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Levels of specificity of *Xylaria* species associated with fungus-growing termites: a phylogenetic approach

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Abstract

Fungus-growing termites live in obligate mutualistic symbiosis with species of the basidiomycete genus *Termitomyces*, which are cultivated on a substrate of dead plant material. When the termite colony dies, or when nest material is incubated without termites in the laboratory, fruiting bodies of the ascomycete genus *Xylaria* appear and rapidly cover the fungus garden. This raises the question whether certain *Xylaria* species are specialised in occupying termite nests or whether they are just occasional visitors. We tested *Xylaria* specificity at four levels: (1) fungus-growing termites, (2) termite genera, (3) termite species, and (4) colonies. In South Africa, 108 colonies of eight termite species from three termite genera were sampled for *Xylaria*. *Xylaria* was isolated from 69% of the sampled nests and from 57% of the incubated fungus comb samples, confirming high prevalence. Phylogenetic analysis of the ITS region revealed 16 operational taxonomic units of *Xylaria*, indicating high levels of *Xylaria* species richness. Not much of this variation was explained by termite genus, species, or colony; thus, at level 2–4 the specificity is low. Analysis of the large subunit rDNA region, showed that all termite-associated *Xylaria* belong to a single clade, together with only three of the 26 non-termite-associated strains. Termite-associated *Xylaria* thus show specificity for fungus-growing termites (level 1). We did not find evidence for geographic or temporal structuring in these *Xylaria* phylogenies. Based on our results, we conclude that termite-associated *Xylaria* are specific for fungus-growing termites, without having specificity for lower taxonomic levels.

Keywords: fungus-growing termite, host specificity, Macrotermitinae, mutualistic symbiosis, phylogeny, *Xylaria*

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Introduction

Symbioses, intimate interactions between different species, are widespread. They range from being beneficial to one species at the cost of the other (parasitic) to being mutually beneficial (mutualistic). Mutualistic symbioses often play a dominant role in ecosystems, as the combined characteristics of two different organisms in a mutualism allow them to exploit previously inaccessible niches (Herre *et al*. 1999).

An impressive example of mutualistic symbiosis is the mutualism between termites of the subfamily Macrotermitinae and fungi of the basidiomycete genus *Termitomyces* (Darlington 1994; Aanen *et al*. 2002). The termites provide *Termitomyces* with faecal pellets of finely comminuted dead plant material and create a climate where *Termitomyces* can thrive on this substrate. In return, *Termitomyces* degrades

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the pellets, and thereby provides digestible and nutritious material for the termites (Sands 1969; Wood & Thomas 1989). The sponge-shaped structure of faecal pellets, called fungus comb, is overgrown with *Termitomyces* (Katoh *et al.* 2002; Moriya *et al.* 2005; Shinzato *et al.* 2005; Aanen 2006). The mutualistic symbiosis between fungus-growing termites and their fungal symbionts is the result of long-term co-evolution (reciprocal genetic adaptation), during which apparently no reversal to free-living state of either of the partners has occurred (Aanen *et al.* 2002).

When symbiotic partners have a high fidelity towards each other, the process of co-evolution may result in co-speciation or co-cladogenesis (Wade 2007). The latter is reflected in similar phylogenetic tree topologies of both partners. In the fungus-growing termite mutualism, where termites and *Termitomyces* are mutually dependent, the tree topologies show signs of co-cladogenesis, mainly at the termite genus level (Aanen *et al.* 2002, 2007; Rouland-Lefèvre *et al.* 2002).

Like in many other symbioses, the focus has so far mainly been on the two most obvious players in the symbiosis. However, the list with examples of multi-partner symbioses is growing. To name just a few, in the lower termite family Rhinotermitidae, there is a three-partner association between termites, protists and bacteria (Noda *et al.* 2007); a parasite has been discovered that plays a stabilising role in the fig-pollinator mutualism (Dunn *et al.* 2008), and in fungus-growing ants even more symbionts co-occur: currently that symbiosis counts five described partners (Little & Currie 2007). It seems that multi-partner symbiosis is not an exception, but rather the rule (Sachs & Simms 2006).

Also in nests of fungus-growing termites, many organisms other than termites and *Termitomyces* have been found: inquiline flies (Gumming 1996), a range of arthropods (Batra & Batra 1979), bacteria (Shinzato *et al.* 2005; Hongoh *et al.* 2006), and many fungi (Sands 1969; Thomas 1987b; Shinzato *et al.* 2005). Especially members of the ascomycete genus *Xylaria* have been frequently reported from fungus-growing termite nests (Ju & Hsieh 2007; Rogers *et al.* 2005; Okane & Nakagiri 2007). Visible structures of *Xylaria* typically occur when termite nests are dead or decaying (Rogers *et al.* 2005). When *Xylaria* species emerge, they cover fungus combs throughout the fungus garden with mycelium, stromata, and synnemata, some with ascomal initials (Rogers *et al.* 2005). When fungus comb from a healthy nest is incubated in the absence of termites, it is often covered by a vigorous mycelium of *Xylaria* within a few days (Batra & Batra 1979; Thomas 1987c; Shinzato *et al.* 2005; Okane & Nakagiri 2007). Could *Xylaria* be a third symbiont in the fungus-growing termite mutualistic symbiosis?

