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Herbert Guedegbe, Edouard Miambi, Anne Pando, Pascal Houngnandan, Corinne Rouland-Lefèvre

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Molecular diversity and host specificity of termite-associated *Xylaria*

Herbert J. Guedegbe[1]

UMR IRD 137 BioSol, Laboratoire d'Ecologie des Sols Tropicaux, Institut de Recherches pour le Développement, 32 avenue Henri Varagnat 93143 Bondy Cedex. 59 70 France, and Laboratoire d'Ecologie Microbienne, Faculté des Sciences Agronomiques (FSA), Université d'Abomey-Calavi, 01 BP 526 Recette Principale Cotonou Benin

Edouard Miambi

Anne Pando[2]

UMR IRD 137 BioSol, Laboratoire d'Ecologie des Sols Tropicaux, Institut de Recherches pour le Développement, 32 avenue Henri Varagnat 93143 Bondy Cedex, France

Pascal Houngnandan

Laboratoire d'Ecologie Microbienne, Faculté des Sciences Agronomiques (FSA), Université d'Abomey-Calavi, 01 BP 526 Recette Principale Cotonou Benin

Corinne Rouland-Lefevre[3]

IRD, Ressources Vivante, 32 avenue Henri Varagnat, BONDY, Seine Saint Denis 93140, France

Abstract: Studies have revealed that some *Xylaria* species were closely associated with fungus-growing termite nests. However this relationship rarely had been investigated and the host specificity of termite-associated *Xylaria* was not yet clearly established. Eighteen *Xylaria* rDNA-ITS sequences were obtained from fungus combs belonging to 11 Macrotermitinae species from eight regions. Low diversity was found between isolates, and nine sequences were retrieved. Termite-associated *Xylaria* were shown to be monophyletic, with three main clades, all including strains from various termite hosts and geographical localities. This new molecular study shows no species specificity with respect to fungus-growing termites, which suggests that there might be substrate specialization.

Key words: fungus combs, host specificity, ITS sequences, phylogeny, termite-associated *Xylaria*

INTRODUCTION

The role of fungus-growing termites on soil functioning is well documented. This group of higher termites, abundant in tropical and subtropical ecosystems, is known to have a significant effect on the physical and chemical properties of soil (Jouquet et al 2006). The impact of fungus-growing termites is largely due to exosymbiosis with a basidiomycete fungus of genus *Termitomyces*, which grows on a special substrate (a fungus comb or fungus garden) built by termite workers (Rouland-Lefevre 2000, Rouland-Lefevre et al 2006) This relationship is a perfect mutualism because the termites create a favorable environment within their nests that promotes the growth of *Termitomyces* and in turn the symbiotic fungus and fungus comb (fecal pellets with poorly digested plant material) provide food for the termites (Rouland-Lefevre 2000). Because of this interesting, rare association several phylogenetic studies focussed on fungus-growing termites and their symbiotic basidiomycete (Aanen et al 2002, Katoh et al 2002, Rouland-Lefevre et al 2002, de Fine Licht et al 2006).

Field observations revealed that *Termitomyces* was the only visible fungus in active nests but that other fungi actively developed when the nest was excavated or abandoned by termites. One fungus, commonly associated with fungus combs abandoned by termites (Sands 1969, Heim 1977, Batra and Batra 1979), was placed within genus *Xylaria* Hill ex Schrank (Xylariaceae family). Fungi in this genus were characterized mainly by perithecial ascocarps and were known to be saprophytic for dead angiosperms and gymnosperms (Rogers 1979, Rogers and Samuels 1986). Some authors considered termite-associated *Xylaria* to be latent saprobes (Sands 1969) or mutualistic symbionts such as *Termitomyces* (Batra and Batra 1979). Nothing was known about their status on fungus combs, and few mycologists had studied *Xylaria* species in termite nests. To our knowledge only two systematic works (Rogers et al 2005, Ju and Hsieh 2007) were conducted to study the *Xylaria* species associated with fungus combs with the latter study restricted to a single termite species from a unique environment. The main problems when studying *Xylaria* species in general were their cosmopolitan status, their highly variable morphology associated with stages of maturity and also probable taxonomic confusion owing to the difficulty in observing ascopore germination sites (Whalley 1996, Lee et al 2000, Rogers et al 2005). It therefore was difficult to identify the *Xylaria* species by means of morphological characters. When they investigated the taxonomy of some *Xylaria* found in termite nests Rogers et al (2005) concluded that only a molecular characterization could clarify the relationship between the two organisms. However the

