Microbial diversity in termite nest

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In the present study, the microbial diversity of termite nest was studied using bacterial tag encoded amplicon pyrosequencing by both culture-dependent and culture-independent approaches. A total of 10,793 and 4,777 high-quality reads were obtained in culture-independent and culture-dependent approaches respectively. The former approach revealed dominant phyla Proteobacteria (32%) and Actinobacteria (20%), whereas the latter approach revealed Firmicutes (74%) and Proteobacteria (22%) as the most dominant phyla. The significant variation in the microbial diversity and composition of termitarium assessed by the two approaches could be due to the fact that culture-dependent approach explored only selected groups of microbial population, whereas metagenomic approach explored complete microbial diversity of termitarium, which provides credence to the application of metagenomic strategy to explore novel microbial species.

Keywords: Metagenome, microbial diversity, pyrosequencing, termitarium.

TERMITES serve as the best model systems for studying the symbiotic association between microbes and animals. The microbial symbionts associated with the termite gut play a pivotal role in lignocellulose digestion, methanogenesis, acetogenesis and nitrogen fixation. Termitarium is the nest of termites comprised of partially digested food materials and faecal matter of termites, enriched with minerals and other organic constituents, which provides a suitable environment for the existence of a huge diversity of microorganisms. The termitarium is highly enriched with humic acids, which serve as terminal electron acceptors for respiration that supports microbial growth[1]. The microbial population of dual origins from both termites and neighbouring soil might result in greater microbial diversity in the termitarium than termite gut or termite-associated soil[2]. Earlier, microbial diversity present in termite gut and soil environments had been well characterized[3]. However, only a few reports are available on the microbial diversity of termitarium[2]. The major limitation associated with conventional cloning and sequencing method is that it is highly time-consuming, labour-intensive and may represent only the predominant microbial populations. Recently, high-throughput next-generation sequencing technologies have bypassed the limitations in traditional sequencing methods. Recent advancement in high-throughput metagenomic sequencing technology had opened up the possibility to explore the total microbial community associated with different environmental niches. The culture-based methods are helpful in understanding the physiological potential of the isolated organisms and their potential applications. However, the existence of unculturable organisms limits their access to the actual microbial diversity in culture-dependent approach. Metagenomic approach can be used to assess the entire microbial community with varying complexity[4]. In the present study, we have investigated the microbial diversity of termitarium by both culture-dependent and culture-independent approaches using bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) method.

The termitarium sample was collected from the Madurai Kamaraj University campus, Madurai, India. The sample was sieved using muslin cloth to remove debris and solid wooden particles. For culture-dependent analysis, 0.1 g of termitarium sample was dissolved in 10 ml of sterile phosphate buffered saline (PBS) and serial ten-fold dilutions were prepared in PBS up to 10^{-7}. Aliquots of 0.1 ml were taken from the different dilutions and plated onto each of the ten different media (soil extract medium[5], AOM1[6], Luria Bertoni agar, nutrient agar, Zobell marine agar, King's B agar, brain heart infusion agar, Trypticase soy agar (0.3%), R2A medium[7] and actinomycete isolation agar) and incubated at 37°C for 7 days. Then the colonies grown on 10^{-4} to 10^{-7} diluted plates in each medium were scraped and homogenized by vortexing. The pooled samples were stored in 10% glycerol at –80°C. From the pooled samples, approximately 1×10^9 CFUs were used for DNA extraction as described earlier[8]. The cells were incubated in lysis buffer (20 mM Tris-Cl, pH 8.0; 2 mM sodium EDTA; 1.2% Triton X-100 and 20 mg/ml lysozyme) overnight at 37°C and DNA was extracted from the lysed cells using DNeasy kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). For culture-independent analysis, metagenomic DNA was extracted directly from the termitarium sample and purified using the method described earlier[9].

