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Journal of Advanced Scientific Research

Available online through http://www.sciensage.info

ISSN
0976-9595
Research Article

ANALYSIS OF SYMBIOTIC MICROBIAL COMMUNITY IN THE GUT AND FUNGUS COMB OF *ODONTOTERMES LONGIGNATHUS* HOLMGREN BASED ON CULTURE DEPENDENT APPROACH

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ABSTRACT

Termites are ecologically important arthropods that mediate carbon turnover and maintain soil fertility in terrestrial ecosystems. They owe their ability to degrade lignocellulose to the microorganisms involved in an obligate symbiotic relationship with them. Higher termites have robust prokaryotic gut populations but do not possess eukaryotic gut protists. Higher termites belonging to Subfamily Macrotermitinae depend on fungal symbionts cultured on special structures of termite-faecal origin known as fungus combs. Culture-dependent analysis of the microbial communities in the gut and fungus comb of the fungus-cultivating termite *Odontotermes longignathus* revealed that the termite gut lacked cellulase producing bacteria. However the bacterial and fungal isolates from the termite fungus comb predominantly belonging to the genus *Bacillus* and division Ascomycota respectively exhibited cellulase activity, suggesting their role in degrading cellulose, the major constituent of the termite diet. Yeast strains were also isolated from the fungus comb. The present study asserts the importance of the fungal symbionts in cellulose degradation within the Fungus combs and the role of yeast and bacterial species in the Fungus combs aiding the fungal potential.

Keywords: *Odontotermes longignathus*, Termite gut microbiota, Fungus comb, Yeast, Bacteria, Fungus

1. INTRODUCTION

Termites are one of the most abundant and ecologically important eusocial insects belonging to the order Blattodea that mediate carbon turnover and maintain soil fertility in terrestrial ecosystems. Like ants, bees, wasps and some aphids, termites are highly social insects that live together in a rigid caste system within the same nest or colony. Among the insects, termites have achieved an outstanding ecological success, with more than 3000extant species in 281 genera, 14 subfamilies and eight families' globally of which about 300 species within seven families have been reported in India [1]. Termites provide major ecosystem services as they play important role in organic decomposition, recycling of wood materials and floral remains, enhancing soil fertility and are potent in converting lignocellulose into industrially valuable biofuels [2]. The enhanced ability of termites to degrade lignocellulose evolved from the mutualistic symbioses with their resident gut microbiota [3-5]. Unlike most other insects, termites have complex and distinctive gut microbial communities responsible for major metabolic processes, including degradation of lignocellulose, homoacetogenesis and nitrogen fixation and recycling [6].

The gut tracts of lower termites are relatively simple and harbor a dense microbial community composed of morphologically diverse prokaryotes and cellulose fermenting flagellate protozoa. On the other hand, higher termites have robust prokaryotic gut populations but do not possess eukaryotic gut protists.A major evolutionary transition took place when higher termite (Termitidae) ancestors adopted a more complex diet than the strictly wooden one, precipitating a cascade of changes in the family. Termites in the subfamily Macrotermitinae, which currently spans over 11 genera and 330 species [7], displays the most sophisticated lifestyle by engaging in an obligate symbiotic relationship with basidiomycete fungi of the genus *Termitomyces* [8]. The termites cultivate their fungal crop on special structures of termite-faecal origin known as Fungus combs, which are built in designated chambers inside the colony. The fungal array provides the termites with nutritious diets from nodules packed with conidia spores and nutrients, in turn, the termites maintains the fungi by continuously providing blended plant material, optimal

growth conditions, and lifetime maintenance [9]. Throughout the termite colony lifecycle, the Fungus combs contribute markedly to lignocellulose degradation. Its precise role, however, in plant substrate breakdown is still debated and may vary between different fungus-growing termite genera [10]. The most speciose genera of fungus-growing termites are *Macrotermes*, *Odontotermes* and *Microtermes* [11].