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[1] Fax: +33 01 48 02

[2] Fax: +33 01 48 02 59 70

[3] Corresponding author. E-mail: rouland@bondy.ird.fr

TABLE I. Taxa used in this study

Species	Strain	Termite symbiont	Origin	GenBank accession Nos.
Xylaria				
Undetermined	AO1	*Odontotermes pauperans*	Ivory coast (Africa)	EU164403
Undetermined	AO6	*Odontotermes sp1*	Benin (Africa)	EU164401
Undetermined	AO7	*Odontotermes sp2*	Benin (Africa)	EU203583
Undetermined	AMS4	*Macrotermes subhyalinus*	Burkina Faso (Africa)	EU164407
Undetermined	AMS10	*Macrotermes subhyalinus*	Cameroon (Africa)	EU203581
Undetermined	AMS9	*Macrotermes subhyalinus*	Cameroon (Africa)	EU203580
Undetermined	AMS13	*Macrotermes subhyalinus*	Chad (Africa)	EU203586
Undetermined	AMB8	*Macrotermes bellicosus*	Benin (Africa)	EU203582
Undetermined	AMB2	*Macrotermes bellicosus*	Burkina Faso (Africa)	EU164406
Undetermined	AMB11	*Macrotermes bellicosus*	Togo (Africa)	EU203587
Undetermined	AMB12	*Macrotermes bellicosus*	Togo (Africa)	EU203588
Undetermined	AAG5	*Ancistrotermes cavithorax*	Togo (Africa)	EU164400
Undetermined	AM3	*Microtermes sp.*	Burkina Faso (Africa)	EU164408
Undetermined	ASMC3	*Macrotermes carbonarius*	Vietnam (Asia)	EU164404
Undetermined	ASMA4	*Macrotermes annandalei*	Vietnam (Asia)	EU203585
Undetermined	ASMG1	*Macrotermes gilvus*	Thailand (Asia)	EU203584
Undetermined	ASMG2	*Macrotermes gilvus*	Thailand (Asia)	EU164402
Undetermined	ASO5	*Odontotermes sp.*	Vietnam (Asia)	EU164405
Xylaria acuta	—	—	MI (USA)	AF163026
Xylaria arbuscula	—	—	California (USA)	AF 163029
Xylaria mali	—	—	Korea (Asia)	AF 163040
Xylaria longipes	—	—	Netherlands(EU)	AF163038
Xylaria enteroleuca	—	—	HI (USA)	AF 163033
Undetermined ascomycete				
Uncultured ascomycete	—	*Odontotermes formosanus*	Japan (Asia)	AB217790
Ascomycota sp.	—	*Odontotermes formosanus*	Japan (Asia)	AB217784
Geniculisynnema termiticola	—	*Odontotermes formosanus*	Japan (Asia)	AB274813
Dyatripe disciformis	—		Spain (EU)	AJ390410

phylogenetic position of termite-associated *Xylaria* was still not defined and it was not clear whether the proliferation of a particular *Xylaria* on fungus combs was due to evolution with termites. It also was not known whether one single *Xylaria* was related to a single termite host or whether a particular *Xylaria* species could occur in nests of various species of termite. The host specificity of termite-associated *Xylaria* needed to be clarified.

The internal transcribed spacer (ITS) region of nuclear ribosomal DNA is a convenient target for phylogenetic analysis in fungi. Characteristics of the ITS region (small size of the region, good intergeneric resolution, weak intraspecific variability, easily amplified using universal primers) associated with the recent expansion of the spacer sequences database make this region extremely useful for resolving lower level relationships (Larena et al 1999, Lee et al 2000, Rouland-Lefevre et al 2002, Belbahri et al 2006, Morakotkarn et al 2007). This study therefore was undertaken to infer the phylogeny of *Xylaria* in the fungus garden of several fungus-growing termite species by using their ITS1-5.8S-ITS2 region sequences. The phylogenetic results were used as the basis for discussion on the host specificity and distribution of termite-associated *Xylaria* species.

MATERIALS AND METHODS

Biological material.—Fungus-growing termite species that built the fungus combs, the localities where they were collected, the isolate codes and GenBank accession numbers of sequences are provided (TABLE I). Fungus combs were kept in a sterile humidified container to promote the formation of *Xylaria* stromata. After 2–5 d at room temperature the elongated stromata were carefully picked up with a sterile stainless steel needle, washed with sterile milliQwater and stored in absolute ethanol at −20 C until DNA extraction was performed.

