ORIGINAL PAPER

Phylogenetic diversity of prokaryotes associated with the mandibulate nasute termite *Cornitermes cumulans* and its mound

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Abstract Phylogenetic analysis of cloned 16S rRNA gene fragments from *Cornitermes cumulans* (T) and its termite mound soil (TM) from the Brazilian Cerrado (Brazilian Savannah) revealed a great diversity of sequences. The bacteria detected in T and TM samples were associated with the following major lineages: *Spirochaetes* (T), *Firmicutes* (T), *Synergistetes* (T), *Cyanobacteria* (T), *Fibrobacteres* (T), *Elusimicrobia* (T), *Chloroflexi* (TM), *Verrucomicrobia* (TM), *Gemmatimonadetes* (TM), *Armatimonas* (TM), *Proteobacteria* (T and TM), *Actinobacteria* (T and TM), *Bacteroidetes* (T and TM), *Planctomycetes* (T and TM), and *Acidobacteria* (T and TM). All archaeal sequences only obtained from TM sample were associated with uncharacterized *Crenarchaeota*. The high values that were obtained for the diversity indices and evenness are indicative of high bacterial diversity from T and TM libraries, whereas the TM archaeal library exhibited low diversity. Therefore, our findings revealed differences between the bacterial communities from termite mounds and those from *C. cumulans*, the latter of which represents a specific bacterial composition when compared to other termite species.

Keywords *Cornitermes cumulans* · Termite mound · 16S rRNA · Prokaryote

Introduction

Soil contains a diverse and vast biota, and the microorganisms that make up this biota perform an essential role within the environment by participating in the cycling and flux of various nutrients and by influencing structure formation and the maintenance of soil quality (Martins 2010). In addition to microorganisms, termites also have a large influence on soil characteristics and are known as "soil engineers".

Termites are a group of insects that have an ecological impact on carbon turnover through cellulose degradation and the cycling of nutrients, which is of the utmost importance to the environment. Different species of termites build unique types of nests, such as galleries and simple chambers, underground nests, arboreal nests, or mounds. The mounds built by these insects are usually made of a mineral matrix that is mixed with feces or saliva, depending on the species of termite. These structures are the leading cause of soil physical changes, including the ability to hold water, density and structural stability, soil chemical modifications, and organic matter content (Fall et al. 2007). These changes can affect the activity and diversity of bacteria, which in agricultural systems are directly influenced by changes in soil (Jangid et al. 2008).

Cornitermes cumulans (Kollar 1932) is a termite species that belongs to the family Termitidae, and only some members construct a nest mound, known as "mound termites," because of the huge nest they build and because of their high prevalence in grazing land. This species is unique to the Neotropics, and their nests are the most abundant in Brazil, especially in the Cerrado (Brazilian Savannah); thus, this termite is an important species for the ecosystem. Using a mineral matrix and saliva, *C. cumulans* can construct mounds that range in height from 50 to 100 cm. The feeding habits of this termite species are based on litter that includes mostly leaves and the dead roots of grasses.

Despite of the known impact of termite activity on the soil affecting the diversity and microbial rhizosphere communities, the prokaryotic composition of these organisms and of their nests has been scarcely explored. Therefore, in

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this study, we sought to illuminate the prokaryotic community diversity associated with *C. cumulans* and its termite mound soil by means of 16S rRNA clone libraries.

Material and methods

Sample collection

C. cumulans workers were collected under aseptic conditions from a nest at the Ecological Station of the Universidade Federal de Minas Gerais (19º52′36″ S - 43°58′16″ W), a region that has been characterized as dry grassland (a type of Brazilian Cerrado), in November 2009. Their termite mound soil (height 1.3 m, TM) and neighbor soil (NS), which was 60 cm from the mound, were also sampled. The opening created in the wall of the mound to collect the soil was 40 cm deep. After 48 h, this gap was refilled, indicating intense termite activity.

Soil samples, abiotic parameters, and the taxonomic identification of the termites

The quantitative mineral composition of TM and NS samples were determined through the quantification of P, Fe, Zn, Mn, and Cu using Mehlich 1 as an extractant; the quantification of Ca, Mg, and Al using KCl (1 mol/L) as an extractant; and the quantification of H+Al using calcium acetate (0.5 mol/L) as an extractant (EMBRAPA 1997). pH and granulometry analyses of the TM and NS samples were also performed (Ruiz 2005). The termite taxonomy (Constantino 1999) was performed at Unidade Laboratorial de Referência em Pragas Urbanas, Instituto Biológico, São Paulo.

