

Gut-specific actinobacterial community structure and diversity associated with the wood-feeding termite species, *Nasutitermes corniger* (Motschulsky) described by nested PCR-DGGE analysis

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Received: 5 August 2008 / Revised: 6 March 2009 / Accepted: 4 May 2009 / Published online: 3 June 2009
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Abstract This comprehensive survey studied the actinobacterial community structure and putative representative members associated with the gut of the wood-feeding termite, *Nasutitermes corniger* (Motschulsky), using nested PCR-DGGE and 16S rDNA sequences analyses. The closest relatives of the actinobacteria inhabiting the gut of *Nasutitermes corniger* were in five families, regardless of the geographical origin of the termite colony: *Propionibacteriaceae*, *Streptomycetaceae*, *Cellulomonadaceae*, *Corynebacteriaceae* and *Rubrobacteraceae*. Feeding termites on beech wood did not result in substantial changes in the actinobacterial community structure as revealed by DGGE banding patterns. Most of the 16S rDNA sequences obtained after excision and sequencing of DGGE bands clustered with those previously retrieved in termite guts. These results confirm the presence of gut-specific actinobacteria.

Except for the 16S rDNA sequences affiliated to *Streptomycetaceae* and *Cellulomonadaceae*, no sequence had more than 97% similarity with the closest isolated strains, indicating the presence of microorganisms that have not yet been cultivated. These results suggest that members of the *Actinomycetales* order account for the largest proportion of the *Actinobacteria* phylum inhabiting the gut of the termite *N. corniger*.

Keywords Actinomycetes · Termite gut · Nested PCR · DGGE

Introduction

Over the past 30 years, increasing attention has been paid to the impact of termites on the turnover of organic matter in tropical and subtropical areas. Termites are important decomposers specializing in the degradation of recalcitrant components of plant residues through their association with symbiotic gut microorganisms (for reviews, see Breznak and Brune, 1994; Brune, 2006). Interest in termite gut microbiota has centered on actinobacteria owing to their ability to degrade lignocellulose. Since the first isolation of *Micromonospora* sp. by Hungate (1946), most of the isolates cultivated from termite guts have been aerobic *Streptomyces* strains (Pasti and Belli, 1985; Bignell et al., 1991). Recently, Kurtböke and French (2007) have cultivated previously undetected actinomycete genera in the termite gut of a lower wood-feeding termite, *Coptotermes lacteus* (Froggatt) using a method that exposed the gut contents to genus- and species-specific phages for the removal of background bacteria to impede the growth of actinomycetes on plates. However, this method is not suitable for assessing bacterial diversity owing to the

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selectivity of cultivation methods. Over the last decade, termite gut microbiota have been extensively studied using the 16S rRNA gene as a molecular marker to characterize the whole bacterial diversity (Ohkuma and Kudo, 1996; Brauman et al., 2001; Hongoh et al., 2003; Nakajima et al., 2005; Hongoh et al., 2006, Schmitt-Wagner et al., 2003). Several 16S rRNA sequences of the *Actinobacteria* phylum falling into different genera were retrieved in termite guts. The analysis of the taxonomic composition showed that the 16S rRNA sequences affiliated with *Actinobacteria* account for a minor part of the gut bacterial microbiota. However, the diversity may have been underestimated since individual taxa present in smaller numbers will not be detected owing to PCR bias (review in von Wintzingerode et al., 1997). This was shown recently by Farris and Olson (2007) who demonstrated bias in universal PCR primers on the detection of actinobacteria from environmental samples. Moreover, a discrepancy was found between the isolates obtained by cultivation and the dominant phylogenetic groups in the clone libraries (Hongoh et al., 2003; Schmitt-Wagner et al., 2003). While culture-independent methods allow an accurate description of dominant phylogenetic groups inhabiting the intestinal tracts of termites, little is known about the community structure and diversity of gut actinobacteria. Although many genes, relevant to cellulose hydrolysis found recently in a wood-feeding termite, were assigned to two groups of bacteria, the fibrobacteres and spirochetes, in functional metagenome analysis of bacteria in the hindgut paunch (Warnecke and Hess, 2009), other microorganisms with cellulase activities, such as actinobacteria, are probably present and located in different gut compartments.