DNA extraction.—Total DNA was extracted with the method described by Rouland-Lefevre et al (2002). A small piece of stromata (200–250 mg) was crushed in a 2 mL sterile Eppendorf tube containing 500 µL CTAB lysis buffer with zirconium balls. The mixture was homogenized by bead-beating (Retsch® MM 200, Germany) for 2 min at maxi-

mum speed (50 Hz) then incubated 30 min at 65 C. After 2 min centrifugation at 14 000 *g* the supernatant was deproteinized by phenol : chloroform : isoamyl alcohol solution (25 : 24 : 1; v/v/v) and washed with chloroform. After centrifugation 15 min at 14 000 *g* the supernatant was transferred to a phase lock gel tube (Eppendorf) with an equal volume of chloroform isoamyl (24 : 1), and the mixture was homogenized and centrifuged (4 min, 4 C, 14 000 *g*). A total of 500 µL of the supernatant was precipitated with a double volume of polyethylene glycol (30% PEG, 1.6 M NaCl). After 30 min centrifugation at 4 C and 14 000 *g* the supernatant was removed and the pellet was rinsed twice in 70% ethanol, air dried and resuspended in 25 µL TE buffer (10 mM Tris-HCl, pH 8.5). The DNA concentration was quantified with a spectrophotometer (NanoDrop® ND-1000 UV-Vis Spectrophotometer). DNA purity was checked at 260–280 nm and before PCR amplification each DNA extract was diluted to a final concentration of 50 ng μL^{-1}.

Polymerase chain reaction and sequencing of the ITS region.—The entire region ITS1-5.8S-ITS2 region was amplified by PCR. The reaction mix was a total volume of 23 µL *Taq Polymerase* Ready-To-Go (Amersham Pharmacia) with 0.2 mL each primer (100 pM) and 2 µL DNA solution. The tubes were placed in a thermal cycler (GenAmp PCR system 2400; Perkin-Elmer) for amplification by 40 cycles of (i) denaturation at 94 C for 15 s, (ii) annealing at 64 C for 30 s and (iii) extension at 72 C for 90 s. PCR amplification was terminated by a final elongation for 10 min at 72 C. The primers for the amplification were ITS5 (White et al 1990) and ITS4A (Larena et al 1999). Amplification products were purified with a QIAquick PCR purification kit protocol (QIAGEN) and electrophoresed on a 1% agarose gel. The purified products were used for the sequencing reaction performed by Society Genome Express (Grenoble, France).

Phylogenetic analysis.—The ITS region of 18 *Xylaria* samples from termite fungus combs, three ascomycetes associated with termite nest, five other *Xylaria* species (chosen according to the phylogeny of *Xylaria* proposed by Okane and Nakagiri 2007) and a related fungus (*Diatrype disciformis*) from GenBank were used (TABLE I). The alignments were performed with the multiple alignment program Clustal X (Thompson et al 1997). The sequence alignment was corrected manually, focusing on gap positions. DNA sequence data was analyzed to provide pairwise percentage sequence divergence. In all analyses *Diatrype disciformis* was used as outgroup. The heuristic search option and the neighbor joining (NJ) method (Saitou and Nei 1987) of PAUP (Swofford 1993) were used respectively for parsimony and distance analyses. All characters were weighted equally, and gaps were treated as missing data. Bootstrap confidence intervals on each branching pattern were calculated from 1000 replications of resampling (Felsenstein 1985).

RESULTS

Several samples studied were found to have ITS sequences with less than 1% divergence. They were considered to have the same sequences. Sequences of AO7, ASO5, AMS9, AMS10, ASMA4, AMB8, MB11, MB12 and MS13 were identical, and sequences of ASMG1 and ASMG2 were similar to each other. According to work on fungal ITS region only one sequence from each group, ASO5 and ASMG2 respectively, was used for the construction of the trees (Rouland et al 2002).

