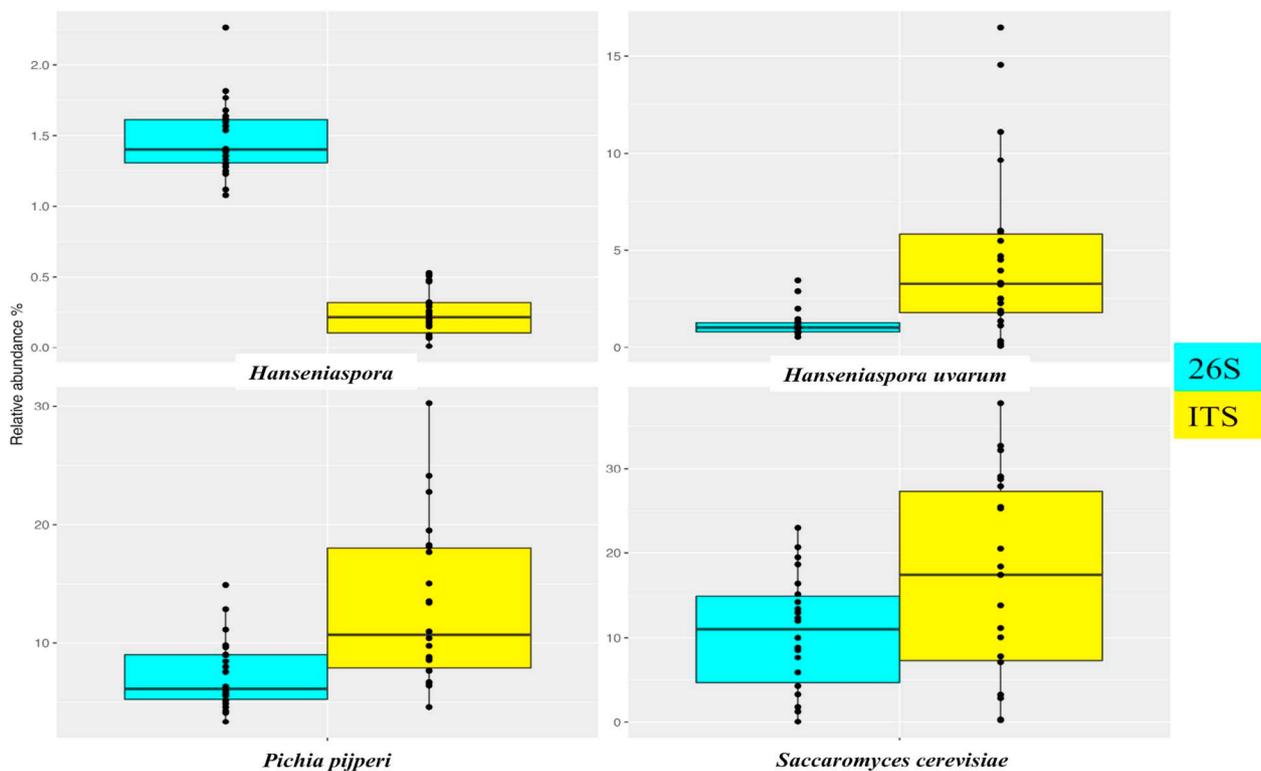
Fig. 4. Boxplots describe statistically different species detected in fermented cocoa bean samples analysed with two different target genes. Individual points and brackets represent the relative abundance and the theoretical standard error range, respectively.

2015). In this study, we performed a comparative evaluation of the two regions as amplicon sequencing targets for the identification of fungi and it also describes the mycobiota community in food matrices.

