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Ascomycete diversity in soil-feeding termite nests and soils from a tropical rainforest

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Summary

Molecular microbial ecology has revealed remarkable biodiversity – prokaryotic and eukaryotic – in numerous soil environments. However, no culture-independent surveys of the termitosphere exists, although termites dominate tropical rainforests. Here, we focused on soil feeders, building nests with their soil-born faeces, enriched with clay–organic complexes, thus contributing to the improvement of soil fertility. In order to assess the fungal community composition of these termitaries compared with soils not foraged by termites, samples of the two types were collected in the Lopé rainforest, Gabon, and processed for generation of fungal internal transcribed spacer (ITS) clone libraries. Although primers were universal, most of the recovered sequences represented Ascomycete that were previously uncharacterized and the proportions of which reached 72.5% in soils and 80% in termitaries. Their affiliation with identified fungi was analysed in performing a phylogenetic tree based on 5.8S rDNA. Furthermore, the ascomycete communities of soil-feeding termitaries and soils shared only 6.3% of sequences. This discrepancy of composition between soil and nest may result from the building behaviour of termites, as the organic matter in the nest is chemically modified, and some vacant ecological microniches are available for more specialized fungi.

Introduction

Although microbial biodiversity has received particular attention since the 1960s, the consequences of biodiversity on ecological processes are still an object of debate and analysis. Soils, the major ecosystem processes of which are mediated by their biota, remain partially described ecosystems, especially tropical rainforest soils, whereas they probably contain among the greatest biodiversities on the planet (Hagvar, 1998; Tiedje *et al*., 1999). Moreover, the direct and indirect interactions between the different soil inhabitants involved in ecosystem mechanisms are far from being completely documented. Among non-associative interactions between species, the ecosystem engineers such as earthworms could play a major role in modifying resource availability for other organisms (Jones *et al*., 1994; Lavelle *et al*., 1997).

Soil-feeding termites (Isoptera, Termitidae) represent around 50% of the known termite species (Noirot, 1992) – about 2600 species – and dominate tropical ecosystems (Collins, 1989). They build their nests using their own faeces enriched with clay–organic complexes formed during gut transit by a physical, chemical and biological rearrangement of the ingested soil (Grassé, 1984). Consequently, the biostructures that comprise the wall nests represent for plants a genuine reserve of nutritive elements, slowly released to the environment by erosion processes. Consequently, in tropical soils where the organic matter turnover is swift, soil feeders largely contribute to soil fertility.

As soil feeders dramatically modify the chemical and physical properties of soils (Garnier-Sillam and Harry, 1995), the question arises as to whether and to what extent they could be considered as metabionts. According to the definition of Waid (1999), metabionts are organisms that modify, maintain or create habitats so that other organisms are able to survive, may be eliminated or fail to grow, while others may adapt or evolve. Our purpose was to investigate how, by altering soil properties, soil-feeding termites also affect microbial diversity and especially soil fungal diversity.

At present, soil fungi have received scant consideration in the discussions on biodiversity. Moreover, interactions between termites and fungi have been poorly characterized. Few works have described either the fungi parasite for termites (Traniello *et al*., 2002) or the mutualistic symbiosis between *Termitomyces* sp. (Basidiomycota) and fungus-growing macrotermitine termites (Aanen *et al*., 2002). Existing studies about non-associative interactions

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are only focused on culture-dependent surveys of fungi in termite nests built by xylophagous or fungus-growing termites (Zoberi, 1979; Mohindra and Mukerji, 1982; Zoberi and Grace, 1990).

In this study, we describe the soil-feeding termite effect on an uncultured fungal community by analysing their internal transcribed spacer (ITS) diversity in the very peculiar soil-borne biostructures, i.e. the nests of soil-feeding termite *Cubitermes* sp. (Termitidae, Termitinae), dominant in the Lopé rainforest (Gabon) compared with surrounding soils. Then, we illustrate the overall molecular ascomycete diversity from the soil-feeding termite nests and tropical soils by performing phylogenetic analyses with the more conserved part of the ITS, i.e. the 5.8S rDNA.