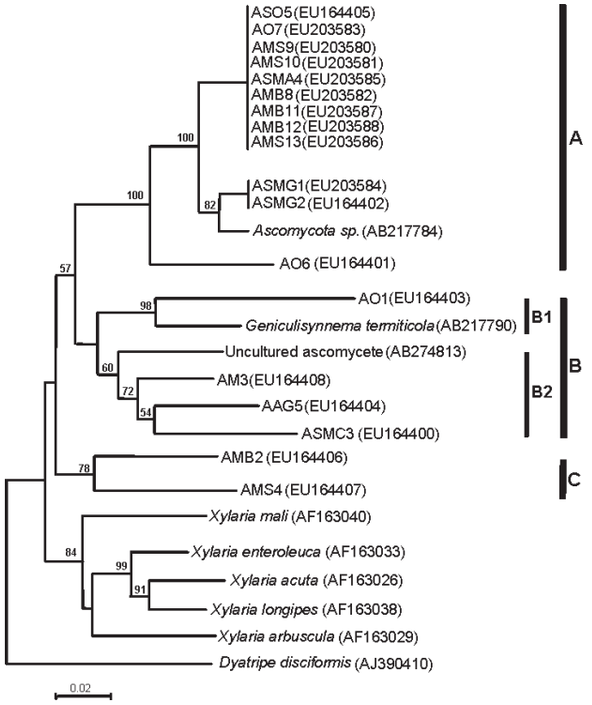

FIG. 1. Phylogenetic tree of termite-associated *Xylaria* inferred by neighbor joining analysis of the ITS1-5.8S-ITS2 sequences. *Diatrype disciformis* (accession No. AJ390410) was used as outgroup. Percentages of 1000 bootstrap resampled datasets are indicated on corresponding branches only for values > 50. Vertical bars and capital letters are used for the three clades (A, B, C) and subclades (B1, B2) as discussed in the text. Sequences obtained during this study are identified by their strain codes and GenBank accession numbers. The sequences of *X. arbuscula*, *X. mali*, *X. enteroleuca*, *X. acuta* and *X. longipes* are taken from Lee et al (2000); uncultured Ascomycete (accession No. AB217790) and Ascomycota sp. (accession No. AB217784) from Shinzato et al (2005) and *Geniculisynnema termiticola* (gen. and sp. nov.) from Okane and Nakagiri (2007).

Phylogenetic relationships inferred from the ITS1-5.8S-ITS2 region sequences of *Xylaria* species and related fungi are shown (FIG. 1), which is the tree produced with neighbor joining. Three equally parsimonious trees were obtained by parsimony analysis. Some differences were noted in the branching pattern for the positions of *Xylaria arbuscula*, which was supported by low bootstrap values, but they

were topologically identical to the NJ tree. The phylogenetic tree produced with neighbor joining showed that all termite-associated *Xylaria* formed a single clade (FIG. 1), although it was not well supported by bootstrap values. This clade was divided into three groups, A, B and C. Group A included 11 of the 18 sequences obtained in this study and one undetermined fungus also isolated from fungus comb. This cluster was well supported by a bootstrap value of 100%. Cluster B contained two subclades, B1 and B2. Subclade B1, which included AO1 (from *O. pauperans*) and *Geniculisynnema termiticola*, was well supported by a bootstrap value of 99%, whereas subclade B2, which included AAG5, AM3, ASMC3 and the uncultured ascomycete (GenBank accession No. AB217790) also isolated from fungus comb, was supported by a bootstrap value of 60%. Clade C, which was fairly well supported by bootstrap analysis, included strains AMB2 (from *M. bellicosus*) and AMS4 (from *M. subhyalinus*). All other *Xylaria* species used in this analysis merged into a single cluster that was clearly distinct from clade ABC (FIG. 1).

DISCUSSION

This study attempted to determine the genetic diversity among termite-associated *Xylaria* species and to define the relationship between them and their termite hosts. The phylogenetic analysis performed in this study clearly demonstrated that termite-associated *Xylaria* formed a monophyletic group, although this was only partially supported by bootstrap values, particularly regarding the position of species from Group B, which remained unstable. These results supported the phylogenetic study of Visser et al (2009), which also showed that all termite-associated *Xylaria* cluster together. Lee et al (2000) showed that all endophytic *Xylaria* species in their study merged into a single clade. These studies indicate clearly that genus *Xylaria* was nonmonophyletic (Brunner and Petrini 1992, Guo et al 2000, Davis et al 2003).