Pyrosequencing was performed at Research and Testing Laboratory (Lubbock, TX, USA) according to the method described earlier[10]. Initially, sequencing library was generated with one-step PCR of 30 cycles using the termitarium metagenomic DNA or genomic DNA isolated from the pooled culture as template using universal bacterial primers 926F (5′AAACTYAAAKGAATTGRCGG3′) and 1392R (5′ACGGGCGGTGTGTRC3′) as described earlier[10] followed by pyrosequencing of the generated sequence library. The bTEFAP procedure comprises of one-step PCR using hot start high-fidelity *Taq* polymerase, and sequencing of amplicons corresponding to V6–V9 region of the 16S rRNA gene. The sequences

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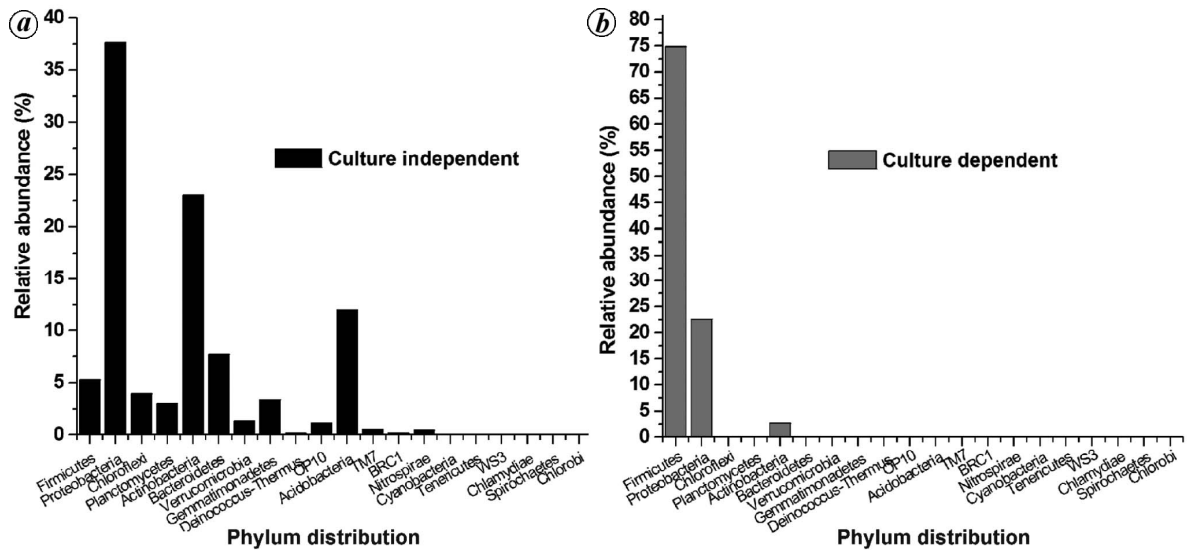

Figure 1. Relative abundance of different phyla observed in the termitarium sample by culture-independent (***a***) and culture-dependent (***b***) approaches.

were deposited in MG-RAST metagenomic analysis server (http://metagenomics.anl.gov) with accession numbers 4478101.3 (culture-independent) and 4529406.3 (culture-dependent) respectively.

The pyrosequencing reads were screened and filtered for quality and length using initial data-processing tool in pyrosequencing pipeline available at ribosomal database project (RDP) server (http://pyro.cme.msu.edu/init/form) with minimum expected average quality score of 20 and minimum sequence length of >150 bp. The high-quality reads were then aligned using aligner tool available at the RDP server. The operational taxonomic units (OTUs) and rarefaction curves were created by aligning unique tag sequences. The richness and diversity indices (Shannon–Weaver and evenness) at each dissimilarity level were calculated using pyrosequencing pipeline available at the RDP server. The ACE and Chao1 diversity indices were determined using Estimate S software (http://viceroy.eeb.uconn.edu/EstimateS). Taxonomic assignments were performed using the RDP Classifier program (http://rdp.cme.msu.edu/) with 80% bootstrap score. The taxonomic composition and abundance of microbial species present in termitarium samples were represented graphically using the visualization tools for taxonomic composition of microbial communities (VITCOMIC)[11].