Results and discussion

Description of fungal ITS clones from soil-feeding termite nests and soils

Two hundred and fifty-three ITS sequences ranging in length from 392 to 558 bp were retrieved from the four soil-feeding termite nests and the three surrounding soils. In order to compare soil-feeding termite nests with soils for their fungal community, we pooled the results for the four soil-feeding termite nests and those of the three soils. Sequences were sorted into 95 phylotypes after comparison against each other using the BLAST search program (>94% identity). Five dominant phylotypes accounted for 40% of all sequences (AY273337, AY273339, AY273323, AY273321, AY273320); the first two were found exclusively in termite nests, whereas the last three were shared by soils and nests (Table 1). About 23% of all sequences showed only a unique representative: 31 from termitaries and 27 from soils. Overall, 44 phylotypes were restricted to termite nests and 45 to soils; only six of the 95 phylotypes were in both representing 6.3%. The retrieved ITS clones showed 40% and 43% homologous coverage of the expected phylotype diversity in the libraries for termitaries and soils respectively. This showed that more than half the fungal community of these samples remained to be explored and raised the question of the number of clones to study to represent any environmental microbial community (Hughes *et al*., 2001).

Identification of the phylotypes

Querying databases with these full-length ITS sequences defined 21 phylotypes similar to known sequences (>94% identity, Table 1): 11 originated from soils and 10 from termite nests. Although primers were universal, within these identified sequences, except one affiliated with Basidiomycota, all were Ascomycota, from the classes Dothideomycete (two phylotypes), Eurotiomycete (three phylotypes), Sordariomycete (14 phylotypes) and an *incertae sedis* class (one phylotype). So the following results focused on Ascomycete diversity. Four of the five numerically dominant phylotypes belonged to these identified sequences and were related to *Penicillium*, *Eurotium*, *Clonostachys* and *Trichoderma* spp. Many sequences, without close ITS BLAST matches, could not be assigned to any known taxa and were designated as unidentified. They could originate from identified fungi with as yet undetermined ITS sequences, from fungi not yet isolated or be of chimeric origin.

Checking for chimeric sequences

The formation of chimeric sequences assembled from different species is a potential risk associated with polymerase chain reaction (PCR) from a mixed population (Wintzingerode *et al*., 1997; Speksnijder *et al*., 2001; Hugenholtz and Huber, 2003). As no existing tool has been adapted to check for the occurrence of chimeras for ITS loci, the ITS1, 5.8S rDNA and ITS2 were independently subjected to BLAST searches on the NCBI database, and three categories of unidentified sequences were observed. For 51% of all unidentified phylotypes, the three parts were similar to some fungal ITS sequences, sometimes very slightly but, on the whole, these observations attested that a chimeric origin was very unlikely. For 41% of the unidentified phylotypes, they matched well with referenced 5.8S rDNA but did not match with either ITS1 or ITS2 sequences from the database, suggesting that these ITS sequences were not in the database. Carter *et al*. (1999) isolated cultivable fungi with such ITS sequences showing that they occur naturally and do not result from rearrangements during PCR between different amplicons. Finally, 8% of the unidentified phylotypes, exhibiting different matched sequences for the three parts, were putative chimeras. But, as they were recovered from different samples analysed independently, it threw their chimeric status back into question. Thus, we could suggest that all the unidentified sequences recovered in our analysis were more likely to be undescribed fungi than chimeras.

