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RESEARCH ARTICLE

YEAST DIVERSITY IN THE INTESTINAL TRACT OF THE FUNGUS-GROWING TERMITE Macrotermes subhyalinus

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Abstract

This study investigated the diversity of yeasts involved in intestinal tract of higher termite *M. subhyalinus* from Côte d'Ivoire. Yeasts were absent from the digestive tract of soldiers and present in the worker termites at a load of 2.1 x 10^5 UFC/mL. Initial molecular identification to the species level was carried out using RFLP of PCR-amplified internal transcribed spacers of rDNA (ITS1-5.8S-ITS4). Three different profiles were obtained from the restriction of PCR products with the seven endonucleases *Hae* III, *Hinf* I, *Pvu* II, *Alu* I, *Xho* I, *BamH* I and *Msp* I. Sequence analysis of the ITS regions allowed us to assign these groups to two different species: *Pichia kudriavzevii* and *Candida tropicalis*. *P. kudriavzevii* was the most commonly species isolated (84.60%). Two genotypic strains were found for this species. The first one (Ia) gave three restriction bands with endonucleases Hae III and Xho I while the second one (Ib) gave two bands and one band respectively with the same enzymes. In addition, these strains showed four and two nucleotides divergence from a reference strains. The sequence data obtained from the PCR products were aligned and compared with other sequences. Homologous sequences of fungi were obtained from GenBank database.

Introduction

Termites are social insects that live in a nest with a structured family organization. They belong to the order Isoptera that are arthropod animals of the class of insects known as "white ants" (klambhampati and Eggleton, 2000). Termites inhabit warm regions of the world and the vast majority live mostly in a particular structure, the mound, which is characteristic of the species (Rouland, 1994). According to the work of Wood and Johnson (1986), known termite species are classified into seven families, namely Mastotermitidae, Kolotermitidae, Termopsidae, Hodotermitidae, Rhinotermitidae, Serritermitidae, and Temitidae. The first six families are collectively known as ''lower termites'' and the seventh family known as ''higher termites''. The main difference between higher and lower termites is that flagellates (protozoa) are present in the gut of lower termites whereas no protozoa were found in higher termites gut (Varma *et al*., 1994**)**. It is also known that higher termites decompose cellulose by using their own enzymes (Ohkuma, 2003).

Termites are small to medium-sized insects that are cryptic in habit. All species live in eusocial colonies. Mature termite colonies contains individuals of remarkably different form and function (Kambhampati and Eggleton, 2000). Each group of individuals that perform the same function is known as a caste. In most species three castes occur: reproductive, soldier, and worker. Immature stages of all castes may also be present in the colony

along with (occasionally) intercastes (Gillott, 2005). Termites are involved in the degradation of complex substances such as cellulose and hemicellulose found in plant (Wood and Sands, 1978; Brown, 1998). These insects have been the subject of many studies because of their importance in tropical environments. These studies relate to their role as ecosystem engineers, their influence on soil properties (Anderson *et al*., 1991; Dangerfield *et al*., 1998; Donovan *et al*., 2001), their contribution to the emission of methane gas (Eggleton *et al*., 1999) and finally their role in the degradation of lignocellulose (Hinze *et al*., 2002).

The digestive tract of termites comprises of several compartments, such as foregut, midgut and hindgut, which the hindgut contains the intestinal microbiota. The most important metabolic activities traditionally attributed to the gut microbiota are, hydrolysis of cellulose and hemicellulose, followed by fermentation of the depolymerization products to short-chain fatty acids, which are then resorbed by the host, and finally, intestinal nitrogen cycling and dinitrogen fixation (Breznak and Brune, 1994; Brune, 1998). The diversity of microbes in the gut of diverse termites includes protozoa, bacteria and fungi (Brune, 2003; Breznak *et al*., 1988). Gut bacteria of termites have been extensively studied. To date, several groups of bacteria are known to inhabit the gut of termite.