For the first time, this study examined the overall composition of actinobacteria in the gut of the higher wood-feeding termite, *Nasutitermes corniger* (Motschulsky) using the nested PCR-DGGE approach. Nested PCR has proved to be an effective means of detecting uncultured *Actinobacteria* in environmental samples (Rheims and Stackebrandt, 1999). It was, therefore, used in this study to increase the specificity and amplification of 16S rDNA fragments of actinobacteria. The amplified fragments were analyzed using the DGGE technique (Muyzer et al., 1993). The PCR-DGGE approach has been used extensively to compare the bacterial community structure of different systems and to monitor temporal changes (review in Nakatsu, 2007). It has also been demonstrated that this culture-independent method may adequately reflect the relative composition of 16S rRNA gene fragments in bacterial communities of different environmental samples (Smalla et al., 2007). Furthermore, this approach obviates the requirement for cultivation and does not require a clone library to be constructed. This study aimed (1) to assess the extent of the gut actinobacteria community structure in two

colonies of the same termite species, *N. corniger*, from different geographical areas, Guadeloupe and French Guiana and (2) to identify putative representative members associated with the microbiota in the intestinal tracts of this termite species.

Materials and methods

Collection of termites, breeding and diets

Two colonies of *Nasutitermes corniger* were used in this study. The nest of a colony was collected in the field near Maripassoula village in French Guiana (NcosD) and kept in artificial conditions (Laboratory of Zoology, University of Bourgogne, Dijon, France) for several years. In contrast, the colony from the *N. corniger* nest NcosG was used directly after field collection in Guadeloupe. To investigate the influence of the exogenous microflora on the gut actinobacterial community structure, 50 worker termites of the NcosD colony were put into sterilized Perspex boxes containing pieces of beech wood that were either unsterilized (C) or sterilized (F). The breeding boxes were kept at 28°C in a simulated tropical atmosphere and mortality was checked each day for 10 days. Pieces of beech wood (W) consumed by the termite workers were also sampled for analysis.

DNA extraction and nested PCR

The gut of ten termites from each box or each nest was removed using sterilized forceps, the intestinal tracts were pulled out and only the hindguts were collected for analysis. Total microbial DNA of the hindgut homogenates was extracted using the method described by Porteus et al. (1997). Specific amplification of 16S rDNA fragments of actinobacteria was performed using the method described by Heuer et al. (1997): a specific primer, F235 (Stach et al., 2003) targeting 16S rDNA fragments of actinobacteria was used with the primer R1378 (Heuer et al., 1997). This step generates an actinomycete template for a second bacterium-specific PCR with primers F984-gc (Nübel et al., 1996) and R1378. The amplification was performed using a Mastercycler personal (Eppendorf, Hamburg, Germany) and PuRe Taq Ready-To-Go® (Amersham Biosciences, Freiburg, Germany). The first amplicons were obtained by initial denaturation at 92°C for 5 min followed by ten cycles of denaturation at 92°C for 45 s, annealing at 60°C for 2 min and extension at 72°C for 2 min. A final extension was performed at 72°C for 30 min. Two microliters of the amplicons were used as a template for the second PCR which was performed under the conditions described above, but with an annealing temperature at 68°C and 30 replication cycles.