Microbial diversity of termitarium sample was investigated by both culture-dependent and culture-independent approaches through pyrosequencing of 16S rDNA hyper variable regions (V6 to V9). A total of 10,793 reads and 4,777 reads with an average length of 366 and 411 bp were obtained in culture-independent and culture-dependent approaches respectively. The selected high-quality reads were classified using RDP classifier, which revealed the existence of a huge microbial diversity in the culture-independent approach.

In culture-independent approach, more than 91.8% of reads represented the bacteria domain and only 0.1% of reads represented archeal group members. It was interesting to note that 8.1% of reads could not be assigned to either bacteria or archaea. Sequencing of full-length 16S rRNA genes (~1.5 kb) may reveal their taxonomic affiliation. The microbial compositions at different phyla for both culture-independent and culture-dependent approaches are shown in Figure 1. A total of 20 different bacterial phyla were observed in culture-independent approach, which represented overall microbial diversity of the termitarium sample. The culture-independent approach revealed dominant phyla Proteobacteria (32%), Actinobacteria (20%), Bacteroidetes (7%), Fibrobacteres/Acidobacteria (6%) and few other less abundant phyla like Gemmatimonadetes, Nitrospirae, Chlamydiae/Verrucomicrobia, Chloroflexi, Firmicutes, Planctomycetes, Tenericutes and Deinococcus. Sequence analysis also revealed the presence of some candidate sequences from phyla OP10 (1%), TM7 (0.4%), WS3 (0.04%) and BRC1 (0.2%). The dominant phylum Proteobacteria includes four major families – Bradyrhizobiaceae, Hyphomicrobiaceae, Phyllobacteriaceae and Rhizobiaceae – which play a major role in nitrogen fixation in termites. These results are in consistent with the occurrence of N_2-fixing gamma proteobacteria in termite gut[3].

The second most abundant phylum present in the termitarium sample assessed by culture-independent approach was Actinobacteria, which comprises of three dominant families – Nocordioidaceae, Microbacteriaceae and Solirubrobacteraceae – which were reported to play major roles in degradation of cellulose, lignin and chitin[2]. Bacteroidetes, Treponema and Spirochaetes were most frequently reported in termite gut, which play a major role in symbiosis[12–14]. In culture-independent approach,

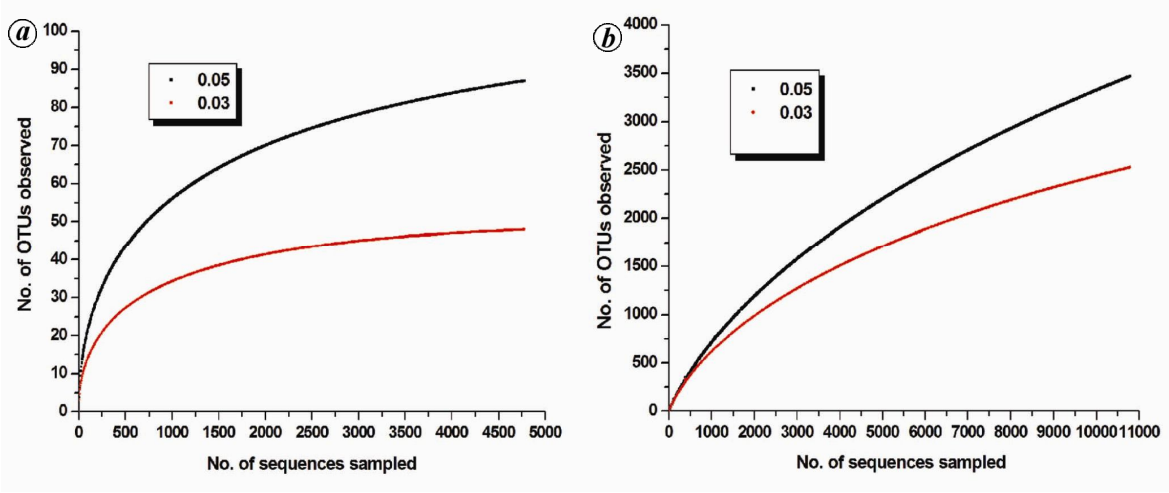

Figure 2. Rarefaction analysis of termitarium bacterial community. Rarefaction curves were used to estimate richness in the sample and sampling effort. The vertical axis shows the number of operational taxonomic units (OTUs) expected after sampling the number of sequences as shown in horizontal axis at 0%, 3% and 5% dissimilarity level. The saturation of rarefaction curves at 3% and 5% dissimilarity level indicates the attainment of maximum sampling effort of the sample. ***a***, Culture-dependent. ***b***, Culture-independent.