In the specific case of the fungus-cultivating termites which belong to the higher termites, the symbiosis between the termite and fungi of the genus Termitomyces is crucial to the digestive process. Despite this symbiosis, the fungus-cultivating termites are also reported to harbor dense populations of bacteria and archaea (Anklin-Mühlemann *et al*., 1995; Brauman *et al*., 2001). Earlier studies involved enumeration of bacterial cells and quantification of metabolite pools in the gut compartments of Macrotermes subhyalinus (Anklin-Mühlemann *et al*., 1995). In another study using group specific probes, *M. subhyalinus* and *M. bellicosus* were also reported to harbor dense populations of bacteria and a smaller population of archaea (Brauman *et al*., 2001). Recently, Hongoh *et al*. (2006) published a comprehensive study of bacterial diversity in *Macrotermes gilvus*. However, little is known about the presence of yeasts in the fungus-cultivating termite guts. Apart from their importance in the nutrition of the termites, these yeasts may also include novel species with potential for exploitation in biotechnology. The present study therefore aims to highlight the diversity of the intestinal yeast community of the higher termite *M. subhyalinus* from Côte d'Ivoire.

Materials and methods

1- Collection of termites

The study was conducted on the fungus-cultivating termites *Macrotermes subhyalinus*, which belong to the higher termites. Termites were collected once from one mound in the savannah of Lamto (Toumodi, central region of Côte d'Ivoire) and Cocody (Abidjan, south of Côte d'Ivoire). Live termites were collected from soil mound and were transported to the laboratory along with mound material. Then they were separated into soldier and worker based on their obvious morphological characteristics. An entomologist from Lamto station identified the termites.

2- Isolation of yeasts

Twenty (20) insects from each caste (worker and soldier) were washed with sterile distilled water, dried on a filter paper and externally sterilized with 70% ethanol. Following the evaporation of ethanol, the hindgut was withdrawn with a sterile forceps under sterile condition. The guts were homogenized in 1 ml of sterile physiological water and serially diluted. Aliquots of 0.1 mL of the appropriate dilution were then plated in triplicate on Sabouraud Chloramphenicol agar medium, followed by incubation at 30°C for 3-5 days. Colonies formed on the agar plates were picked and streaked on fresh Sabouraud Chloramphenicol agar plates for further purification. Pure yeast isolates were obtained by subsequent streaking on fresh Sabouraud Chloramphenicol agar plates.

3- PCR-RFLP identification of yeast isolates

3-1- DNA extraction and purification

For DNA extraction, the yeast isolates were grown on Sabouraud Chloramphenicol medium for 48 hours. A distinct colony was picked and mixed with 1 mL of sterile distilled water. An extraction and purification kit (Instagen Matrix, Bio- Rad, USA) was then used according to the manufacturer's instructions.

3-2- Amplification of the ITS region

Amplification reactions of the 5.8S rDNA -ITS region were conducted under the following conditions: 50 µL of each reaction mixture contained 20 µL of PCR Master Mix 1X (5PRIME Hot MasterMix 2,5X DOMINIQUE Dutscher, France), 1 µL of DNA (about 50 ng), 0.2 µM of each primer (ITS1 and ITS4, Eurofins, France) and PCR water (pure filtered water) to complete the reaction volume to 50 µL. The primers ITS1 (5'-

TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATA-3') described by **White *et al*. (1990)** were used.

Amplification was performed in a thermocycler Thermalcycler 2720 type (AB Applied Biosystems, Syngapore) programmed as follows: initial denaturation at 94°C for 2 min followed by 30 cycles each comprising a denaturation step at 94°C for 1 min, an annealing step at 50°C for 30 s and elongation at 65°C for 2 min. A final extension at 65°C for 7 min marks the end of the PCR reaction.

The products of PCR reaction were separated by electrophoresis on 1% agarose gel. Electrophoresis was performed in 0.5 X TAE at 120 V for 1 h 30 min. Once electrophoresis is completed, the gel was stained in a solution of TAE (0.5X) containing ethidium bromide at a concentration of 1 µg/mL and then washed in distilled water for 5 min. The profiles obtained were visualized under ultraviolet light using a UV plate (UVITEC, UK). The gel was photographed using a camera type ALPHA DigiDoc RT (USA) and printed with a printer Digital Graphic printer (UP-D897 SONY, USA).