DGGE-analysis

Amplified fragments obtained by nested PCR were separated in a denaturing gradient gel. DGGE was performed using a Dcode™ Universal Mutation Detection System (Biorad, Richmond, CA), using the method described by Muyzer et al. (1993). Amplicons (500 ng) were loaded onto 8% (w/v) polyacrylamide gels in 0.5× TAE with a narrow gradient of urea and formamide, around 52–58%. After overnight migration under 75 V at 60°C, the gels were stained with ethidium bromide (0.5 mg L⁻¹) for 15 min, rinsed for 20 min in milli-Q water and then photographed with UV transillumination with the GelDoc 2000 system (BioRad, Richmond, CA). To assess the taxonomic affiliation of the actinobacteria, pieces of DGGE bands were excised with a sterile scalpel and placed in tubes containing 20 µL of sterilized milli-Q water. The DNA of each band was allowed to diffuse into the water overnight at 4°C. The eluate (2 µL) was used as a DNA template in a new PCR reaction under the same conditions as described above. The amplicons were analyzed by DGGE to confirm that the expected products were isolated. Amplicons with a single band co-migrating with that of the original sample were excised and amplified and then sequenced by Genome Express laboratories (Grenoble, France). The CHECK_CHIMERA command of the RDP facilities was used to detect chimeric sequences. The 16S rDNA sequences were compared with the closest sequences referenced in the public databases. In order to assign each sequence to the correct taxonomical affiliation, the closest relatives were selected and aligned to construct a phylogenetic tree. The nearest sequences from GenBank listed as isolated from guts of other termites were also processed in the tree construction. All sequences were aligned using the Clustal W algorithm (Thompson et al., 1994) in the BioEdit 7.0.1 sequence editor (Hall, 1999) and corrected manually. Phylogenetic analyses were then performed with MEGA 4.0 (Tamura et al., 2007) using the Neighbor-Joining and Parsimony methods. For constructing Maximum Likelihood tree, we used MultiphyI online (<http://distributed.cs.nuim.ie/multiphyI.php>).

Results

Nested PCR-DGGE analysis of the actinobacteria community structure

A comparative analysis of the DGGE profiles obtained from the gut contents of worker caste termites was performed on the two *N. corniger* colonies. Band patterns on the three DGGE profile replicates of each treatment showed good resolution although the amplified fragments

were approximately 600 bp long (Fig. 1). Taking into account the presence and absence of individual bands, Jaccard dissimilarity distance ($\Delta = 0.098$) did not indicate significant differences in DGGE band patterns between the treatments. About nine bands were found in the three DGGE profile replicates of the worker termites from the NcosD colony, whereas the gut contents of the NcosG colony analyzed directly after field collection displayed about six bands. However, each of these six bands had a corresponding band in the DGGE profile of the gut contents of the NcosD colony. Ncos0 and Ncos1 exhibited the same intensity in common bands.

As the exogenous food microflora may have influenced the composition of the microbial gut community, the

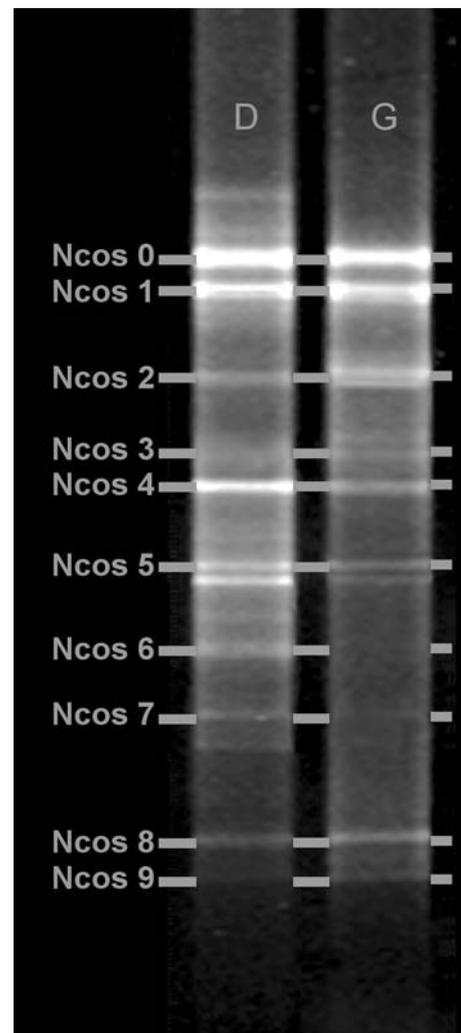


Fig. 1 DGGE banding pattern of 16S rDNA amplified fragments of actinobacteria from the hindguts of a higher wood-feeding termite, *N. corniger*: lane *D* worker caste termites of the nest from French Guiana kept in laboratory for several years; lane *G* worker caste termites of nest from Guadeloupe processed after field collection. Annotations on the left of the picture indicate the codes of excised bands and their migration position on the gel