DNA extraction

Total DNA from the TM sample was extracted using the UltraClean Mega Soil DNA Isolation® kit (MoBio Laboratories, USA) according to the manufacturer's instructions. Isolated DNA was stored at −20 °C until further processing. The extraction of total DNA from termites was performed according to Corby-Harris et al. (2007). Prior to DNA extraction, the exterior of termites was washed with the following (in order): sterile distilled water (five times), 70 % ethanol (five times), 6 % sodium hypochlorite (five times), and sterile distilled water (five times). The termites were then macerated in liquid nitrogen.

PCR amplification of the 16S rRNA gene, cloning, and sequencing

Fragments of the 16S rRNA gene were amplified from *C. cumulans* (T) and TM genomic DNA for the construction of bacterial and archaeal clone libraries. The bacterial 16S rRNA gene fragment was amplified by touchdown PCR according to Freitas et al. (2008), using the conserved primer set 8f (5′AGAGTTTGATCMTGGCTCAG 3′) and 907r (5′ACGGHTACCTTGTTACGACTT 3′) (Lane 1991). The sequences of the archaeal primers were 16SAf (5′TTATTGGGCCTAAAGCRTC3′) and 1400Ar (5′CGGCGAATTCGTGCAAGGAGCAGGGAC3′) (Clementino et al. 2007). The 16S rRNA gene was amplified by PCR according to Clementino et al. (2007). The amplicons were confirmed using agarose gel electrophoresis. Five separate PCR amplifications were pooled before cloning. The PCR products were purified using a DNA extraction kit (Fermentas, Canada) for cloning.

Clone libraries were constructed for bacterial and archaeal communities present in the TM and bacteria present in the T by cloning the PCR products that were obtained with the different primer sets. These purified PCR products were cloned into the pJET1.2/blunt cloning vector using the CloneJET ™ PCR Cloning Kit (Fermentas) according to the manufacturer's instructions. Electrocompetent *Escherichia coli* XL1 Blue were transformed with the ligations according to the manufacturer's instructions. Plasmid DNA was extracted using the GeneJET™ Plasmid Miniprep Kit (Fermentas).

DNA sequencing reactions were performed with the DYEnamic ET Dye Terminator Cycle Sequencing Kit, and the products were run on a MegaBACE 1000 capillary sequencer (GE Healthcare, UK) according to the manufacturers' instructions. Partial 16S rRNA gene sequences were checked for quality, aligned, and edited to produce a consensus using the programs Phred v. 0.020425 (Erwing and Green 1998), Phrap v. 0.990319 (Green 1994), and Consed 12.0 (Gordon et al. 1998). Chimeras were checked using Bellerophon software (http://comp-bio.anu.edu.au/bellerophon/bellerophon.pl). The sequences were compared with the Silva database (release 108) (Pruesse et al. 2007) and the approximate phylogenetic affiliations were conducted with ARB software (Ludwig et al. 2004) using the neighbor-joining method (Saitou and Nei 1987).

Operational taxonomic units (OTUs) were determined by using DOTUR software (Schloss and Handelsman 2005); similarity levels between sequences of at least 97 % have been proposed for classifying a microorganism at the species level (Drancourt et al. 2000). Coverage of the clone libraries was calculated using the equation $C = 1 - (n/N) \times 100$, where n is the number of unique OTUs and N is the number of sequences analyzed in the library (Good 1953). The nucleotide sequences from OTUs were deposited in the GenBank database with accession numbers JN830961-JN831088.

Results and discussion

Physicochemical properties of the samples

The TM and NS samples had different physicochemical compositions (Table 1). All minerals, except for Al, were enriched in the TM compared to the NS sample. The lower concentration of Al found in the TM can be explained by the complexes formed by this ion with soil organic matter (Sarcinelli et al. 2009) because it is known that termite mounds contain more organic matter than soil. The granulometry analysis revealed that the TM and NS samples contained 50 and 41 % clay content, respectively; a characteristic property of the Cerrado soil. Therefore, the data are consistent with studies from other research groups that have revealed an increased richness in clay and minerals from termite mounds with respect to the neighboring soil (Holt and Lepage 2000; Ndiaye et al. 2004). According to Holt and Lepage (2000), this enrichment occurs because the termites select neighboring soil particles that are rich in minerals to build their nests. Previous study showed that lower clay and organic matter content of soils around mounds suggest that termite activity can reduce the bioavailability of soil nutrients for plants (Adekayode and Ogunkoya 2009).