3-3- Restriction analysis

The enzymes used for digestion of the amplified DNA are: *Hae* III, *Hinf* I, *Pvu* II, *Alu* I, *Xho* I, *BamH* I and *Msp* I. The reaction mixture (25 µL) was composed of 11 µL of sterile deionized water, 2.5 µL of 10X buffer supplied with the enzyme, 0.5 µL of acetylated BSA 10 mg /L, 1 µL of enzyme 10 U/L (Promega) and 10 µL of PCR product. The digestion was carried out in Eppendorf tubes placed at 37°C water bath for 5 hours. The restriction fragments obtained were separated by size by electrophoresis in a 3% agarose gel (w/v). The gel was then stained 15 min in BET (ethidium bromide), rinsed in distilled water for 5 min and photographed under ultraviolet light using a UV plate (UVITEC, U.K). A photo of the gel was taken with a camera type ALPHA DigiDoc RT (USA) and printed with a printer Digital Graphic printer (UP-D897 SONY, USA) category.

4- Sequencing of the amplified DNA

The amplified DNA was sequenced to determine the taxonomic position of the strains of yeast. Sequencing was carried out on the amplicons obtained after different PCR reactions. Thus, the amplicons were sent to the sequencing platform cochin Eurofins MW operon (France). The nucleotides which were not incorporated during the PCR amplification were removed by filtration of the amplification products. The purified DNA was first quantified by electrophoresis on 0.8% agarose gel (w/v), using a molecular weight marker whose concentration is known. The sequencing reactions were performed using the Sanger method with the automatic sequencing kit ABI 3730xl DNA Analyzers-96-capillary.

The sequences obtained were compared to those of NCBI (National Center for Biotechnology Information) genes bank by using the ' BLAST search option. Percentages of similarity were determined between the isolates and closest sequences listed in GenBank. Sequences are considered similar when they have a percentage of similarity at least 97%.

The set of sequences (from this study or databases) were aligned using the ClustalW program (Thompson et al., 1994) via the interface BioEdit (**Hall, 1999**). This sequence alignment was corrected manually, including the length of the sequences studied. The algorithm for constructing phylogenetic chosen is that of Neighbor -Joining (**Saitou and Nei, 1987**) proposed by the MEGA 5 (**Tamura *et al.*, 2011**) software and software MABL (phytlogeny.fr).

Results

Yeast identification by PCR-RFLP

Yeasts were found only in the intestinal tract of worker termites at a load of 2.1 x 10^5 UFC/mL. These yeasts were isolated for identification purpose.

As shown in figure 1, PCR of the ITS regions revealed two groups of yeasts: those with amplified band size of 500 bp were named group I, and those with amplified band size of 550 bp were named group II. The digestion of the PCR products with the enzymes *Hae* III , *Hinf* I, *Pvu* II , *Alu* I, *BamH* I, *Xho* I and *Msp* I yielded one to three fragments. Figures 2 and 3 show the profiles obtained with *Hae III* and *Msp I*. Fragments ranged in size from 90 to 450 bp for Hae III, from 100 to 500 bp for Xho I, from 200 to 350 bp for Msp I, from 150 to 250 bp for Hinf I and from 400 to 500 bp for Alu I (Table 1). The restriction enzymes Msp I, Hinf I and Alu I confirmed the two groups obtained with PCR. Group I showed fragments size of 250 bp with Msp I, 150 and 250 bp with Hinf I and 400 bp with Alu I while group II showed fragments size of 200 and 350 bp with Msp I, 250 bp with Hinf I and 500 bp with Alu I. The restriction enzymes Hae III and Xho I allowed to distinguish two sub-groups within the group I. these sub-groups were named Ia and Ib. group Ia showed fragments size of 100, 150 and 250 bp with Hae III and Xho I while group Ib showed fragments size of 100 bp, 400 bp with Hae III and 500 bp with Xho I. Yeasts from group Ib were the most frequent isolates found in the intestinal tract of *M. subhyalinus* worker (61.53% of isolates) followed