Recently the 26S region has been studied using the Roche 454 technology (De Filippis et al., 2017b). However, this platform has been shown to result in high sequencing errors due to A and T rich homopolymers (Luo et al., 2012), while Illumina does not present this sequencing error (Erlich et al., 2008). Our results and a previous study (De Filippis et al., 2017b) reveal that 26S gene is a reliable target site for both NGS technologies (Roche 454 and Illumina) for eukaryotic species. In order to evaluate the effect of the target gene used we compared the sequencing results of both biological samples and mock communities. In our study, different relative abundances were obtained for both type of samples and these differences were based on the PCR target used. We observed in such cases that ITS2 target region led to underestimations of species with longer fragments (*S. pombe*, and *H. opuntiae*), while an overestimation of shorter fragments occurred (*F. vercillioides*, *A. fumigatus* and *P. brevicompactum*). However, it should be pointed out that this correlation between amplicon length and estimation is not valid for all the species analysed. Apart from amplification length, other parameters that influence relative abundance calculations of taxa within samples could be considered. Sampling errors, different primers alignment efficiencies during PCR amplification, performance of degenerate primers used during PCR amplification, result in wrong representation in terms of relative abundance of microbial populations (Ihrmark et al., 2012; Polz and Cavanaugh, 1998).

In addition to underestimation of abundances, "identification bias" is also common to amplicon-based analyses, where minor groups are poorly represented (Koljalg et al., 2013). The lack of updated reliable public reference data set and the discrepancies to refer to fungal species have been recently demonstrated for the ITS sequences (Koljalg et al., 2013). This is also in accordance with our results, suggesting that our new database for the 26S, validated by the widely used SILVA, proved to be a curated and rich database to be used. Differences between the two databases regarding taxonomic classification of sequences were

obtained. The newly constructed 26S database delivered a more precise taxonomic assignment of the sequences. This could be due to the fact that SILVA database comprised also non-microbial sequences, incomplete sequences and sequences with unassigned taxonomy. In contrast, each taxonomy in our database was double checked to get the higher taxonomic resolution, obtaining clearly more robustness results in terms of taxonomic assignment from the biological samples. Special attention must be paid on the miss-identification of fungal strain on the current available database. This current issue is pointed out in this study, in which *S. cerevisiae* and *H. uvarum* were misidentified from fermented samples, using SILVA database.

Given the intricate nature of PCR, the amplification of biological samples has been problematic (Polz and Cavanaugh, 1998). Our results exhibited high proportion of fungal coverage (98–96%) by both primer pair sets, which suggested that fungi account for roughly the complete eukaryote rRNA in the studied fermented cocoa beans on average. The results also highlight a lower biodiversity of fungal communities for ITS2 compared with the 26S region in fermented cocoa beans, which contradicts with previous studies where ITS region has been used in NGS studies as the universal primer set for fungi (Bokulich et al., 2012, 2014; Tedersoo et al., 2015). Such discrepancies between outcomes of different studies may arise on account of the biased quantification of relative abundance of taxa due to the uneven length of ITS fragments (Bellemain and Carlsen, 2010), the preferential amplification of rRNA genes for certain taxa by PCR, sequencing bias due to unequal amplification of the target gene or due to inaccurate taxonomic classification of reference databases (Simon and Daniel, 2011). Despite these challenges, the greater recovery trends in the community composition in the 26S target region observed here, have been supported from previous studies, where higher discrimination power of species identification in early diverging lineages of LSU compared with ITS was reported (Schoch et al., 2012).

Our study suggested that the 26S as a target gene showed greater biodiversity in biological samples compared with the universal primer ITS. However, it should be noted that the present study shows the

performance of a new pair of primers targeting the 26S region for fungal strains. Therefore, the novelty of these primer sets is also our limitation, that can be successfully overcome through future research focusing on the use of small fragments of the LSU region to target fungal species, that could support our observations. Therefore, the combination of both target genes, where species identification can be performed applying ITS and phylogenetic analysis with 26S, is highly recommended and the use of both will depend on the purpose of taxa investigation (Klaubauf et al., 2010; Schoch et al., 2012). From a molecular microbial ecology perspective, in terms of classification of marker-gene sequences, there is evidently a need for more extensive testing of primers targeting different genes and *loci*, to support and identify all fungal species in NGS studies. Clearly, the benefits of characterizing fermented microbial diversity may bring important advancements to the food industry, such as discrimination of starter culture to improve food quality or to accelerate processes. This study provides new insight into the selection of better primers and taxonomical assignment to study fungal ecology, which should enable food research to gain a broader view of the microbial diversity present in a range of fermentations avoiding biases. One also notes, the limited availability of updated databases to assess ecological populations.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2018.10.010.

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