relationship between the structure of actinobacteria community in the gut contents of *N. corniger* and in the beech wood used as natural food was analyzed. Comparative analysis of DGGE band patterns was performed on the three replicates of the worker caste termites (NcosD) kept in containers and fed with beech wood that was either unsterilized or sterilized (Fig. 2). Visual differences in the DGGE band patterns could be observed according to the treatments. However, the DGGE band patterns of the three replicates for each treatment were similar. It appeared that none of the bands detected in the termite guts had the same migration distance as those found in the beech wood. One intense band and a large number of low intensity unresolved bands were observed in the DGGE band patterns of actinobacteria 16S rDNA fragments from beech wood. However, four intense bands (Ncos1, Ncos3, Ncos4 and Ncos5) were present in the profiles of the termite gut content for both types of beech wood. The mortality of termites after 10 days was around 24%.

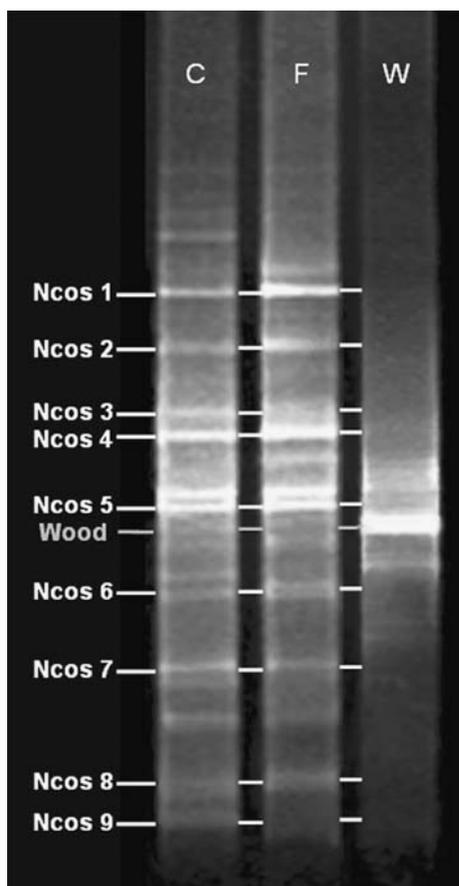


Fig. 2 Comparison of DGGE banding pattern of 16S rDNA amplified fragments of actinobacteria from the hindguts of worker caste termites fed with unsterilized beech wood (C), the hindguts of worker caste termites fed with sterilized beech wood (F) and unsterilized beech wood used as natural feed (W). Annotations on the left of the picture indicate the codes of excised bands and their position on the gel

Sequence analysis of the 16S rDNA fragments and assignment to phylogenetic groups

16S rDNA sequence analysis of the bands excised from DGGE gels revealed that most of the bands were affiliated with the *Actinobacteria* phylum. The sequences retrieved fell into five families (Table 1) including *Propionibacteriaceae* (Ncos3, Ncos4 and Ncos8 bands), *Cellulomonadaceae* (Ncos6 and Ncos7 bands), *Corynebacteriaceae* (Ncos2 band), *Streptomycetaceae* (Ncos9 band) and *Coriobacterineae* (Ncos5 band). However, the sequence retrieved from the Ncos1 band was affiliated with *Opitutaceae* (*Verrucomicrobia* phylum), and was unexpectedly bound by the primers used for actinobacterial 16S rRNA gene amplification. The Ncos0 band could not be sequenced. The identity of comigrating bands was confirmed by DNA sequence analyses. The sequences corresponding to these bands shared more than a 97% 16S rRNA gene sequence similarity. Cluster analysis of the sequenced DGGE bands showed that the majority of sequences retrieved were grouped with termite gut actinobacteria sequences available in public databases (Fig. 3). Furthermore, except for the Ncos9 band which was closely related to *Streptomyces alanosinicus*, the sequences corresponding to the Ncos1, Ncos2, Ncos3, Ncos4, Ncos5 and Ncos8 bands were genetically distant (identity <93%) from those of characterized strains.