Undescribed fungi

In the studied samples, unidentified ascomycete ITS (74/95 phylotypes) diversity reaches 72.5% of the total diversity in soils (37/51 phylotypes) and 80% in termite nests (40/50 phylotypes) with only 4% shared (3/75 phylotypes). These results could only be compared with the data of the few available culture-independent fungal surveys concerning temperate soils, none of these studies having yet investigated either forest soil or termite nests in the trop-

Table 1. Phylogenetic affiliations determined by BLAST of fungal ITS phylotypes – represented at least twice among samples or identified (identity >94%). The number of clones per phylotype are given for soil and termite nest libraries. The results were pooled for the three soils and the four nests. The fungal phylogroups defined by the phylogenetic analysis on 5.8S rDNA were also done.

ITS clones	Closest identified relative				5.8S rDNA group	No. of clones in libraries	
Accession no.	Phylum	Class	Designation	% Identity		Nests	Soils
AY273298	Ascomycota	Dothideomycete	*Lacazia loboi*	97	Lacazia	0	1
AY273297			*Curvularia inaequalis* CBS 185.47	96		0	1
AY273309			ND[a]	ND[a]	Curvularia	0	2
AY273319			ND[a]	ND[a]		1	1
AY273338		Eurotiomycete	ND[a]	ND[a]		2	0
AY273299			*Aspergillus terreus*	99		0	2
AY273320			*Eurotium niveoglaucum* NRRL 137	99	Aspergillus-Penicillium	25	3
AY273307			ND[a]	ND[a]		3	0
AY273339			*Penicillium westlingii* NRRL 800	95		10	0
AY273318		Sordariomycete	*Metarhizium anisopliae*	97	Metarhizium	0	3
AY273335			*Metarhizium* sp. KACC 40230	96		2	0
AY273317			*Colletotrichum gloeosporioides*	98	Colletotrichum	0	2
AY273330			*Bionectria rossmaniae*	95		1	0
AY273321			*Clonostachys candelabrum* CBS 504.67	99	Bionectriaceae	13	12
AY273329			ND[a]	ND[a]		2	0
AY273328			*Cordyceps heteropoda*	97		2	0
AY273326			ND[a]	ND[a]	Cordyceps	3	0
AY273327			*Cordyceps nutans*	99		1	0
AY273313			ND[a]	ND[a]		0	7
AY273306			*Calonectria kyotensis* Cam13	98		0	2
AY273305			*Fusarium* sp. NRRL22574	97		0	2
AY273314			ND[a]	ND[a]	Fusarium-Verticillium	0	4
AY273332			*Nectria haematococca*	98		4	0
AY273334			*Verticillium chlamydosporium*	99		1	0
AY273322			ND[a]	ND[a]		3	1
AY273324			ND[a]	ND[a]		1	1
AY273333			ND[a]	ND[a]	Trichoderma	2	0
AY273323			*Trichoderma* sp. ZAUT031	100		14	9
AY273304			*Phialocephala xalapensis* CBS 218.86	100	Phialocephala	0	1
AY273301			ND[a]	ND[a]		0	2
AY273325			ND[a]	ND[a]		4	0
AY273303			ND[a]	ND[a]	Xylariales	0	6
AY273302			*Pestalotiopsis* sp.	99		0	1
AY273310			ND[a]	ND[a]		0	2
AY273296		incertae sedis	*Oidiodendron tenuissimum* UAMH 8512	95	Oidiodendron	0	2
AY273308		Unidentified	ND[a]	ND[a]	I	0	5
AY273315			ND[a]	ND[a]		0	2
AY273300			ND[a]	ND[a]	II	0	7
AY273316			ND[a]	ND[a]	III	0	3
AY273336			ND[a]	ND[a]		2	0
AY273331			ND[a]	ND[a]	IV	2	0
AY273337			ND[a]	ND[a]		14	0
AY273311			ND[a]	ND[a]	V	0	4
AY273312			ND[a]	ND[a]		0	2
AY353055	Basidiomycota		*Udeniomyces pyricola* CBS 6754	97	Bas	0	1

The accession no. in bold letters define the five most abundant sequences among the samples.
a. Not determined, corresponds to an identity ≤94%.

ics. In temperate environments, Smit *et al*. (1999) recovered 86–92% unidentified fungi by 18S rDNA analyses from wheat rhizosphere, while Viaud *et al*. (2000) revealed an unidentified fungi rate of 86% from a soil by ITS analysis. In accordance with these authors, we assumed that the gaps within current databases prevented the linking of a majority of our cloned sequences to established taxa.