The physicochemical differences between TM and NS may influence the composition of their microbial communities. Indeed, the dendrogram generated from the ARDRA fingerprint revealed that the TM and NS environments harbored distinct bacterial communities (data not shown). It is well known that the plant species are the dominant factors influencing the composition of the rhizosphere microbial communities (Barret et al. 2011). However, it should be noted that the termite activity leads to nutrient depletion of soil possibly disturbing this microbial community, which has a plant growth-promoting effect.

General features of the libraries

To obtain information on the composition and structure of the TM and T prokaryotic communities, three clone libraries of 16S rRNA genes were constructed, and 328 randomly selected clones from the three libraries were sequenced (Table 2). Of the 328 sequences, 286 were used for further analyses after quality control and the removal of chimeric sequences.

The clone libraries constructed from the T and TM samples were evaluated by rarefaction analysis, when the OTUs were defined at ≥97 % sequence identity (data not shown). The rarefaction curves of bacteria were far from saturation, and the coverage values of clone libraries were 63 and 68 %, for T and TM, respectively, indicating that further sequencing of more clone libraries would have revealed additional diversity. In contrast, the rarefaction curve of archaea (TM library) reached an asymptote, indicating that most of the diversity of archaea was detected in the sample (Good's coverage 95 %).

Diversity indices are a valuable tool with which to quantify diversity in a community and describe its numerical structure (Hill et al. 2003). They reflect the richness and the relative abundance of OTUs in each environment. Values from the Shannon and Simpson indices demonstrated a high diversity of bacteria in the TM and T libraries compared to the archaeal TM library (Table 2). These data indicated a relatively even OTU distribution, whereas the low Shannon and high Simpson diversity indices in the archaea from the TM library are due to the presence of a few dominant OTUs. Additionally, both the Chao1 and ACE values indicated a greater diversity of bacterial species in the TM and T libraries.

Phylogenetic composition of bacterial clone libraries

Soil habitats contain a great diversity of macro- and microorganisms, and bacteria are the largest group with respect to species richness and community size (Neher 1999). The phylogenetic analysis of 16S rRNA gene sequences constitutes an important tool for the assessment of complex microbial diversity. The phylogenetic diversity of the 16S rRNA gene clones from the TM and T libraries is shown in Fig. 1. The two libraries showed that OTUs belong to several bacterial phyla. Furthermore, five (one OTU from T library and four OTUs from TM library) of the 127 bacterial OTUs detected were considered to be unclassified at the phylum level and, thus, may represent new bacterial taxa.

Table 1 Quantification of the physical and chemical parameters of the termite mound and the neighboring soil

Soil samples	Parameters										
	pH	P (mg/Kg)	K (mg/Kg)	Ca^{2+} (cmol$_c$/Kg)	Mg^{2+} (cmol$_c$/Kg)	Al^{2+} (cmol$_c$/Kg)	H+Al (cmol$_c$/Kg)	Zn (mg/Kg)	Fe (mg/Kg)	Mn (mg/Kg)	Cu (mg/Kg)
NS	5.23	1.6	40	0.98	0.28	0.67	4.9	1.21	27.4	5.3	0.32
TM	5.7	1.5	138	3.64	0.89	0.2	6.1	4.61	128.2	49.5	1.27

NS neighboring, *TM* termite mound

Table 2 Analysis of the sequence diversity and coverage of the bacterial and archaea libraries of the termite mounds and the termite

Samples	TMB	TB	TMA
Clones	151	137	40
Chimeras[a]	25	15	2
OTUs	67	58	2
Libraries coverage (%)	65	68	95
Shannon – Weaver	3.94	3.39	0.12
Simpson	0.014	0.080	0.94
Chao 1	409.5	128.9	2
ACE	271.5	154.8	0

TMB termite mound bacteria, *TB* termite bacteria, *TMA* termite mound archaea

[a] Chimeric sequences were included in the total number of clones, but excluded from phylogenetic analysis

Actinobacteria and *Acidobacteria* comprised the largest fractions of the TM clone library, whereas *Verrucomicrobia*, *Gemmatimonadetes*, and *Armatimonadetes* were minor members. Members of *Proteobacteria*, *Actinobacteria*, and *Acidobacteria* have been reported to constitute the largest groups of bacterial communities derived from a variety of soils, including Cerrado soil. Thus, for the most part, our findings corresponded to the results from the studies of Hugenholtz et al. (1998), Kent and Triplett (2002), Zhang and Xu (2008), and Quirino et al. (2009).