Eighteen taxa of 11 fungus-growing species from eight localities were used in this study. Only nine strains of *Xylaria* were found on termite fungus combs with one strain closely related to five termite genera, according to the sequences obtained. This indicated that only a few *Xylaria* species occurred on the comb. It is interesting to note that no sequences from *Xylaria* matched any of those isolated by Ju and Hsieh (2007) from *Odontotermes formosanus* nests. This could indicate that the *Xylaria* isolated from the termites comb were different from those present in the nest. One termite-associated *Xylaria* was able to grow on the fungus comb of several termite species. The occurrence of one particular *Xylaria* species in a given nest therefore is not restricted to one single fungus-growing termite species. No evidence supported the occurrence of two or more *Xylaria* taxa on the comb of one particular termite species collected from one particular environment. However Okane and Nakagiri (2007) found two xylariaceous fungi together on an *Odontotermes formosamus* comb. The first was identified as *X. angulosa* and the second as *Geniculisynnema termiticola* gen. and sp. nov. This new genus and species used in our study was clustered with the termite-associated Xylariaceae group as well as both ascomycete fungi taken from one particular *O. formosamus* colony (Shinzato et al 2005). The fact that, unlike the other endophytic *Xylaria*, genus *Geniculisynnema* was clustered with the clade of the strains isolated during this study raised the question about the effective affiliation of termite-associated Xylariaceae to genus *Xylaria*. Several authors believe that, on the basis of taxonomic characteristics, xylariaceous fungi found associated with fungus-growing termite nests are significantly different from other *Xylaria* species and that they therefore should be placed within another genus (see reference in Rogers et al 2005). Furthermore nucleotide divergence comparisons (data not shown) revealed a significant difference between *Xylaria* species associated with fungus-growing termite nests and those commonly found as wood endophytes. The new molecular data provided by our study and the special niche (i.e. fungus-growing termite comb) strongly support placing termite-associated *Xylaria* at least in a specific subgenus.

The main hypothesis for this study was that the *Xylaria* species found on fungus comb might have evolved with termites, but the findings appear to show no co-evolution between *Xylaria* and hosts. Furthermore, when geographical origins were correlated with the *Xylaria* taxa, the results suggested that geographical location did not play a major role in the distribution of *Xylaria* species on fungus comb and one particular *Xylaria* species was found to be associated with several termite genera collected from different environments. Visser et al (2009) concluded the same but their samples, originated from South Africa only, were less informative on this topic. Based on our results, termite-associated *Xylaria* cannot be considered termite symbionts like *Termitomyces* spp. Sreerama and Veerabhadrappa (1993) also suggested, with regard to enzymatic activity, that there might not be any symbiotic association between the termite gut-associated fungus *Xylaria nigripes* and its termite host *Odontotermes horni*. However the other *Xylaria*, most of which are endophytic species, were vertically and/or horizontally transmitted through seeds of their hosts (Bayman et al 1998, Davis et al 2003).

In a comparable insect mutualistic association (fungus-growing ant symbiosis) some parallels were drawn between termite-associated *Xylaria* and an Ascomycete fungus of genus *Escovopsis*, which infected leaf-cutter ant gardens to the detriment of colony growth and crop productivity (Mueller and Gerardo 2002). Phylogenetic studies had shown that *Escovopsis* was a specialized parasite of fungus-growing ant symbiosis and evolved with the hosts (Currie et al 2003), but this study did not reveal any evidence of a similar pattern between *Xylaria* and fungus-growing termites.

Does the fungus select the termite nest as their particular type of habitat? It could be argued that the *Xylaria* species found on fungus comb unlike other *Xylaria* are specialized for this substrate and therefore probably are found in saprophytic association with termite nests. Furthermore grooming, engineering activity and contact between termite species helped to disperse *Xylaria* spores to a number of nests. It also should be noted that, as *Xylaria nigripes* had already been isolated from termite guts (Sreerama and Veerabhadrappa 1993), winged reproductive termites might also transmit termite-associated *Xylaria* and inoculate new colonies. Termite-associated *Xylaria* on fungus combs might play a role similar to that played by other *Xylaria* species (i.e. awaiting host senescence and further decomposition of the substrate). This strategy has the advantage over other saprophytic species because it claims the cell materials before the start of decomposition (Davis et al 2003). This could explain why termite-associated *Xylaria* were not observed on active combs and why they seemed more competitive and rapidly overgrew only when the combs had been removed from the nest or abandoned by the termites. Wood and Thomas (1989) also suggested that *Xylaria* species might be in a dormant state on fungus combs and become active only if combs died or were removed from the nest. However no evidence shows *Xylaria* species either in mycelium form or in ungerminated spores in active combs (i.e. with termites). It might be that the comb microclimate conditions (temperature, humidity, CO_2, etc) are more favorable to the growth of *Xylaria* species when termites leave the combs.

This work was the first attempt to infer the molecular phylogeny of termite-associated *Xylaria* and to investigate the relationship between termite-associated *Xylaria* and hosts. However the isolation technique used to obtain *Xylaria* strain did not let us cover combs unable to produce *Xylaria* stromata. It is possible therefore that these samples also contain *Xylaria* species that were not detected under experimental conditions. Specific probes designed with the new molecular data provided by this study and DNA extraction directly from Macrotermitinae fungus combs and worker gut contents might be a further line of research.

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