Putative ascomycete phylogroups from termite nests and soils

We performed phylogenetic analyses that aimed at representing the overall molecular ascomycete diversity of our samples and designing putative fungal phylogroups. Owing to the high variability of ITS discussed by Bridge and Spooner (2001), we restricted our analysis to the 5.8S rDNA, which provides enough information to check gross linkages of unidentified sequences to established taxa. In order to have a representative set of phylotypes, we included 44 of the retrieved ascomycete phylotypes (those identified and those represented at least twice) plus the 21 matched known sequences. The phylograms constructed by the neighbour-joining distance analysis (Fig. 1) and by the maximum parsimony method were congruent except for the relative position of AY273300, AY273337 and AY273331. Twelve ascomycete phylogroups were identified and included 35 phylotypes (Fig. 1). Five other phylogroups remained unidentified (I to V) and were extremely diverse, as revealed by their spreading across the phylogram. The *Fusarium–Verticillium* and *Aspergillus–Penicillium* groups showed the highest diversity (eight and five phylotypes respectively). Moreover, the *Fusarium–Verticillium* group was divided into two distinct clusters underlining its problematical taxonomy (Guadet *et al*., 1989; Bidochka *et al*., 1999).

Putative ecological roles of Ascomycota from termite nests and soils

Soil fungi are known to mediate many biochemical interactions (Bridge and Spooner, 2001), and our PCR-based approach facilitates the identification of a wide range of putative ecological actors from soil-feeding termite nests and soils. Some of the genera affiliated to our clones are known to be parasitic for insects (*Cordyceps*, *Metharizium*, *Verticillium*), obligate or facultative; others display a variety of interactions with plants: pathogens (*Curvularia*, *Fusarium*, *Phialocephala*), biocontrol agents (*Colletotrichum*, *Bionectria*) or root-associated fungi (*Oidiodendron*), and the other genera could be saprophytes, necrophilia and even coprophile (*Aspergillus*, *Penicillium*, *Xylaria*). It is worth noting that six phylogroups were exclusively associated with soils: unidentified groups I, II and V and *Oidiodendron*, which is a plant root-associated fungi, *Colletotrichum* and *Phialocephala*, two phytopathogenic fungi, whereas only two associated with termitaries: unidentified group IV and *Cordyceps*, an entomopathogenic fungi.

Impact of soil-feeding termites on Ascomycete community composition

Despite the fact that the method used presents some limitations such as lack of discrimination between living and dead or active and dormant fungi, bias towards the most prevalent organisms and shortage of authenticated reference sequences, we pointed out the low overlap (only 6.3% of phylotypes shared) between ascomycete communities from soils and soil-feeding termite nests. Fungi are known to be constrained by climatic factors such as moisture and temperature, by vegetation, resource quality and local processes such as predation and competition and may also be disturbed by the engineering effects of other biota. This last constraint might be the most important factor affecting fungal diversity within the same region and vegetation type (for a review, see McLean and Parkinson, 1998; 2000). Clearly, without increasing or decreasing soil ascomycete diversity, soil-feeding termites entail such rearrangements of ascomycete community composition in their nests as they are really distinct from those of the surrounding soils.

Few studies have described soil fungal diversity by molecular tools, and fewer compared soils or examined the effect of some stress on soil fungal community. Ranjard *et al*. (2001), using ribosomal intergenic spacer analysis (RISA) to compare five soils from different geographical origins and exhibiting contrasting physical and chemical properties, revealed qualitative differences between the fungal communities. By identification of ITS cloned sequences, Chen and Cairney (2002) demonstrated that the effect of a perturbation such as prescribed burning in Australian forest soils affected the fungal composition. Landeweert *et al*. (2003), examining the soil basidiomycete community in different soil horizons by ITS sequencing, showed that they differed along the profiles: some phylotypes were exclusively found in the organic horizon, whereas others were in the mineral one.