The presence of microfungi also has been detected in the Fungus combs. Members of the class *Sordariomycetes* in the division *Ascomycota, Pestalotiopsis maculans* and *Xylaria*, both in the order *Xylariales,* have been detected growing on the fungus comb [12]. The bacterial communities harbored in the Fungus combs of the fungus growing termite *Odontotermes formosanus* contributed to cellulose-hemicellulose hydrolysis, gut fermentation, nutrient production and the breakdown of the fungus comb [12]. The bacterial isolates predominantly belonging to the genus *Bacillus* (Phylum Firmicutes) apart from lignocellulosic degradation, also suppressed the growth of the microfungus *Trichoderma harzianum* (genus Hypocrea), which grew voraciously in the Fungus combs in the absence of termites [12]. These in vitro studies suggested that *Bacillus* sp. may function as mutualists in the termite gut fungus comb microbial ecosystem.

Termites harbour a distinctive gut microbiota shaped through diet and co-evolution with their hosts [6]. It is estimated that 10^6 to10^7 microorganisms per µl gut volume reside in the 2-3 mm of a termite gut [13-14]. These microbial communities are transmitted vertically from older individuals to new offspring. Compared to bee and ant gut microbiotas, termites show a higher degree of complexity in their gut microbial communities [11-15]. The adoption of fungal symbionts by Macrotermitinae markedly rearranged their gut microbiota compositions [16-18]. Several cloning-based studies first reported that some *Odontotermes*, *Macrotermes* and *Microtermes* species harbour distinct gut microbial communities compared to other termites [19-20]. Moreover, macrotermitine guts contain an assemblage of gut bacterial communities that are more similar to ancestral cockroach guts than to non-fungus-growing termite microbiotas [11, 16].

Apart from harbouring bacteria that target the fungal cell wall, gut metagenomics in two fungus-growing termite species, *Odontotermes yunnanensis* and *Macrotermes natalensis*, suggest a shift in carbohydrate degradation capacities to target less complex polysaccharides compared to other termite taxa. This fits with the hypothesized process of diet decomposition in fungus-growing termites: Fungus is the main decomposer of complex polysaccharides in the fungus combs, whilst termite gut microbiotas degrade the simpler polysaccharides [21-22]. Proteobacteria, Spirochaetes, Synergistetes, Planctomycetes, Deferribacteres and Actinobacteria have been identified from macrotermitinae gut microbiota [8]. The present study is an attempt to isolate and identify cultivable bacterial strains from termite gut microbiota and bacterial as well as fungal colonies from the Fungus combs. The identified bacterial and fungal isolates were assessed qualitatively for their hydrolytic enzyme potential.

2. MATERIAL AND METHODS

2.1. Collection and identification of the termite

The adult specimens of termite were collected from termitarium from Cherupuzha, Kannur district, Kerala, India (Lat – Long: 12.2728 N 75.3672 E). The preliminary morphological identification was done by using authentic identification keys and guides [23]. The Fungus combs from the termitarium were aseptically collected in polypropylene tubes in icebox and frozen at -20°C for the microbial analysis. Molecular identification of the termite based on the mitochondrial cytochrome oxidase subunit I (COI) gene sequencing approach was performed to supplement and support morphological identification [1]. The termite specimens were washed in running water and then 2-3 times in distilled water. The COI gene in the isolated genomic DNA was amplified separately using the specific set of PCR primers developed by Folmer [24], Fom F 1498 forward (5'-GGTCAACAAATCATAAAGATATTGG-3') and Fom R 2190 reverse (5'-TAAACTTCAGGGTGACCAAAAA ATCA-3'), which are considered the universal primers for the amplification of the 658 bp region located at the upstream of the COI gene [25]. The PCR purified amplicon was sequenced from both ends using the Sanger's dideoxy chain termination sequencing method [26] with ABI 3730XL automated sequencer. The sequences were analyzed for the gaps and nonsense codons, and aligned manually by using Bioedit software [27]. The sequences that are conspecific and congeneric were taken from NCBI GenBank for further phylogenetic analysis with Neighbour joining method in MEGA6 software [28].