by yeasts from group II (23.07% of isolates) and group Ia (15.38% of isolates). One of the three different PCR-RFLP profiles (group II) was identified to the species level after comparing the molecular mass of the restriction products with those previously described in the literature (Fernandez-Espinar et al., 2000; Llanos et al., 2004; Jeyaram et al., 2008.). This group corresponded to *Candida tropicalis*. The other PCR-RFLP profiles (groups Ia and Ib) were not found to correspond to any of the species reported by previous studies.

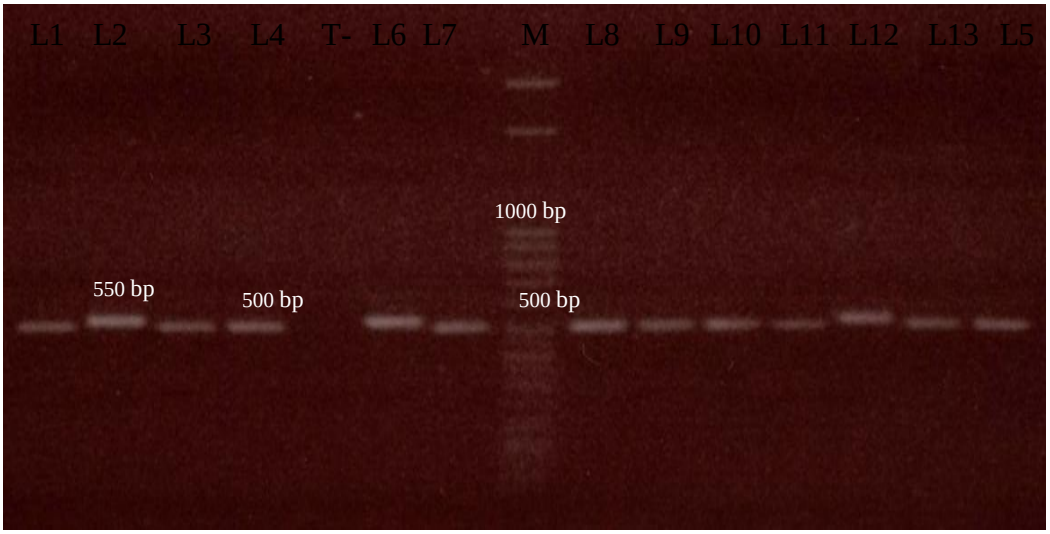

Figure 1: PCR profile of ITS region of yeasts isolated from the intestinal tract of *Macrotermes subhyalinus* worker.
L1, L3, L4, …… L13: Yeast isolates; T-: negative control; M: 100 bp ladder molecular size marker