The only data exploring the influence of ecosystem engineering on fungal community are culture-dependent studies concerning earthworms. Some mesocosm or temperate field studies have demonstrated, with plating methods, that the epigeic earthworm *Dendrobaena octaedra* (McLean and Parkinson, 1998; 2000) and the endogeic *Octolasion tyrtaeum* (Scheu and Parkinson, 1994) affected fungal species composition, whereas contrasting data were obtained on the widespread anecic earthworm *Lumbricus terrestris* (Tiunov and Scheu, 2000). Numerous mechanisms, including the alteration of physical and

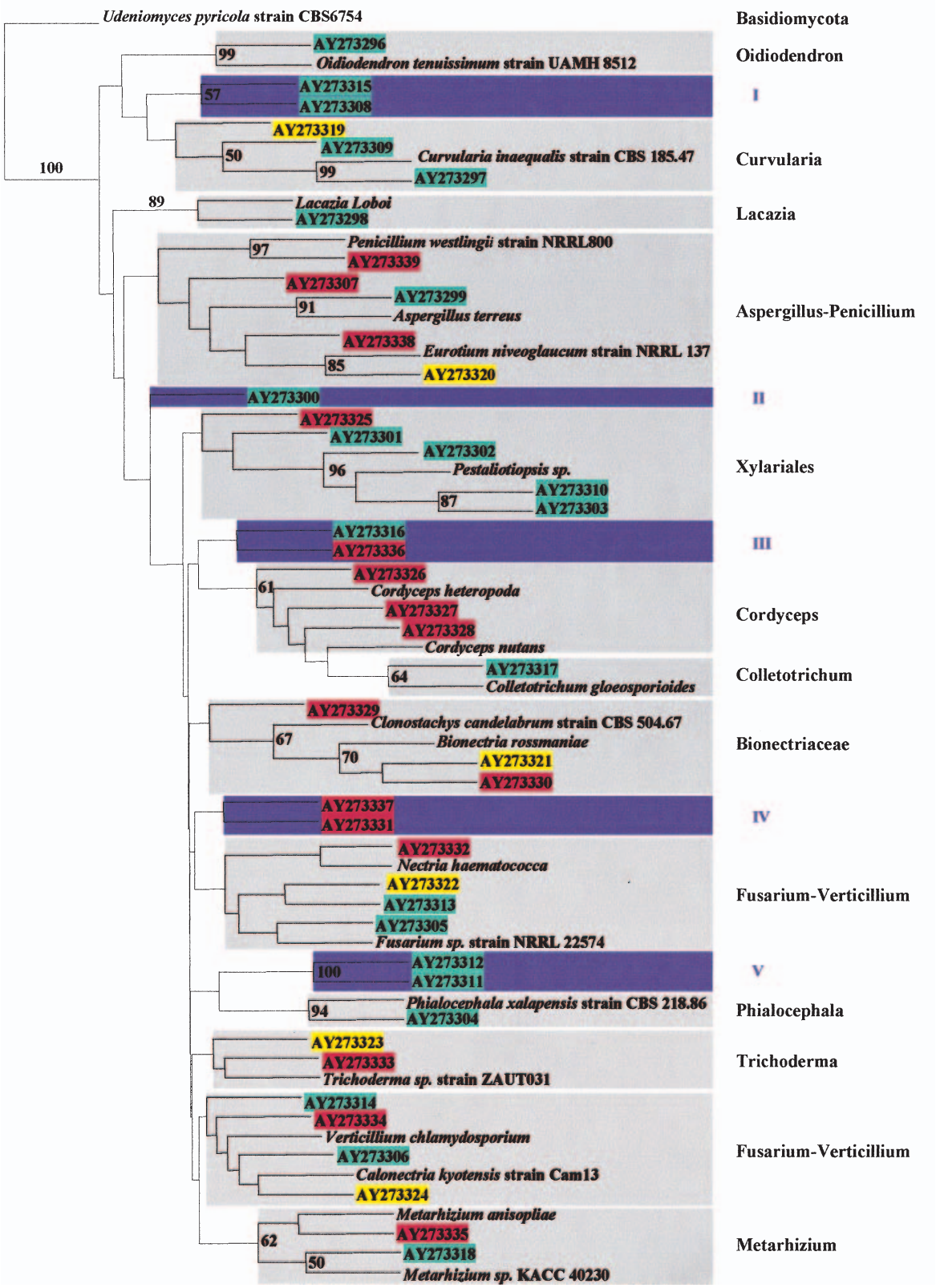

Fig. 1. Phylogram displaying the relationships between ascomycete 5.8S rDNA associated with *Cubitermes* nests and surrounding soils and their 21 matched known sequences. The tree shown, rooted with Basidiomycota, was derived from the neighbour-joining distance analysis. Bootstrap values >50% are indicated. Grey boxes delimit putative fungal groups and blue boxes, the unidentified fungal groups (I to V). Phylotypes are highlighted in red or blue whenever exclusively found in termite nests or in soils, respectively, and in yellow whenever common to both samples.

chemical properties of the environment, translocation of litter, dispersal of fungal propagules and grazing of fungi, could be attributed to ecosystem engineers. Here, the marked discrepancy observed between termite nests, which are faeces made, and soil ascomycete communities most probably arises from the building behaviour of the soil-feeding termites resulting in modifications of the physical and chemical parameters of the habitat (Garnier-Sillam and Harry, 1995). Three major mechanisms could be involved. First, the biological composition of the ingested soil, transformed into faeces, may be drastically modified by the passage through the termite gut because of the very alkaline pH (> 12) observed in *Cubitermes* soil feeder gut, unique in biological systems (Brune and Kuhl, 1996). Some fungi may be destroyed during gut passage, whereas others may be favoured, as suggested by Tiunov and Scheu (2000) for *Trichoderma* spp. in earthworm faeces. Secondly, in