Previous studies on bacterial diversity in termite nests have been reported (Fall et al. 2007, Long et al. 2010). In our sample, the bacteria found in TM library were associated with nine phyla (Fig. 1). In other studies, using DGGE (Fall et al. 2007) to analyze the variation of the internal transcribed spacer region, coupled with the construction of

16S rRNA gene clone libraries and subsequent sequencing (Long et al. 2010), the bacteria were associated with six and four phyla, respectively. *Verrucomicrobia*, *Planctomycetes*, and *Gemmatimonadetes* were detected in our analysis, although they were not observed in the analyses by Long et al. (2010) and Fall et al. (2007). In contrast to our work, the phylum *Firmicutes* was dominant in the study by Long et al. (2010).

Thirty percent of all classified sequences from the T clone library corresponded to *Spirochaetes* (which were represented exclusively by *Treponema* genus), *Firmicutes*, and *Actinobacteria*. Other important minor bacterial phyla included *Planctomycetes*, *Cyanobacteria*, *Fibrobacteres*, *Acidobacteria*, and *Elusimicrobia*, the latter being a typical phylum from termites gut (Ikeda-Ohtsubo et al. 2010). Most OTUs (67.2 %) from the T clone library were associated with other sequences that had previously been obtained from other termite species, and the remaining OTUs from soil and gut of other insects. Furthermore, all bacterial phyla found in T library were previously reported as symbionts of other species of termites (Hongoh 2010). The phyla *Spirochaetes* and *Fibrobacteres* are known to act in the hydrolysis of lignocellulose and are considered essential symbionts of the termites gut (Hongoh 2011).

Previous cultivation-independent studies have been performed on the bacterial diversity of the lower and higher termite gut with different feeding habits. Among these, *Macrotermes michaelseni* (Mackenzie et al. 2007) and *Macrotermes gilvus* (Hongoh et al. 2006) have been reported to feed on litter and are found in savannah regions, similar to *C. cumulans*. Despite eating habits and habitat similarities between these two species and *C. cumulans*, the bacterial communities among them were very distinct. The bacteria found in the gut of *M. michaelseni* were less diverse, and
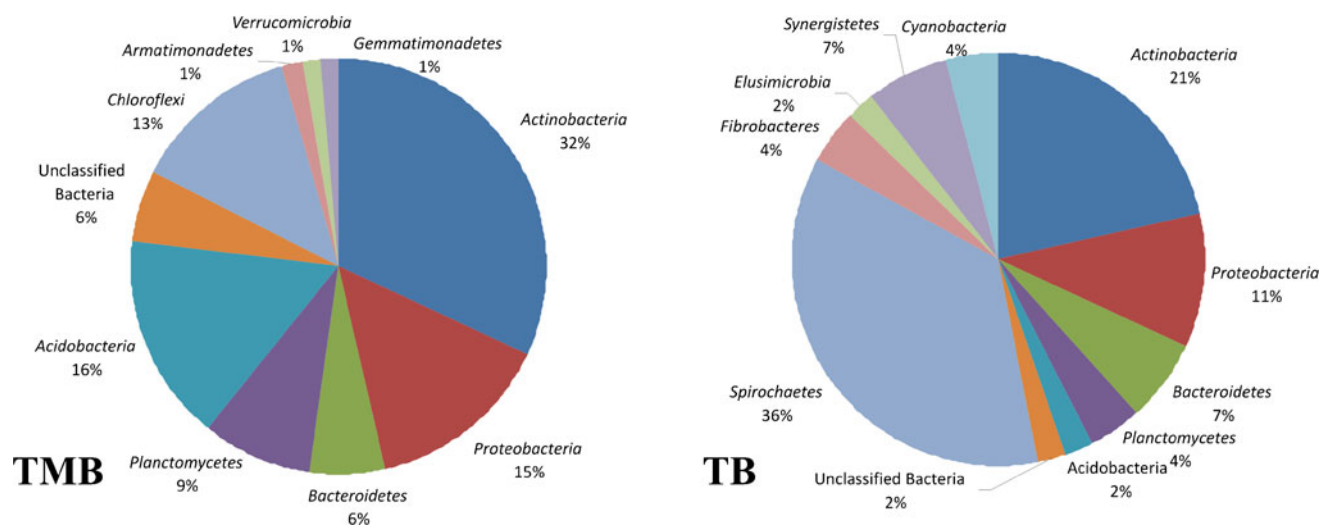
Fig. 1 Phylogenetic distribution of bacterial 16S rDNA sequences generated from T and TM samples