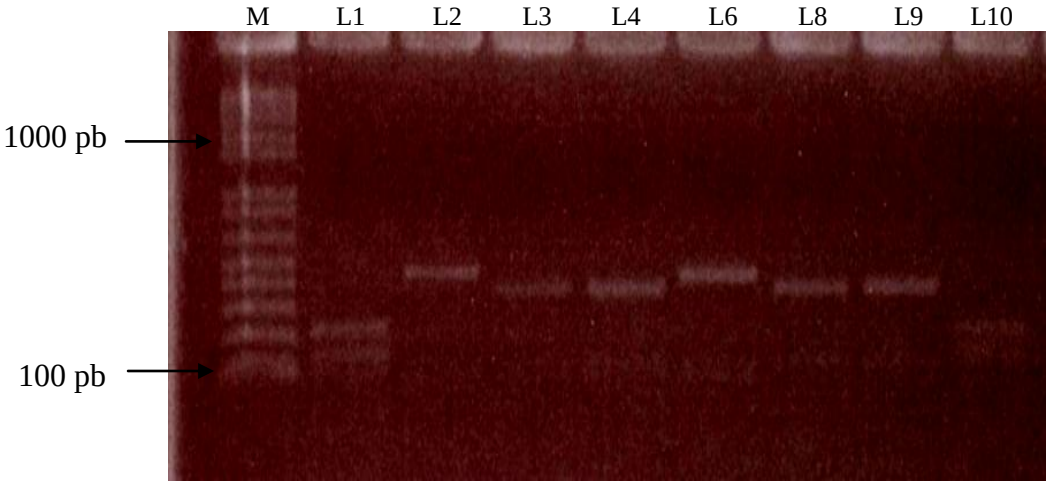

Figure 2: Restriction profile of ITS region obtained with the enzyme *Msp I* for yeasts isolated from the intestinal tract of *Macrotermes subhyalinus* worker.
L1, L3, L4, L8, L9 and L10: isolates from group I; L2 and L6: isolates from group II; M: 100 bp ladder molecular size marker

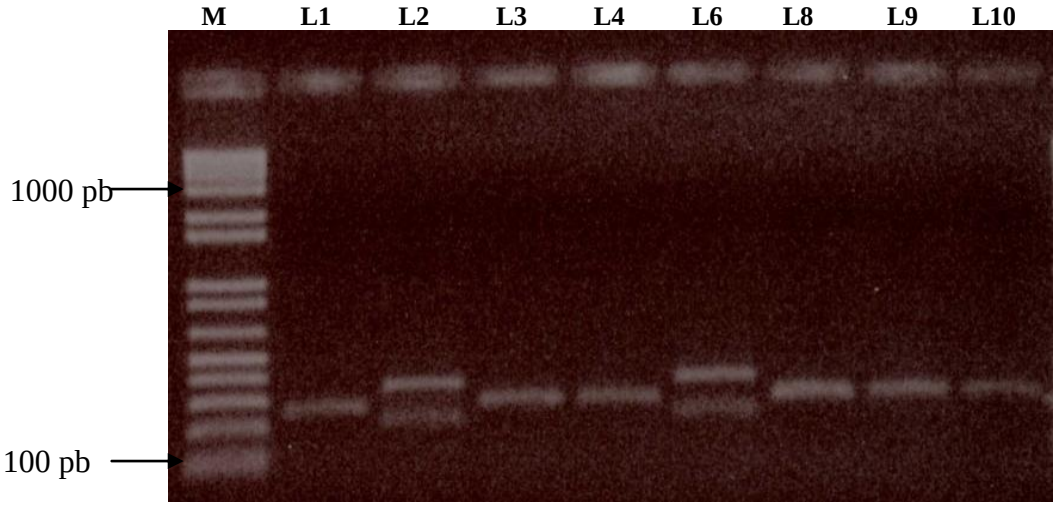

Figure 3: Restriction profile of ITS region obtained with the enzyme *Msp I* for yeasts isolated from the intestinal tract of *Macrotermes subhyalinus* worker.
L1, L3, L4, L8, L9 and L10: isolates from group I; L2 and L6: isolates from group II; M: 100 bp ladder molecular size marker

Yeast identification by sequencing

In order to confirm the identity of group II and to find identity of the unknown groups, representative strains of each profile were identified by sequencing of the 5.8S rDNA-ITS region. As it can be seen in Table 2, the size of the sequences obtained for all the strains was ranged between 450 and 512 bp. Table 2 shows also the homology between the nucleotide sequences of the yeast strains obtained after sequencing and those available in the Genbank database. Thus, sequences of groups Ia and Ib showed 99% homology respectively with *Issatchenkika orientalis* WM 03.103 (synonymously known as *Pichia kudriavzevii*) and *Pichia kudriavzevii* ATCC 6258 in the database. Group II showed 98% homology with *Candida tropicalis* ATCC 750.

The sequences of yeast species identified in this study have been deposited in the GenBank database of NCBI under KF806463 numbers (group Ia), KF806465 (group Ib) and KF806464 (group II) and may serve as a reference in the context of identification of novel strains of yeast. These sequences led to the realization of a phylogenetic tree.