termite gut, the organic matter is subjected to fundamental rearrangements, as a result of physical, chemical and biological actions. Clay and organic particles are intimately mixed creating stable organo-mineral complexes, while the occurrence of some humification processes produces more complex molecules such as humic acids (Garnier-Sillam and Harry, 1995). Consequently, in faeces and hence in the nests, the heterogeneity of the organic matter, the creation of new substrates or reduced access for fungi in such clay–organic complexes could favour some specialized fungal species. Thirdly, the occurrence of fungal inhibitors cannot be excluded (Chen *et al*., 1998; Lamberty *et al*., 2001).

Conclusions

In the studied samples, we demonstrated that a high proportion of the biodiversity remained undescribed and that, by modifying the environment, soil-feeding termites drastically affect the soil ascomycete community structure. The observed gap most probably results from the building behaviour of the termites because, in the nest, the organic matter is chemically transformed, and some vacant ecological microniches are available for more specialized environmental fungi. As a consequence, soil-feeding termites could be considered, according to Waid (1999), as true metabionts, because they create habitats and supply resources to dependent organisms such as fungi that may adapt, evolve and hence diversify. Nonetheless, we should take care about the fact that the sampling of the microbial community was not exhaustive in this study. Further analyses are required to assess whether such ascomycete diversity modifications in nests of *Cubitermes*, the most abundant genus in some African forests, may affect some biogeochemical cycles, and this leads us to question the putative link between molecular diversity and functional redundancy. Furthermore, in such environmental biotopes, tropical rainforest soils are among the best candidates able to shelter the highest fungal diversity (Christensen, 1989; Hawksworth and Rossman, 1997; Bridge and Spooner, 2001), and termite nests could confer resilience to ecosystem function facing putative environmental changes.