Bacteroidetes (formally referred to as *Cytophaga-Flexibacter-Bacteroides*) was the dominant group (Mackenzie et al. 2007), whereas *Bacteroidetes* and *Firmicutes* were the phyla predominant in *M. gilvus* (Hongoh et al. 2006).

Most OTUs affiliated with the phyla outlined in Figs. 2 and 3 were phylogenetically related to uncultured bacteria from other termite species and from the soil. Nevertheless, some OTUs related to the *Actinobacteria*, *Proteobacteria*, *Bacteroidetes*, and *Acidobacteria* could be classified at the genus level, i.e., *Marmoricola*, *Nocardioides*,

Planosporangium, *Conexibacter*, *Microlunatus*, and *Actinomadura* (*Actinobacteria* of the TM clone library); *Rhodococcus*, *Luteococcus*, and *Propionibacterium* (*Actinobacteria* of the T clone library); *Caulobacter*, *Phenylobacterium*, *Altererythrobacter*, *Pedomicrobium*, and *Burkholderia* (*Proteobacteria* of the TM clone library); *Chromobacterium*, *Paracoccus*, *Pantoea*, and *Novosphingobium* (*Proteobacteria* of the T clone library); *Flavisolibacter* (*Bacteroidetes* of the TM clone library); and *Edaphorobacter* (*Acidobacteria* of the TM library). Some
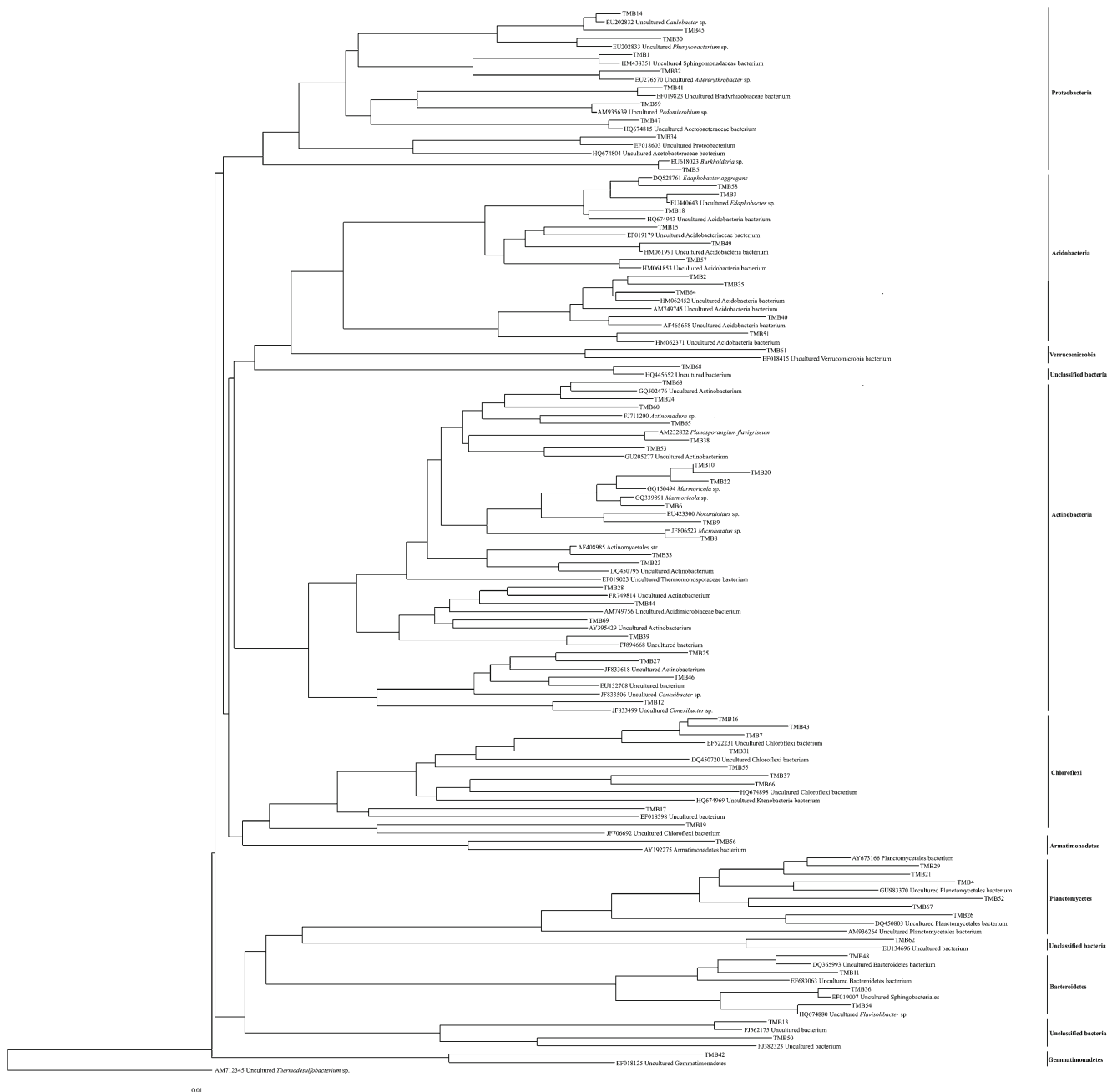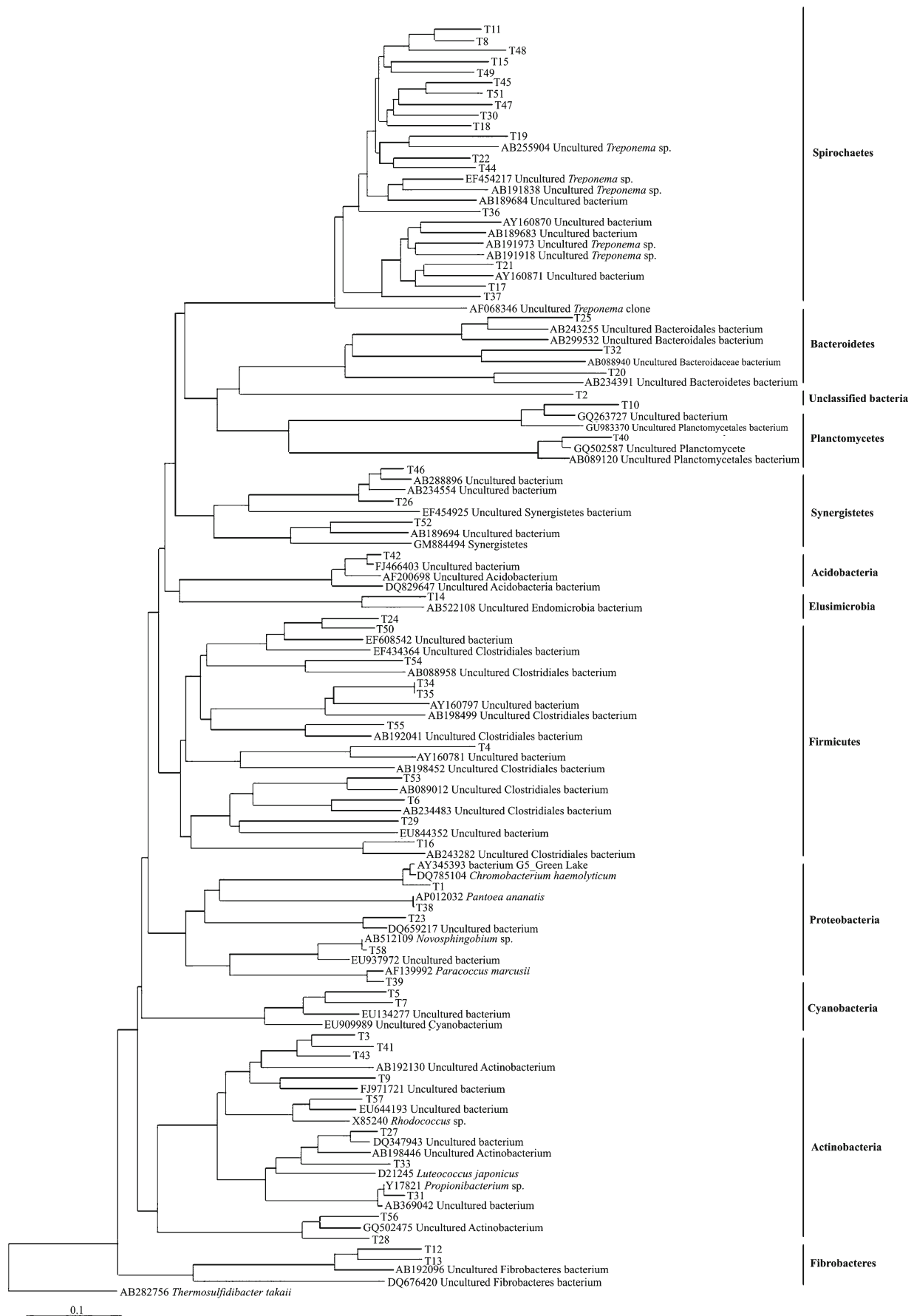