the relative abundance of Bacteroidetes, Firmicutes and Spirochaetes was considerably low. The difference in the abundance of these bacterial species might be due to the difference in the physico-chemical properties that exist between two different environments, i.e. termite gut and termitarium. The steep increase in the pH between the midgut and hindgut region of termite has been reported to alter the microbial community[13]. Earlier, it was reported that the abundance of Bacteroidetes was found to be 1.7% at pH < 4 and 17% at pH > 8 (ref. 15). Another distinctive feature of the termitarium sample was the presence of *Deinococcus-Thermus*, a highly hazard-resistant group of bacteria, which has not been reported to be associated with the termite gut. *Deinococcus* sp. has been reported from extreme environments such as hot springs and radioactive waste-disposal sites[16].

In recent years, termitarium has been used as a biofertilizer to improve rice production in paddy fields[17]. The organic matter present in the termitarium is highly rich in nitrogen, phosphorus and sulphur, which facilitates the growth of microorganisms such as nitrogen fixers (*Rhizobium*), decomposers (*Pseudomonas*) and sulphur oxidizers (*Thiobacillus*). The genera *Rhizobium*, *Pseudomonas* and *Thiobacillus* were also observed in the termitarium samples assessed by culture-independent approach. The existence of genera *Desulfovibrio*, *Clostridium* and *Enterobacter* indicated the presence of CO_2-reducing acetogen bacteria in termitarium. It was also interesting to note that *Geobacter* sp. represented 8% of the total bacterial community whose occurrence in termitarium seems to be unusual. The culture-independent approach also revealed certain archael group members such as Crenarchaeota and Nitrososphaera, which play a major role in nitrogen fixation. The occurrence of these soil beneficial organisms in termitarium soil supported the use of this soil as biofertilizer.

The culture-dependent approach has revealed the presence of only three different phyla – Firmicutes (74%), Proteobacteria (22%) and Actinobacteria (3%). Among the members of Proteobacteria, Gammaproteobacteria was found to be abundant in termitarium sample, whereas the Alphaproteobacteria and Betaproteobacteria were poorly represented in culture-dependent approach. Actinobacteria belongs to the order Actinomycetales, which includes seven genera – *Streptomyces*, *Microbacterium*, *Sinomonas*, *Kocuria*, *Micrococcus*, *Arthrobacter* and *Rhodococcus*. Of these, Firmicutes comprised of the family Bacillaceae (68%) and Proteobacteria comprised of Enterobacteriaceae (20%). Of the family Bacillaceae, the species *Bacillus subtilis* was found to be predominant, which has been reported to be involved in lignin degradation.

Earlier studies on the microbial diversity associated with termite gut using the conventional methods such as denaturing gradient gel electrophoresis (DGGE) and 16S rDNA sequencing have reported the presence of major phyla Proteobacteria, Actinobacteria, Bacteroidetes/chlorobi and Fibrobacteres/Acidobacteria[16]. Similar results were also observed in termitarium sample by culture-dependent approach, implying that these phyla form the major constituents of termite intestinal microflora, involved in cellulose degradation, nitrogen acquisition and acetogenesis in termites species. The culturing method also favoured the growth of obligate aerobic members that belonged to the phylum Firmicutes. Some bacteria belonging to genera *Klebsiella*, *Salmonella*, *Sodalis* and *Staphylococcus* were obtained only in culture-dependent approach and not found in culture-independent approach. Not all bacterial genera identified in culture-dependent approach were recovered by the culture-independent method. However, culture-independent method provides more complete data of the bacterial

Table 1. Prediction of species diversity by richness estimators at two dissimilarity levels comparing culture-dependent and metagenomic approaches.