Experimental procedures

Sample collection

Four termite nests from soil-feeding *Cubitermes* sp. and three surrounding soils without noticeable termite activity were collected from the rainforest in Lopé, Gabon. Each termite nest studied was composed of samples taken from three different parts of the same construction, and each surrounding soil was composed of samples taken from five different points of the topsoil (1 m^2). Samples were air dried, crushed and sieved (1 mm). For soils and termitaries, total carbon and nitrogen contents, cation exchange capacity, clay percentage, pH and water retention capacity are described in Table 2. According to their particle size distribution, soils can be defined as loamy sand to sandy clay loam (soil classification of the US Department of Agriculture).

Isolation of ITS sequences from samples

Total DNA was extracted using a FastDNA® SPIN kit for soils (BIO 101) as described by the manufacturer with slight modifications. Samples were submitted to two rounds of bead-beating lysis, and a supplementary purification step with CTAB (Porteous *et al*., 1997) was applied. DNA amplification of the fungal full-length ITS (ITS1 + 5.8S + ITS2) was performed using ≈50 ng of soil microbial DNA in a 50 μl volume containing 1.5 mM $MgCl_2$, 16 mM $(NH_4)_2$, 67 mM Tris-HCl, pH 8.8, 0.01% Tween 20, 5 pmol of each nucleotide, 50 pmol of each universal primer, PN3 and PN34 (Viaud *et al*., 2000) and 2 units of Silverstar DNA polymerase (Eurogentec). The PCR was carried out using four linked profiles: (i) denaturation at 95°C for 2 min; (ii) 15 cycles: denaturation at 95°C

Table 2. Physical and chemical properties of soil and termite nest samples.

	C	N	CEC	Clay	pH	WRC
Termite nests	30.53 ± 11.94	2.67 ± 0.81	9.13 ± 2.12	41.98 ± 9.31	5.3 ± 0.5	36.3 ± 7.6
Soils	27.55 ± 20.31	1.93 ± 1.12	4.44 ± 2.14	18.33 ± 8.36	4.9 ± 0.2	32.3 ± 12.1

The mean values ± standard deviation were expressed for total carbon and nitrogen contents in g kg^{-1}, for the cation exchange capacity in cmol H^+ kg^{-1} and for the clay and water retention capacity (WRC) as a percentage.

for 1 min, annealing at 65°C for 1 min, extension at 72°C for 1 min 30 s; (iii) 15 cycles: denaturation at 95°C for 1 min, annealing at 65°C for 1 min, decreased by 0.7°C per cycle, extension at 72°C for 1 min 30 s; (iv) 10 cycles: denaturation at 95°C for 1 min, annealing at 54.5°C for 1 min and extension at 72°C for 1 min 30 s. Purified amplicons were cloned in pGemT vector (Promega) as described by the manufacturer. Clones with inserts of the expected size – from 390 to 766 bp (Ranjard *et al*., 2001) – were sequenced with primers PN3 or PN10 (Viaud *et al*., 2000). Sequences were submitted to NCBI database using the BLAST search program (Altschul *et al*., 1997).

Phylogenetic analyses

Multiple alignments were performed using the DIALIGN program, version 2.2.1 (http://rna.icmb.utexas.edu/linxs/main.html). Phylogenetic inferences were determined by neighbour-joining analysis with Kimura genetic distance and by parsimony analysis using the software PHYLIP (Felenstein, 1989). Bootstrap analyses were performed on 1000 data sets.

Diversity index

For each gathered library (soils and termitaries), we calculated the parameter C as homologous coverage with the following formula: $C = [1–(n1/N)] \times 100$, where n1 is the number of phylotypes containing only one sequence and N the total number of clones analysed (Good, 1953).

Nucleotide sequence accession numbers

Full-length ITS sequences have been deposited in the GenBank database under accession numbers AY273296 to AY273339 and AY353055.

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