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Use of Fragments from D1/D2 Domain of 26S rRNA Gene to Select *Saccharomyces cerevisiae* from Palm Wine

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Author's contribution

The sole author designed, analyzed, interpreted and prepared the manuscript.

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Short Communication

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ABSTRACT

Aims: To determine if the 600 bp PCR amplicon of D1/D2 domain region of 26S rRNA genes in *Saccharomyces cerevisiae* can be used for pre-selection and identification of *S. cerevisiae.*
Study Design: *In vitro* analytical study.
Place and Duration of Study: University of Nottingham, United Kingdom; Study was carried out between March and September 2013.
Methodology: Polymerase chain reaction amplification and restriction digestion using *Hae*III restriction endonuclease.
Results: The restriction profile of *S. cerevisiae* was different from that of *Pichia kudriavzevii*, *Candida ethanolica* and *C. tropicalis*. The profile was the same with known *S. cerevisiae* strains NCYC 1406 and S288c and yielded three fragments of approximately 306,156 and123 bp.
Conclusion: *Hae*III digestion of D1/D2 domain of 26S rRNA gene of *S. cerevisiae* confirms that

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pre-selection and sequencing can be performed with one PCR reaction instead of two in palm wine yeast identification studies.

Keywords: Yeasts; restriction fragments; 26S rRNA; D1/D2 domain; palm wine; Saccharomyces cerevisiae.

1. INTRODUCTION

Palm wine is described as the collective name for a group of alcoholic beverages produced by the natural fermentation of the sap obtained from various tropical plants of the *Palmae* family [1]. The drink is consumed around the world especially in Africa, South Asia and parts of South America [2,3]. The drink plays an important role in the socio-cultural activities of people from south eastern Nigeria because no traditional marriage is complete until the drink is served. The physico-chemical condition of palm wine is a function of the metabolic activities of the inherent yeasts [4] and in the last four years, there has been an increase in the identification and molecular characterization of *S. cerevisiae* from palm wine. Molecular characterization has been carried out on yeast isolates from Burkina Faso [5], Mexico [6] and Nigeria [7].

In the aforementioned studies, investigators carried out yeast species identification with two PCR reactions. First restriction fragment lenght polymorphism (RFLP) analysis of PCR amplified ITS1- 5.8S rDNA-ITS2 regions [8] was performed as a preliminary identification step to pre-select isolates after which NL1/NL4 primers covering the D1/D2 Domain of 26S rRNA [9] were used for sequencing to confirm identities and generate sequences that were submitted to the gene bank for curation. It is known that divergence in the region is generally sufficient to resolve individual species [10]. The NL1/NL4 primers covering this region are now the primers of choice for most researchers working with yeasts and other fungi in many traditional food and drink. The primers have been used in yeast identification [11] from domestic *ragi* (used as a dry starter in food fermentation) and determination of diversity of yeast species during fermentative process contributing to Chinese Maotai-flavour liquor [12]. In other investigations, the primers have been used to perform phylogenetic analysis of biofuel yeasts [13] and investigation of fungal flora in raw milk [14].

S. cerevisiae is the dominant yeast in palm wine from several locations and requires more studies to capture the diversity of the organism, discover novel sub-species and establish the species distribution in palm wine of different regions around the world where the drink is consumed. Identification of new yeasts from palm wine in Africa may lead to the discovery of new strains possessing novel properties like the novel non-conventional yeast *Dekkera bruxellensis*, a distant relative of *S. cerevisiae* that has gained attention in the food industry and academic research because it is a spoilage organism in the wine and bioethanol industry but contributes to the flavour profile of beer [15]. One reason that could slow down these type of investigations in developing countries may be the availabilty of resources, hence a quicker way of selecting *S. cerevisiae* for further studies is desireable. The possibility of using one PCR amplicon for preselection and sequencing has not been explored. Therefore, the objective of this work was to determine if restriction fragments of the amplicon generated by the widely used NL1/NL4 primers can be used to pre-select *S. cerevisiae* from other yeasts found in the palm wine drink.

2. MATERIALS AND METHODS

Eighteen yeast isolates sourced from palm wine of two tree species [7] and 2 reference yeast strains NCYC 1406 and S288c in storage at the Microbial Investigation Center, University of Nottingham, United Kingdom (Table 1) were used. The reference *S. cerevisiae* yeast strains were used as positive control while other yeast strains served as negative control. The set of strains which were under cryo-preservation at -20℃ were allowed to thaw and then grown by streaking cryo preservation beads on Rose Bengal Chloramphenicol agar (CM0549; Oxoid, Basingstoke, UK) prepared with the supplement (SR0078; Oxoid). Colonies that emerged after 72 hours growth at 28℃ were used for DNA extraction.

Template DNA was extracted from yeast colonies with manufacturer's kit (Thermo Scientific, Illinois, USA) after which amplification of the D1/D2 domain was carried out using the NL1/NL4 primers as previously described [8].