Discussion

Community structure and diversity in actinobacteria associated with the gut contents of *Nasutitermes corniger*

Despite numerous 16S rDNA sequences of bacteria retrieved from termite guts, very few comprehensive surveys have been carried out into the structure of gut actinobacterial communities, including the identification of putative representative members. As the nested PCR approach successfully detected uncultured *Actinobacteria* in environmental samples (Rheims and Stackebrandt, 1999), this technique was used to amplify 16S rDNA fragments of actinobacteria inhabiting the gut of a wood-feeding termite species, *Nasutitermes corniger*. The resultant amplified 16S rDNA fragments were then separated by DGGE analysis to determine the actinobacteria community structure in the gut. Putative representatives were identified by sequence analysis of excised DGGE bands. The DGGE analysis revealed that all six bands visible in the banding patterns of the gut contents from the NcosG colony were found in the NcosD colony. These results suggest that the geographical origin of termites does not have a significant influence on the structure

Table 1 Homology of sequences from DGGE bands compared to public database sequences

Band code	Sequence Accession	Closest relatives		Identity
		Taxonomical affiliation	Accession	
Ncos -1	EU089690	Verrucomicrobia		0.947
		Opitutaceae		
Ncos -2	EU089691	Uncultured bacterium PBg1-128	AY791245	0.926
		Actinobacteria		
Ncos -3	EU089692	Corynebacteriaceae		0.960
		Uncultured <i>Corynebacterium</i> MTcory14R	AF115940	
Ncos -4	EU089693	Propionibacteriaceae/Nocardiaceae		0.967
		Uncultured bacterium BOf2-16	AB288888	
Ncos -8	EU089697	Uncultured bacterium BOf2-16	AB288888	0.973
		Cellulomonadaceae		
Ncos -6	EU089695	Micrococccineae strain Ellin124	AF408966	0.949
		Uncultured bacterium BOf2-16	AB288888	
Ncos -7	EU089696	<i>Cellulomonas composti</i> TR7-06	AB166887	0.976
		Streptomycetaceae		
Ncos -9	EU089698	<i>Streptomyces alanosinicus</i> NBRC 13493	AB184442	0.984
		Unclassified Rubrobacterineae		
Ncos -5	EU089694	Uncultured bacterium SZB15	AM176851	0.981
		Mycobacteriaceae		
Wood	EU089699	<i>Mycobacterium</i> sp. strain 439/93	X80770	0.927

of the termite gut actinobacteria community. These results did not support the statement that actinomycete flora of termites depends largely on geographical origin (Watanabe et al., 2003). This raised the question of the relationship between the composition of microbial community in the gut and in the host food. It was, therefore, checked whether the actinobacteria found in the beech wood overlapped those detected in the termite gut. It has already been shown that the variations of artificial carbon sources in diets result in changes in the termite gut microbial community (Tanaka et al., 2006; Miyata et al., 2007). Therefore, beech wood was used in the present study as natural food instead of artificial carbon sources to be closer to the feeding conditions of these termite species in the field. Our hypothesis was that if foreign microbiota could be introduced into the gut microbial community along with the food, the corresponding 16S rDNA fragments would be detected in DGGE banding patterns of termites fed with unsterilized beech wood. This study demonstrated that there was no obvious relationship between food microflora and gut actinobacterial community. This agrees with previous studies which stated that there was no substantial overlap between gut microbial communities in soil-feeding termites and the surrounding soils used as natural food (Donovan et al., 2004; Fall et al., 2007). Furthermore, since the identity of comigrating bands was confirmed by nucleotide sequencing of DGGE bands, the large number of fragments detected in the DGGE profiles can be considered as an indication of the presence of diverse

actinobacteria taxa in the gut. However, this diversity seems to be restricted to taxa belonging to the *Actinomycetales* order and most of the sequences of putative representative members were affiliated to the families *Corynebacteriaceae*, *Streptomycetaceae*, *Cellulomonadaceae*, *Propionibacteriaceae* and *Coriobacterineae*.