2.2. Isolation of cultivable bacteria from termite gut microbiota and Fungus combs

Twenty worker termites were surface sterilized with 70% ethanol and degutted using sterile forceps [29]. The dissected out gut was homogenized with saline solution in glass tissue homogenizer and further dilution was made from this up to 10^{-3}. 0.1ml of inoculum from 10^{-1} dilution was spread plated on nutrient agar medium. The plates were incubated at 28±2°C for 24 hours. The collected Fungus combs sample from termitarium was homogenized and serially diluted (10^{-1} to 10^{-3}). The samples were spread plated on to nutrient agar plates. The plates were incubated at 28±2°C for 24 hours. The individual colonies were picked from the plate, purified by quadrant streaking and transferred to nutrient agar slants for further studies.

2.3. Isolation of cultivable fungi and yeast from the termite Fungus combs

Portions of the fungus comb were homogenized and serially diluted (10^{-1} to 10^{-3}) and spread plated onto Sabouraud dextrose agar (SDA) [30]. The plates were incubated at 28±2°C for 72 to 120 hours. Individual colonies were selected and purified on separate SDA agar plates and incubated at 28±2°C for 72- 120 hours.

2.4. Molecular identification of the isolated bacteria and fungi

DNA from bacterial and fungal strains were isolated from 18 hour old pure culture using genomic DNA isolation kit (Mo Bio Laboratories) following manufacturer's instructions. The concentration and purity of total DNA isolated fromthe termite gut and fungus comb were measured using a NanoDrop-1000 spectrophotometer (NanoDrop Technologies Inc.). The DNA was then purified using DNA purification kit (Qiagen) to remove the humic acid contaminants.

For bacterial identification, isolated genomic DNA was used as template in the PCR amplification for 16S rRNA gene using the universal primers 27 forward (5'-AGAGTTTGGATCMTGGCTCAG-3') and 1492 reverse (5'-CGGTTACCTTGTTACGACTT-3'). For yeast and fungal identification, the nuclear ribosomal ITS region comprising internal transcribed spacer 1 (ITS1), 5.8S and internal transcribed spacer 2 (ITS2) was amplified using standard primers ITS1 forward (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 reverse (5'-TCCTCCGCTTATTGATATGC-3').

The gel purified PCR products were then sequenced following Sanger's dideoxy chain termination method with AB1 3730XL Automated sequencer (Sanger and Coulson, 1975). The obtained forward and reverse sequences were trimmed and aligned by using Clustal W. The consensus thus obtained was taken for searching similarity with other sequences in NCBI database using the BLAST tool [31].

2.5. Enzyme assays of the isolated and identified bacterial, yeast and fungal strains

The bacterial, yeast and fungal isolates were qualitatively tested for the production of enzymes including cellulase, lignin peroxidase, laccase, chitinase, pectinase and protease using media CMC agar, Crawford's agar, 0.01% Guaiacol agar, 5% Chitin agar, Pectin agar and 2% Casein agar respectively. The plates were incubated at 28±2°C for 24 to 48 hours. The presence of a halo zone or clear zone around the colonies was considered as positive.

3. RESULTS

3.1. Host termite, *Odontotermes longignathus* Holmgren, 1914

The termite specimen collected for the present study was identified morphologically using authentic identification keys up to the genus level as *Odontotermes* [23]. Molecular identification was done using mitochondrial COI gene sequencing for the confirmation of its taxonomic identity as *Odontotermes longignathus* Holmgren, 1914 and the obtained final sequence was submitted in NCBI GenBank (Accession No: MN 205551) for public accession. The phylogenic status of the collected specimen was also inferred with Neighbour joining method in MEGA6 software (Fig. 1).

3.2. Bacterial community structure analysis by 16S rRNA sequencing

A total of 8 bacterial isolates from gut and 5 bacterial isolates from the Fungus combs of *O. longignathus* were obtained by culture-dependent approaches. The isolated bacterial strains were identified using 16S rRNA gene sequencing method followed by BLAST in NCBI database (Table 1).

3.3. Yeast and fungal community structure analysis by ITS sequencing

A total of 7 yeast strains and 3 fungal strains were isolated from the Fungus combs of *O. longignathus* termitarium by culture-dependent approaches. The isolated strains were identified by sequencing ITS region (Table 2).