The phylogenetic tree constructed from multiple sequence alignments of the 5.8S rDNA-ITS region of the three groups of isolated yeasts and yeast sequences present in GenBank is shown in Figure 4. This phylogenetic tree led to yeast species following: *Candida tropicalis* 18S, *Candida tropicalis* ATCC 750, *Candida tropicalis* E80, *Issatchenkia orientalis* WM 03 103 and *Pichia kudriavzevii* ATCC 6258. Thus, groups Ia and Ib correspond to *Pichia kudriavzevii*, with bootstrap values of 100% each, whereas the yeasts belonging to group II correspond to the species *Candida tropicalis*.

Table 1: Length in bp of the PCR amplified products of 5.8S rDNA-ITS region and ITS-RFLP obtained with seven restriction endonucleases of yeasts isolated from intestinal tract of *Macrotermes subhyalinus* worker

ITS-RFLP group*	**ITS size (pb)**	**Restriction fragments size (bp)**							**Corresponding species ****	**Percent of isolation**
		***Hae* III**	***Xho* I**	***Msp* I**	***Hinf* I**	***Pvu* II**	***Alu* I**	***BamH* I**		
Ia	500	250 150 100	250 150 100	250	250 150	500	400	500	ND	15.38
Ib	500	400 100	500	250	250 150	500	400	500	ND	61.53
II	550	450 90	500	350 200	250	500	500	500	*Candida tropicalis*	23.07

* The Roman numeral indicates the group and the letters a and b indicate the subgroups from the same group

** Yeasts were identified to species level by comparison of pattern obtained for each group with the pattern of the strains described by Fernandez-Espinar et al, 2000.; Llanos et al, 2004.; Jeyaram et al., 2008.

ND: Not Determined

Table 2: Yeasts isolated from the intestinal tract of *Macrotermes subhyalinus* worker nucleotides of the ITS region identity percent compared to the sequences in GenBank

ITS-RFLP group	Genbank corresponding species	Number of nucleotide compared	Number of nucleotide divergence	Percent of identity
***Ia (KF806463)**	*Issatchenkia orientalis* WM 03.103 (EF568013)	450	4	99
Ib (KF806465)	*Pichia kudriavzevii* ATCC 6258 (KC601852)	471	2	99
II (KF806464)	*Candida tropicalis* ATCC 750 (AY939810)	512	7	98

* The Roman numeral indicates the group and the letters a and b indicate the subgroups from the same group; brackets, the accession numbers of the species.