Method	No. of sequences	Distance	OTUs	ACE	Chao1	Shannon	Evenness
Metagenomic	10,793	0.03	3469	6924.27	6504.51	7.42914	0.91137
		0.05	2527	4103.64	3904.94	6.9846	0.89149
Culture dependent	4,777	0.03	87	99.74	92.76	2.55298	0.57166
		0.05	48	50.52	49.66	1.88139	0.486

Figure 3. Comparative mapping of culture-independent and culture-dependent microbial diversity in the termitarium sample using VITCOMIC. Blue dots indicate the relative abundance of the sequences in the culture-independent method and red dots indicate the relative abundance in the cultured population. Grey dots indicate the occurrence of specific species in both culture-independent and culture-dependent approaches. The dots in the innermost circle (i) represent 80–85% identity with the existing 16S rRNA gene sequences in databases; Similarly, other circles represents higher levels of identity with the existing sequences in the database as follows: (ii) 85–90%; (iii) 90–95% and (iv) 95–100%.

community, including viable but not culturable bacteria and also nonviable bacteria. In other cases, culturable methods favour the growth of certain bacteria that are less abundant in the environment, which could not be detected in metagenome by culture-independent methods. Recently, Shade *et al.*[8] have reported that culturing captures the rare bacterial community from the soil samples, which was poorly represented in whole metagenome-based analysis.

The sampling effort and the richness of the microbial diversity in the termitarium by both culture-dependent and culture-independent approaches were estimated by rarefaction analysis (Figure 2). In culture-dependent approach, the rarefaction curves attained complete saturation level, and required no further sampling, whereas the rarefaction curves did not attained saturation level for culture-independent approach. The clustering analysis has revealed that the sequences were clustered into 3469 and 3537 OTUs for culture-independent and 87 and 48 OTUs for culture-independent approaches at 3% and 5% dissimilarity levels respectively (Table 1). Diversity index is a mathematical measure of species diversity in a community.

Diversity indices provide important information about rarity and commonness of species in a community. The ability to quantify diversity is important for biologists to understand the community structure. The Shannon diversity index (*H*) is commonly used to characterize species diversity in a community. The index accounts for both abundance and evenness of the species present. The diversity and evenness indices for culture-independent approach are much higher than those for the culture-dependent approach, which indicated that the culture-independent approach not only has a greater number of species, but the individuals in the community are distributed more equitably among these species.

VITCOMIC tool was used to classify the bacterial populations at species level using BLASTn analysis. The results are represented in a circle map where each circle is assigned to different similarity levels starting from 80% followed by 85%, 90%, 95% and 100%. Each dot indicates the average similarity and the size of the dots represents the relative abundance of the sequences. The overall taxonomic composition of the microbial community of culture-independent and culture-dependent analysis is shown in Figure 3. From the total reads obtained in culture-independent approach, 57% of sequences showed 91–95% similarity level and only 19% of sequences showed 96–100% similarity with existing rRNA gene sequences in the database. A total of 23% of sequences showed only 85–90% of similarity. Approximately 1% of the reads showed less than 85% similarity with existing sequences. In culture-dependent method, 96% of sequences showed 96–100% similarity level and only 4% sequences were assigned at 91–95% similarity. No sequences were observed in the range between 80% and 90% similarity levels. In Figure 3, each species in the reference database was placed in circles with ordered phylogenetic relatedness. Physical distances between the nearest species in the plot indicate the genetic distances of 16S rRNA genes between them. The circle in the plot indicates the boundaries of BLASTn average similarities. Each dot represents average similarities of each sequence against the nearest relative species in the reference dataset. The size of these dots indicates the relative abundance of sequence in the sample.

To conclude, the microbial diversity associated with termitarium habitat was assessed by both culture-independent and culture-dependent approaches. Both the approaches provided knowledge of a wide spectrum of termitarium-associated microbial populations. Further sequencing efforts with higher sequencing depth may help in obtaining further insights into microbial diversity of the habitat.