Fig. 2 Phylogenetic tree showing the affiliation of bacterial OTUs from TM library. Phylogenetic relationships were inferred with ARB software with Silva database (release 108) using the neighbor-joining method

of these genera, such as *Burkholderia* and *Bradyrhizobium*, are important for soil nitrogen fixation and the stimulation of plant growth, respectively (Hennecke 1990; Preisig et al. 1996; Pandey et al. 2005).

Archaeal community composition in termite mounds

The construction of the archaeal 16S rRNA gene clone library was successfully obtained only for the sample TM, despite 10 attempts to do with the sample T. In an endeavor to obtain the amplification of the 16S RNA gene fragment (T sample), different archaeal primers set were used namely: 16SAf and 1400Ar (Clementino et al. 2007); 25 F and 1492R (Lane 1991); 21 F and 958R; (DeLong 1992); and mcrA-F and mcrA -R (Luton et al. 2002). According to Hongoh (2010), the abundance of archaea in the gut of termite species that have already been studied ranged from 0 to 10 %. Therefore, our results can be explained by the absence or low abundance of archaea in the gut of *C. cumulans*. Moreover, according to Brune (2006), the abundance of archaea in the gut of termites is apparently related to diet. In termites that feed on litter, such as *C. cumulans*, the abundance of archaea is lower (0.1–1.7 %) than in termites feeding soil (1.4–3.1 % archaea).

The phylogenetic analysis of the TM archaeal 16S rRNA gene revealed a total of two OTUs, indicating that the archaeal community was poorly diverse. Both OTUs were affiliated with the phylum *Crenarchaeota*. In addition, one OTU gathered 37 clones, whereas another OTU contained only one clone. It should be mentioned that all OTUs were uncharacterized archaea.

The identification of archaea from non-extreme environments is important for understanding their ecological role in different environments. The incidence of archaea inhabiting the soil is extremely low, ranging from 0.5 to 3.8 % of all prokaryotes that inhabit this environment (Ochsenreiter et al. 2003; Timonen and Bomberg 2009). Although members of the phylum *Crenarchaeota* have previously been isolated from extreme environments, they are often found in many less-extreme environments, including different soil types, such as that of the Cerrado (Bintrim et al. 1997; Ochsenreiter et al. 2003; Clementino et al. 2007; Timonen and Bomberg 2009). Our results are in agreement with the work of Tagliaferro (2005) which only detected clones belonging to the phylum *Crenarchaeota*.

Similar to previous studies of termite species, this survey detected a wide variety of phylogenetic taxa. The analysis of 16S rRNA gene sequences revealed that a specific

microbiota is associated with the termite species and not with the feeding habits or habitats of the termites. These data support the hypothesis of Hongoh et al. (2006) that microorganisms in the intestinal tracts of termites have coevolved with their hosts. The survey also detected a broader phylogenetic breadth of taxa than any previous study of termite mounds, including several taxa that had not been previously detected in termite mounds. The results of this study provide the first insights into the bacterial diversity in the gut of a member of the mandibulate nasute grass-feeding termite, a group unique to the Neotropics and its nest.

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