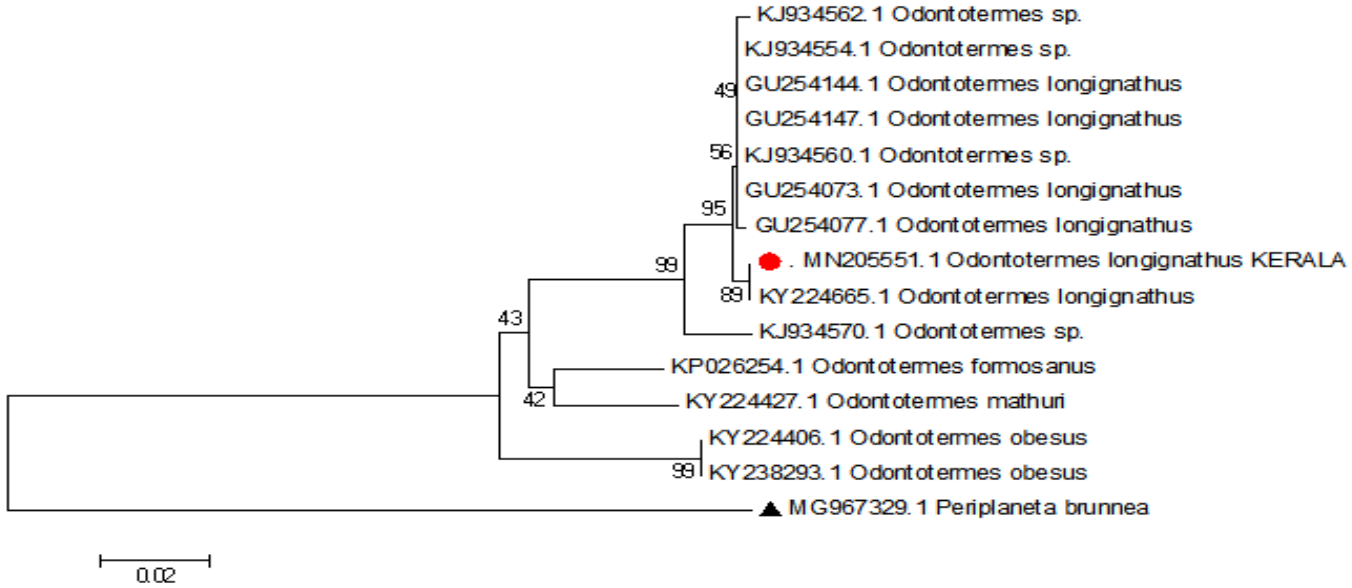

Fig. 1: Phylogenetic similarity of *Odontotermes longignathus* KERALA (MN 205551) of the present study with closest members in the NCBI GenBank constructed by Neighbour joining method

Table 1: Bacterial strains obtained from gut and Fungus combs of *O. longignathus* by aerobic culture-dependent approaches with the percentage of 16SrRNA sequence similarity to the nearestneighbor accessed from NCBI database

Source	Bacterial strain	GenBank Accession No.	BLAST sequence similarity
Termite gut	*Achromobacter xylosoxidans* AAJ -03	MN 197738	100%
	Micrococcus yunnanensis AAJ -04	MN 197740	99.88%
	Serratia marcescens AAJ -05	MN 197739	99.86%
	Serratia marcescens AAJ -10	MN 197741	99.79%
	Serratia marcescens AAJ -11	MN 197743	99.91%
	Serratia marcescens AAJ -14	MN 197744	99.34%
	Serratia marcescens AAJ -21	MN 197745	99.23%
	Serratia marcescens AAJ -23	MN 197746	99.41%
Fungus combs	*Bacillus cereus* AAJ –F1	MN 229571	100%
	Bacillus subtilis AAJ –F2	MN 229572	99.78%
	Bacillus flexus AAJ –F3	MN 229740	100%
	Bacillus megaterium AAJ –F4	MN 230100	100%
	Fictibacillus phosphorivorans AAJ –F5	MN 230872	100%