1. Lovley, D. R., Coates, J. D., Blunt-Harris, E. L., Phillips, E. J. P. and Woodward, J. C., Humic substances as electron acceptors for microbial respiration. *Nature*, 1996, **382**, 445–448.
2. Fall, S., Hamelin, J., Ndiaye, F., Assigbetse, K., Aragno, M., Chotte, J. L. and Brauman, A., Differences between bacterial communities in the gut of a soil-feeding termite (*Cubitermes niokoloensis*) and its mounds. *Appl. Environ. Microbiol.*, 2007, **73**, 5199–5208.
3. Ohkuma, M. and Kudo, T., Phylogenetic diversity of the intestinal bacterial community in the termite *Reticulitermes speratus*. *Appl. Environ. Microbiol.*, 1996, **62**, 461–468.
4. Rajendhran, J. and Gunasekaran, P., Strategies for accessing soil metagenome for desired applications. *Biotechnol. Adv.*, 2008, **26**, 576–590.
5. Hamaki, T., Suzuki, M., Fudou, R., Jojima, Y., Tabuchi, A., Sen, K. and Shibai, H., Isolation of novel bacteria and actinomycetes using soil extract agar medium. *J. Biosci. Bioeng.*, 2005, **99**, 5485–5492.
6. Stott, M., Michelle, C., Bruce, M., Angela, S., Shaobin, H., Maqsudul, A. and Peter, D., Isolation of novel bacteria, including a candidate division, from geothermal soils in New Zealand. *Environ. Microbiol.*, 2008, **10**, 2030–2041.
7. Reasoner, D. J. and Geldreich, E. E., A new medium for the enumeration and subculture of bacteria from potable water. *Appl. Environ. Microbiol.*, 1985, **49**, 1–7.
8. Shade, A., Hogan, C. S., Klimowicz, A. K., Linske, M., McManus, P. S. and Handelsman, J., Culturing captures members of the soil rare biosphere. *Environ. Microbiol.*, 2012, **14**, 2247–2252.
9. Manjula, A., Sathyavathi, S., Gunasekaran, P. and Rajendhran, J., Comparison of seven methods of DNA extraction from termitarium for functional metagenomic DNA library construction. *JSIR*, 2011, **70**, 945–951.
10. Dowd, S., Sun, Y., Secor, P., Rhoads, D., Wolcott, B., James, G. and Wolcott, R., Survey of bacterial diversity in chronic wounds using pyrosequencing, DGGE, and full ribosome shotgun sequencing. *BMC Microbiol.*, 2008, **8**.
11. Mori, H., Maruyama, F. and Kurokawa, K., VITCOMIC: visualization tool for taxonomic compositions of microbial communities based on 16S rRNA gene sequences. *BMC Bioinformat.*, 2010, **11**, 332.
12. Hongoh, Y., Ohkuma, M. and Kudo, T., Molecular analysis of bacterial microbiota in the gut of the termite *Reticulitermes speratus* (Isoptera; Rhinotermitidae). *FEMS Microbiol. Ecol.*, 2003, **44**, 231–242.
13. Lilburn, T., Schmidt, T. and Breznak, J., Phylogenetic diversity of termite gut spirochaetes. *Environ. Microbiol.*, 1999, **1**, 331–345.
14. Schmitt-Wagner, D., Friedrich, M., Wagner, B. and Brune, A., Axial dynamics, stability, and interspecies similarity of bacterial community structure in the highly compartmentalized gut of soil-feeding termites (*Cubitermes* spp.). *Appl. Environ. Microbiol.*, 2003, **69**, 6018–6024.
15. Lauber, C., Hamady, M., Knight, R. and Fierer, N., Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. *Appl. Environ. Microbiol.*, 2009, **75**, 5111–5120.
16. Makarova, K., Aravind, L., Wolf, Y., Tatusov, L., Minton, W., Koonin, V. and Daly, J., Genome of the extremely radiation-resistant bacterium *Deinococcus radiodurans* viewed from the perspective of comparative genomics. *Microbiol. Mol. Biol. Rev.*, 2001, **65**, 44–79.
17. Miyagawa, S. *et al.*, Indigenous utilization of termite mounds and their sustainability in a rice growing village of the central plain of Laos. *J. Ethnobiol. Ethnomed.*, 2011, **7**, 24.

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