Table 1. Yeast strains from palm wine used in this study showing palm tree source

S/n	Accession no	Yeast species	Palm tree source
1.	HG425325	*Pichia kudriavzevii*	*Elaeis* sp.
2.	HG425326	*S. cerevisiae*	*Raphia* sp.
3.	HG425327	*S. cerevisiae*	*Elaeis* sp.
4.	HG425328	*S. cerevisiae*	*Elaeis* sp.
5.	HG425329	*S. cerevisiae*	*Raphia* sp.
6.	HG425330	*S. cerevisiae*	*Raphia* sp.
7.	HG425331	*S. cerevisiae*	*Raphia* sp.
8.	HG425332	*Candida ethanolica*	*Raphia* sp.
9.	HG425333	*P. kudriavzevii*	*Elaeis* sp.
10.	HG425334	*C. tropicalis*	*Raphia* sp.
11.	HG425335	*P. kudriavzevii*	*Raphia* sp.
12.	HG425336	*C. ethanolica*	*Elaeis* sp.
13.	HG425337	*S. cerevisiae*	*Elaeis* sp.
14.	HG425338	*S. cerevisiae*	*Raphia* sp.
15.	HG425339	*S. cerevisiae*	*Raphia* sp.
16.	HG425340	*S. cerevisiae*	*Elaeis* sp.
17.	HG425341	*S. cerevisiae*	*Elaeis* sp.
18.	HG425342	*S. cerevisiae*	*Elaeis* sp.
19.	NCYC 1406	*S. cerevisiae*	Control
20.	S288c	*S. cerevisiae*	Control

Briefly, PCR amplicons were generated with the primers NL1 (5′-GCATATCAATAAGCG GAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACG G-3′) using the following cycling program:- Initial denaturation at 94℃ for 5 min, followed by 30 cycles at 94℃ for 90 s, 53℃ for 30 s and 72℃ for 90 s before carrying out the final extension at 72℃ for 7 min. The unpurified amplified PCR fragments (25 µl) were placed in eppendorfs after which 0.5 µl of *Hae*III enzyme (Promega, Madison, USA) was added. The mixtures were incubated for 3 hours at 37℃ following which the amplicons were viewed in 3% agarose gel. In addition to a 100 bp molecular marker, a gel imaging analysis software (Qingqi Wang, Version 1.10) was used to determine band sizes.

3. RESULTS AND DISCUSSION

In this work, amplification of the D1/D2 domain of 26S rRNA (600 bp) and digestion of the amplicon was carried out with *Hae*III endonuclease. The enzyme was used because of its high discrimination power. Following the digest (Fig. 1), it was found that all the strains of *S. cerevisiae* e.g strain HG425338 (Lanes 3) and strain HG425337 (Lane 7) showed the same profile with positive control *S. cerevisiae* reference strains S288c (Lane 8) and NCYC1406 (Lane1) and produced estimated bands (306+156+123 bp) which was a different profile to other non-saccharomyces yeast *P. kudriavzevii* (Lane 2) and *C. ethanolica* (Lanes 4, 6). This indicates that this method may be used to select *S. cerevisiae* from palm wine. To the best of the author's knowledge, the profile consisting of the specific bands (306+156+123 bp) found in this study have not been reported.

In a previous study Zanol et al. [16] used primers NL1 and L6 to amplify a region of the 26S rRNA from 53 yeast species and obtained a fragment size of 1100-1150 bp that included the D1/D2 domain. Then, the fragment was digested with restriction endonucleases (*Apa*I, *Hinf*I, *Mse*I, *Hae*III and *Cfo*I) in order to differentiate yeast species frequently isolated from grape surfaces, wine and cellar equipments. The restriction enzyme *Hae*III had high discriminatory power and digested all strains including *S. cerevisiae* 252^{NT} and produced 24 restriction profiles overall from the different yeast species tested.

Studies of phylogenetic importance on the large ribosome subunit have made significant progress in the last two decades. Following Van der Auwera et al. [17] study on the structure of the large ribosomal subunit RNA of the fungi *Phytophthora megasperma*, and phylogeny of the oomycetes, sets of primers which allow sequencing and PCR amplification of eukaryotic large ribosomal subunit RNA genes of a wide range of phylogenetically distant organisms were developed and paved way to its application in a wide variety of yeast characterization studies.

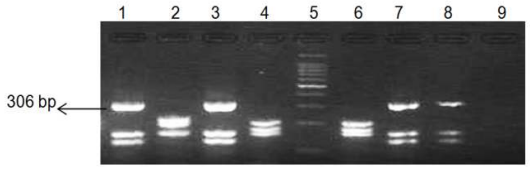

Fig. 1. Restriction fragments of D1/D2 domain of the 26S rDNA using the enzyme *Hae*III. Image shows *S. cerevisiae* strain NCYC1406 (Lane1), *P. kudriavzevii* (Lane 2), *S. cerevisiae* HG425338 (Lanes 3) *C. ethanolica* (Lane 4), 100 bp ladder (Lane 5), *C. ethanolica* (Lane 6), *S. cerevisiae* strains HG425337 (Lane 7) and S288c (Lane 8). PCR reaction negative control (Lane 9)

This led to analysis of the restriction profiles of a PCR amplicon of the large subunit of rDNA (26S rDNA), comprising the D1/D2 region in order to develop a routine methodology to examine wine yeast species [18,15].

Also Dagar et al. [19] presented the suitability of D1/D2 domain of large-subunit ribosomal DNA for differentiation of anaerobic fungi *Orpinomyces joyonii* and *Orpinomyces intercalaris* based on PCR-RFLP and demonstrated adequate heterogeneity in the large-subunit ribosomal DNA for species-level differentiation. In the last 5 years, studies on the amplicon generated with NL1/NL4 primers have increased and more analysis using other yeast species with this method may be beneficial.

4. CONCLUSION

The results obtained from this study revealed that the PCR amplicon generated from the D1/D2 domain of the 26S rDNA investigated can be used for RFLP analysis first to pre-select candidate strains and also for sequencing to confirm the identity of the strain of interest. Adopting such an approach will help save time and materials. A wider study to determine if the method is suitable for selection of a large number of wild yeasts from other traditional drinks still needs to be carried out. Also, further work to determine if the method can differentiate between *S. cerevisiae* and other *Saccharomyces* species like *S. paradoxus, S. uvarum, S. boulardii* and *S. bayanus* will be beneficial.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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