Table 2: Yeast and fungal strains obtained from the Fungus combs of *O. longignathus* by culture-dependent approaches with the percentage of ITS region sequence similarity to the nearest neighbor accessed from NCBI database

Type	Organism and isolate number	GenBank Accession	BLAST sequence similarity
Yeast	*Wickerhamomyces anomalus* AAJ –Y1	MT 108722	100%
	Kodamaea ohmeri AAJ –Y2	MT 111914	100%
	Kodamaea ohmeri AAJ –Y3	MT111911	99.78%
	Kodamaea ohmeri AAJ –Y5	MT 111913	99.72%
	Candida orthopsilosis AAJ –Y4	MT 108785	100%
	Candida orthopsilosis AAJ –Y8	MT 108787	99.94%
	Candida parapsilosis AAJ –Y7	MT 108792	100%
Fungi	*Paecilomyces formosus* AAJ –FU2	MT 108799	100%
	Hypoxylon anthochroum AAJ –FU3	MT 108847	100%
	Corynespora cassiicola AAJ –FU4	MT 108846	100%

Table 3: Qualitative analysis of hydrolytic enzyme production capacity of the bacterial, yeast and fungal strains isolated from gut and Fungus combs microbiota of *O. longignathus* by culture dependent approach

Isolate strain	Source and type	Hydrolytic enzyme activity					
		Cellulase	Protease	Lignin Peroxidase	Laccase	Pectinase	Chitinase
AAJ -03	GTB	-	+	-	-	-	-
AAJ -04	GTB	-	+	-	-	-	-
AAJ -05	GTB	-	-	-	-	-	-
AAJ -10	GTB	-	+	-	-	-	-
AAJ -11	GTB	-	+	-	-	-	-
AAJ -14	GTB	-	+	-	-	-	-
AAJ -21	GTB	-	+	-	-	-	-
AAJ -23	GTB	-	+	-	-	-	-
AAJ –F1	FCB	+	+	-	-	-	-
AAJ –F2	FCB	+	+	-	-	-	-
AAJ –F3	FCB	+	+	-	-	-	-
AAJ –F4	FCB	+	+	-	-	-	-
AAJ –F5	FCB	+	+	-	-	-	-
AAJ –Y1	FCY	+	-	-	-	-	-
AAJ –Y2	FCY	-	-	-	-	-	-
AAJ –Y3	FCY	-	-	-	-	-	-
AAJ –Y5	FCY	-	-	-	--	-	-
AAJ –Y4	FCY	+	-	-	-	-	-
AAJ –Y8	FCY	+	-	-	-	-	-
AAJ –Y7	FCY	+	-	-	-	-	-
AAJ –FU2	FCF	+	+	-	-	-	-
AAJ –FU3	FCF	+	+	-	-	-	-
AAJ –FU4	FCF	+	+	-	-	-	-

GTB: Gut bacteria; FCB: Fungus comb bacteria; FCY: Fungus comb yeast; FCY: Fungus comb fungi + denotes Positive; - denotes Negative

3.4. Enzyme assays of bacterial, yeast and fungal isolates

The identified bacterial, yeast and fungal isolates were qualitatively tested for their hydrolytic enzyme activities like cellulase, lignin peroxidase, laccase, chitinase, pectinase and protease (Table 3).

4. Discussion

The gut and the fungus comb of termites under subfamily Macrotermitinae harbor wide variety of novel symbiotic microbes [29, 32]. Because of the limitations in the traditional morphological and biochemicalapproach in identifying members of the insect gut microbiome and soil microorganisms, culture dependent molecular methods were used [12].