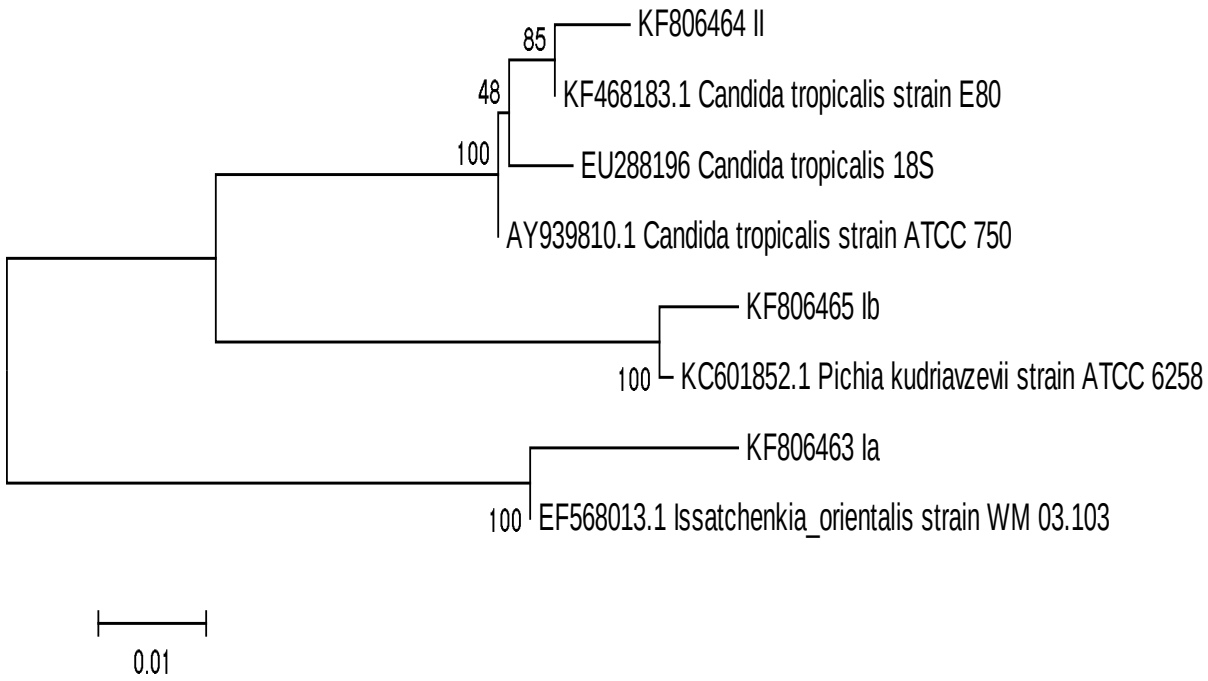

Figure 4: Phylogenetic tree of yeasts isolated from the intestinal tract of *Macrotermes subhyalinus* worker according to sequences of the 5.8S rDNA-ITS region (dendrogram produced with MEGA 5 program by the model of Neighbor-joining with a bootstrap value of 100).

Discussion

Yeasts have been isolated and identified from lower termites many times. In fact, Prillinger et al. (1996) isolated strains from six species of lower termites. These strains were assigned to the genera *Candida*, *Debaryomyces*, *Pichia* and *Sporothrix*. Molna et al. (2004) identified the species *Trichosporon mycotoxinivorans* sp. Nov. from the hindgut of *Mastotermes darwiniensis* (Mastotermitidae). For higher termites, up until now, most microbial investigations have focused on bacteria and the association between *Termitomyces*, a mycelial *Basidiomycete* genus, and some higher termites in the Macrotermitinae (Aanen et al., 2002; Rouland-Lefevre and Bignell, 2002). By contrast, very little is known about yeasts. Here, we report yeasts in the intestinal tract of *M. subhyalinus*. The absence of yeasts in soldiers and their presence in workers may indicate that yeasts play an important role in the diet of *M. subhyalinus* worker. Indeed, it was suggested that the enzymes produced by termite associated-yeasts aid in the digestion and detoxification of the diet ingested by their hosts and/or the yeasts directly provide essential nutrients to the hosts, such as amino acids, vitamins, and lipids (Dowd, 1992; Vega and Dowd, 2005). Schafer et al. (1996) and Wenzel et al. (2002) reported that some yeast isolates were able to hydrolyze cellulose or xylan.

Yeast count found in *M. subhyalinus* worker was about 2.1 x 10^5 UFC/mL. This count is lower than bacteria count reported by Anklin-Mühlemann et al. (1995). These authors revealed a bacterial density of 10^9-10^{11} cells/µL in the intestinal tract of *M. subhyalinus* worker. The negligible quantity of yeasts is the main argument for scientists who state that yeasts harbouring the digestive tract of animals have only minor importance for the host. Nevertheless, yeasts may be of physiological relevance, even though they are present to a much lesser extent than bacteria. In fact, yeasts could provide a relevant biomass, as they have a cell volume 30- to 100-fold higher than bacteria (Gatesoupe, 2007). Commensal yeasts may interact with intestinal bacteria and due to this interplay affect microbial diversity and host organism (Urubschurov et al., 2011).