Relevance of the retrieved 16S rDNA sequences

Numerous monophyletic lineages comprising exclusively termite gut clones from diverse host species have already been reported (e.g., Hongoh et al., 2005; Shinzato et al., 2005). This study found that most sequences retrieved clustered with those of termite gut clones available in public databases. The phylogenetic positions of the related representatives were the same whatever the phylogenetic reconstruction methods (NJ, ML analysis). However, the affiliation of Ncos5 cannot be determined precisely. Indeed, the sequence alignment analysis revealed that its closest relative sequence belonged to one member of the *Rubrobacterineae* family. This family, phylogenetically positioned among environmental clone sequences at the root of the class *Actinobacteria*, has not been found in termite guts so far. In contrary, based on the results of phylogenetic trees, it appeared that Ncos5 is more related to *Coriobacterineae*. Nevertheless since most the sequences retrieved clustered with those of termite guts previously described, our results confirm the conclusions of previous

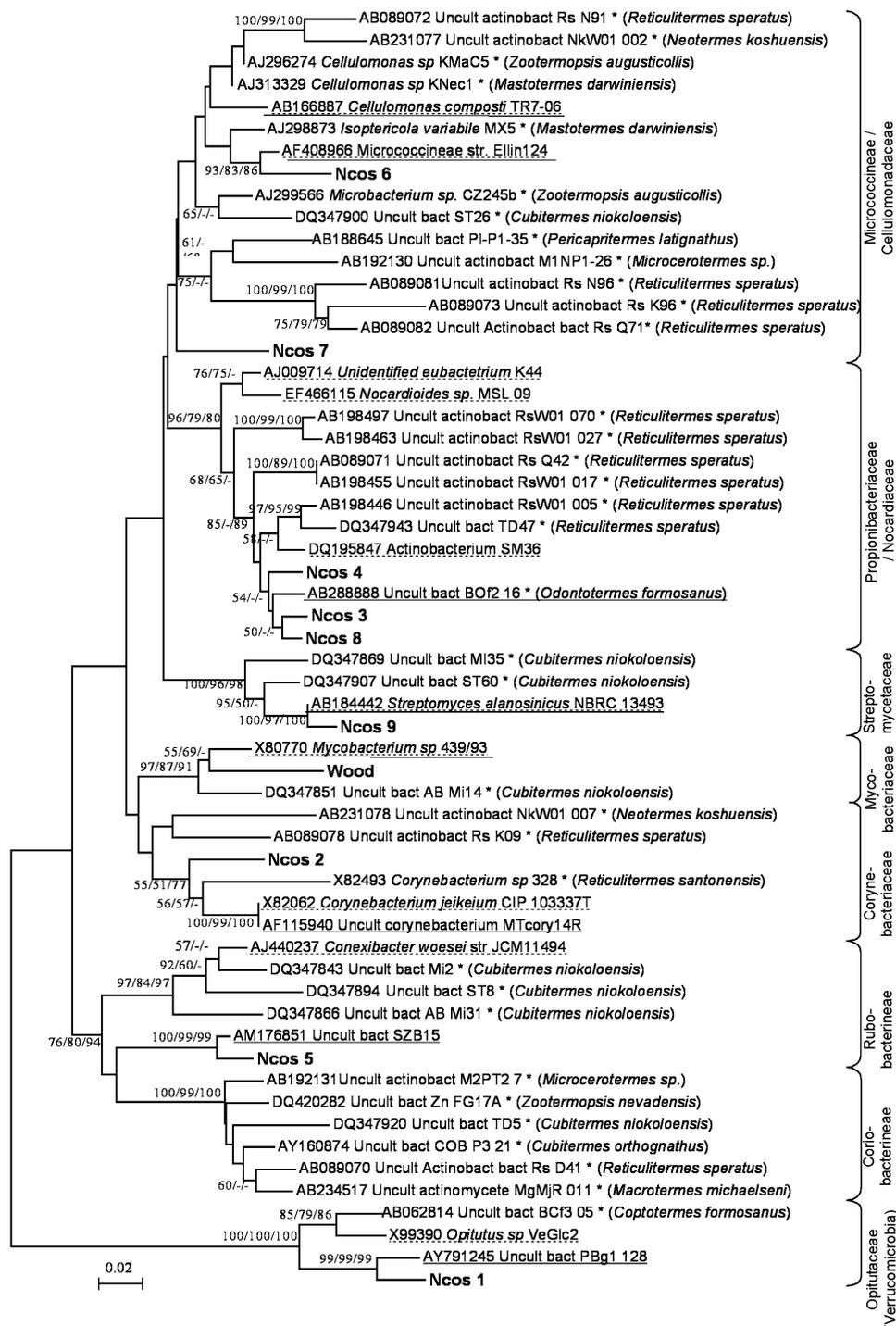


Fig. 3 Phylogenetic tree showing the relationship between the sequences of DGGE bands. *solid line* sequences from uncultured strains; *dashed line* sequences from characterized isolate strains; (*): sequences retrieved in termite gut. The bootstrap percentages are indicated for NJ/MP/ML

works that gut-specific symbionts that have coevolved with host termites (Schmitt-Wagner et al., 2003; Hongoh et al., 2006; Shinzato et al., 2007). This study also revealed that the largest part of the *Actinobacteria* phylum inhabiting the gut of the wood-feeding termite *N. corniger* was composed of *Actinomycetales*. This is consistent with the taxonomic

affiliation of numerous actinobacteria strains isolated from termite guts using cultivation-based methods. Cellulolytic, ligninolytic and lignin-solubilizing activities were detected among these actinobacteria strains (Pasti and Belli, 1985; Kuhnigk and König, 1997). Kawase et al. (2004) demonstrated that family 19 chitinases (EC 3.2.1.14) occurred