The 16S rRNA gene based molecular identification of bacterial isolates from the gut of *O. longignathus* by sequencing 16S rRNA gene revealed that the isolates comprised mainly of three species of bacteria, namely *Serratia marcescens, Achromobacter xylosoxidans* and *Micrococcus yunnanensis*. *S. marcescens* is a species of rod shaped, gram negative bacteria, that is also a facultative anaerobic organism, classified as an opportunistic pathogen in the family Enterobacteriaceae. *M. yunnanensis* is a gram positive cocci belonging to the Micrococcaceae family; usually occur in irregular clusters, tetrads and pairs. Actinobacteriae have been already reported from macrotermitinae gut microbiota [8]. As there are reports of *M. yunnanensis* isolated from the roots of trees [33], these bacteria might have entered the termite gut through the ingestion of tree roots which might be a major source of their diet. *A. xylosoxidans* is a gram negative, rod shaped bacteria belonging to phylum Proteobacteria. *A. xylosoxidans* have previously been identified from the gut microbiota of termites belonging to Macrotermitinae subfamily [34]. There are many reports on the

cellulolytic enzyme potential of these bacterial species isolated from various sources, including termite gut microbiota [12, 35]. But the results obtained in the present study reveal that the bacterial strains isolated from the gut microbiota of *O. longignathus* did not exhibit cellulase activity implicating the possibility that the bacteria in the gut of fungus cultivating termites underwent an evolutionary change in their cellulase producing capacity. Since most of the cellulose and lignocellulose food source in Macrotermitinae are degraded by the Fungus combs symbionts, the bacteria just assists the digestion and breakdown of the resultant simple polysaccharides only. Majority of the strains were found to be positive in proteolytic enzyme production that confirms their functional role in Macrotermitinae gut microbiota.

The dominant group of bacterial composition of termite Fungus combs belongs to the phylum Firmicutes. By anaerobic culturing, the *Lactococcus* and *Clostridium* species can be cultured from the fungus comb [12]. But in the present study, the culture method for Fungus combs bacterial isolates was aerobic type only. Molecular identification of bacterial isolates from the termite Fungus combs by sequencing 16S rRNA gene revealed that the isolates comprised mainly of five species of bacteria: *Bacillus cereus, B. subtilis, B. flexus, B. megaterium* and *Fictibacillus phosphorivorans.* All the five species are gram positive, rod shaped bacteria. *Bacillus* species have been detected in the gut of soil termites and other invertebrates [36]. Also, multiple species of *Bacillus* are reported to be readily cultured from bulk and rhizopheric soil [37]. Many of them function as antagonists against various fungal and nematode pathogens of plants by secreting various kinds of antibiotics [38]. Therefore, it is a possible that the *Bacillus* species inhabiting the fungus comb could probably function as mutualists with the native fungal colony in the termitarium of macrotermitine termites. All the *Bacillus* species exhibited cellulase and protease activity. As the fungus comb *Bacillus* could enzymatically degrade cellulose, they could probably cooperate along with fungal species in the degradation of cellulose substances [39].

Mainly four different species of yeasts have been identified by sequencing ITS region, namely *Wickerhamomyces anomalus, Kodamaea ohmeri, Candida parapsilosis* and *C. orthopsilosis*. All these species have been reported to be present in the paunch region of both lower and higher termites and are noted for their capacity to degrade hemicelluloses and lignocelluloses [40]. *W. anomalus, C. orthopsilosis* and *C. parapsilosis* showed cellulose and lignocellulase activity. Three different species of fungi could be isolated from the Fungus combs of *O. longignathus.* They are *Paecilomyces formosus, Hypoxylon anthochroum* and *Corynespora cassicola*, all belong to Division Ascomycota. Fungal species belonging to Ascomycota have been previously isolated from the Fungus combs of the termite *Microtermes diversus* [41]. All the fungal isolates exhibited cellulase activity. These fungal species might aid the termites in the digestion of cellulose which is the primary content of their diet. It has been shown that cellulolytic fungi play positive role in termite nutrition [9, 11-12, 42]. *Termitomyces* could not be isolated in the current experiment even though other cellulase producing fungal species were isolated.

The present study asserts the importance of the fungal symbionts in cellulose degradation within the Fungus combs and the role yeast and bacterial species in the Fungus combs play in aiding the fungal species. *Odontotermes longignathus*, being a higher termite, lack the flagellated protists that assist the cellulolytic degradation in lower termites. It is clear that the termite gut bacterial communities do not take part in lignocellulose degradation and has taken over the function of degrading simpler polysaccharides.

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