The molecular methods based on PCR-RFLP of the ITS region of rDNA and sequencing of the ITS regions were used to identify yeasts isolated from the intestinal tract of *M. subhyalinus* worker. PCR-RFLP was used as very efficient tools in many cases of yeasts identification (Suarez et al., 2007; Jeyaram et al., 2008). This method is mostly used because it is fast and simple (Guillamón et al., 1998; Esteve-Zarzoso et al., 1999). However the restriction enzymes used vary from one author to another. A lot of restriction enzymes allow more comparison. But often, in this number, few enzymes really permit to make a distinction between the species (McCullough et al., 1998). In this study, we chose the most used (*Hae* III, *BamH* I and *Hinf* I) and four other (*Xho* I, *Msp* I, *Pvu* II and *Alu* I). The ITS profile showed three groups (Ia, Ib, II) among yeast isolates. These three groups were confirmed by the sequencing of the ITS regions. Indeed, the subgroup Ia showed four nucleotides divergence from *I. orientalis* WM 03.103 (now known as *P. kudriavzevii*) while the subgroup Ib showed two nucleotides divergence from the type strains *P. kudriavzevii* ATCC 6258. This result may indicate that there are two genotypic strains of *P. kudriavzevii* in the intestinal tract of studied termites.

All the two species identified belonged to ascomycetous yeasts and *P. kudriavzevii* was the most commonly species isolated (84.60%). Yeasts from this phylum are considered as ubiquitous microorganisms, which can be found in a vast variety of different ecological systems associated with terrestrial and underwater flora and fauna (Rosa and Peter, 2006). De Almeida et al. (2012) also reported *P. kudriavzevii* as the most frequent yeast species in the rumen fluid of dairy cattle. In the intestinal tract of termites, this yeast might be involved in the utilization of xylose, a subunit of the plant cell wall carbohydrate hemicellulose, as its genome contains genes encoding enzymes involved in xylose utilization and the pentose phosphate pathway for bioethanol production (Chan et al., 2012). This species is also a potential producer of phytases, enzymes useful in food processing and agriculture. So it has been isolated from food and fruit sources, such as sourdoughs (Meroth et al., 2003), fermented butter-like products (Ongol and Asano, 2009), the starter culture of Tanzanian fermented togwa (Mugula et al., 2003), the African fermented cassava lafun (Padonou et al., 2009), a Ghanaian fermented cocoa bean heap (Daniel et al., 2009), fermented pineapple juice (Chanprasartsuk et al., 2010), orange juice (Arias et al., 2002), and grape (Zott et al., 2010). Furthermore, *P. kudriavzevii* showed excellent antibacterial activity against several pathogens of human health significance such as *Escherichia coli*, *Enterococcus faecalis*, *Klebsiella* sp., *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Pseudomonas alcaligenes* (Bajaj et al 2013), a property which could confer selective advantages to the insect host against possibly parasitic or pathological bacterial species.

The relatively small number of yeast species isolated from the termites investigated (two species) may indicate either a limited biodiversity in the intestinal tract of the workers, or could be due to the difficulty of cultivating many species outside of the insect host. It has been shown that a newly isolated yeast endosymbiont associated with the wood-louse *Armadillidium vulgare* (Latr.) has an absolute requirement for exogenous siderophores, which are produced by some common soil fungi (Thanh et al., 2000). As a result, this ascomycete (labelled wood-louse symbiont) can be cultivated only on synthetic media supplemented either with filtrates from certain fungal cultures or with ferric cloride ($FeCl_3$). Analogously, the presence of growth factors produced either by the host insect itself or

by other microbial endosymbionts may be necessary in order to elucidate the entire yeast microflora of this habitat. In addition, oxygen low concentration in the termite hindgut may explain the small number of yeast species isolated. Redox potentials ranging from -230 to -270 mV were registered, i.e., practically anaerobic conditions were revealed by this method (Breznak, 1983).

Conclusion

In this study, using various molecular techniques, we were able to study yeast diversity in the intestinal tract of higher termites *M. subhyalinus*. Only two species were found: *P. kudriavzevii* and C. *tropicalis*. *P. kudriavzevii* was the most commonly species isolated with two genotypic strains. This species apparently contains some neat enzymes which make it very important in the nutrition of the termites and in biotechnology for processes such as the production of bioethanol and phytases used to increase the uptake of phosphorus by plants (biofertilizer). So further investigations should be conducted on these strains for their potential use in biotechnology.

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