among actinobacteria especially in the *Actinomycetales* order. Furthermore, although nested PCR were performed using an *Actinobacteria*-specific primer F235, designed by Stach et al. (2003), which matched the sequences of the majority of *Actinobacteria*, two prominent monophyletic actinobacterial clusters comprised exclusively of *Reticulitermes speratus* gut clones were absent: one containing Rs-F20 and Rs-N91 clones, for example, and another containing an Rs-H69 clone (Hongoh et al., 2003). This discrepancy may be explained by the 2 to 4 mismatches exhibited by the F235 primer. However, this observation may simply indicate the absence of taxa belonging to the related *Actinobacteria* families in the gut of *N. corniger*, since the bacterial community structure in the gut was shown to be basically consistent within a genus of termites (Hongoh et al., 2005). This study also revealed the presence in the termite gut of new microbial species that have not yet been cultivated. This is supported by the fact that most of the sequences retrieved (except *Streptomycetaceae* and *Cellulomonadaceae*-related sequences) did not have close relatives among known isolated species.

In conclusion, since PCR-DGGE provided a better description of microbial communities than that provided by cultivation-based methods, the *Actinobacteria* community structure described in this study may give a reasonably accurate picture of the diversity of this specific bacterial group in the gut of *N. corniger*. However, the abundance of the taxa as displayed by intensive bands may not reflect the absolute quantitative composition of actinobacterial community owing to the PCR-based method biases (von Wintzingerode et al., 1997). Members of the *Actinobacteria* phylum inhabiting the termite gut on *N. corniger* are diverse and widely distributed among different families of the *Actinomycetales* order. Most of the actinobacterial-related sequences revealed the presence of new bacterial species that result from indigenous gut microbiota. Further investigations are required (1) to explore changes in the actinobacteria community in the gut with respect to the food components and (2) determine the resultant impact on the degradation of lignocellulose materials in termite guts.

Acknowledgments We are grateful to Dr Gladys Loranger-Merciris, from the University Antilles-Guyane (Point-à-Pitre, Guadeloupe, France), and to Dr Alain Robert and Dr Christian Bordereau, from the University of Bourgogne (Dijon, France), for their valuable help in providing and identifying termites.

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