The nature of *Xylaria* in the nests of fungus-growing termites has been a point of debate. Thomas (1987a) observed that all fungi isolated from a fungus comb also occurred in the surrounding soil, except for *Termitomyces* and *Xylaria*, which suggests specificity of these two types of fungi for fungus-growing termites. Sannasi (1969) described *X. nigripes* as the cultivated symbiont of *Odontotermes redemanni*, without mentioning *Termitomyces*. Batra & Batra (1979) claimed that *Xylaria* is an additional symbiont, growing in the comb and enhancing the breakdown of lignin by *Termitomyces*. In contradiction with a beneficial role, there are records stating that *Xylaria* is being suppressed in the fungus garden (Thomas 1987c), and thus may be seen as an antagonistic instead of a beneficial symbiont (Moriya *et al.* 2005). Beneficial or not, Rogers *et al.* (2005) posed that certain *Xylaria* species (e.g. *X. escharoidea*, *X. furcata* and *X. nigripes*) have co-evolved with termites, while other species may be associated with termites as saprotrophs or in other less- specific ways. The latter *Xylaria* species could behave as opportunistic weeds, competing with *Termitomyces* for substrate and benefiting from the unique, relatively competition-free niche. There is thus still no consensus about the nature of fungus-growing termite-associated *Xylaria*.

Here, we investigate whether *Xylaria* is specialised on fungus-growing termites. In other words, do certain *Xylaria* species specifically and perhaps exclusively occur in nests of fungus-growing termites? Do termite-associated *Xylaria* show signs of co-evolution with fungus-growing termites like the cultivated *Termitomyces* does? We approach these questions about *Xylaria* specificity for fungus-growing termites by estimating the phylogenetic relationships between *Xylaria* isolates from termite nests and *Xylaria* isolates that are not associated with termites. We test the specificity of *Xylaria* for fungus-growing termites at four levels: (1) fungus-growing termites (Macrotermitinae), (2) termite genera, (3) termite species, and (4) termite colonies (nests).

Materials and methods

Collecting field samples and general methods

Xylaria was isolated from field samples collected in 2003, 2005 and 2007 at 12 different sites across the northeastern part of South Africa (Table 1). Comb samples were taken from nests of eight species of fungus-growing termites belonging to the genera *Macrotermes*, *Microtermes* and *Odontotermes*. Sampling to isolate *Xylaria* was done down to the scale of fungus combs within a nest and sections within a fungus comb.

Material from the field was stored at 5 °C, and processed within 2 days after collecting. All fungal isolations were done on malt–yeast–agar plates (20 g/L malt extract, 2 g/L yeast extract, 15 g/L agar). All incubations were at 25 °C. The first fungus comb samples of 2003 were split; one piece was incubated in light and the other in the dark. Since no differences in growth of *Xylaria* were observed, all further incubations were in the dark.

Table 1 Origin of sequences of *Xylaria* isolates from South African fungus-growing termite nests. 'ITS OTU' codes in bold indicate isolates of which also the LSU region was sequenced. *Full site descriptions: Pretoria1 = L.C. de Villiers sports grounds, University of Pretoria; Pretoria2 = PPRI-farm, Pretoria; Pretoria3 = Rietondale, Pretoria; Estcourt1 = between White Mountain lodge and Estcourt; Estcourt2 = along road to Estcourt; Badplaas = Vijgeboomdam, Badplaas; Blairbeth = farmland northwest of Blairbeth; Naboomspruit = Amsterdam farm, Naboomspruit; Pienaar's River = SABS farm Radium, Pienaar's River; Pietersburg = dam, New Pietersburg

Date	Nest	Comb	Isolate	Termite taxon	Site*	ITS type	ITS OTU	LSU type	GenBank Accession no.
2003-01-29	317		317	*Odontotermes*	Pietersburg	1.01	1		
2003-01-31	320		320	*Odontotermes latericius*	Pretoria2	1.01	1		
2003-01-31	324		324	*Odontotermes latericius*	Pretoria2	1.01	1		
2003-01-31	327		328	*Odontotermes latericius*	Pretoria2	1.01	1		
2003-02-06	352		353	*Odontotermes transvaalensis*	Pienaar's River	1.01	1		
2007-02-18	707	E	707.E3	*Macrotermes natalensis*	Badplaas	1.01	1		
2003-01-31	326	L	326.L	*Odontotermes latericius*	Pretoria2	1.01	1		
2005-11-22	501	3	501.3a	*Macrotermes natalensis*	Pretoria2	1.01	1		
2005-11-22	501	3	501.3c	*Macrotermes natalensis*	Pretoria2	1.01	1		
2005-11-24	502	2	502.2b	*Odontotermes*	Nabooomspruit	1.01	1		
2005-11-24	504	3	504.3j	*Odontotermes*	Nabooomspruit	1.01	1		
2005-11-24	505	15	505.15j	*Macrotermes natalensis*	Nabooomspruit	1.01	1		
2005-11-24	505	17	505.17j	*Macrotermes natalensis*	Nabooomspruit	1.01	1		
2005-11-29	512	1	512.1a	*Odontotermes*	Pienaar's River	1.01	1		
2007-02-14	702	A	702.A	*Macrotermes natalensis*	Pretoria3	1.01	1		
2007-02-14	702	C	702.C	*Macrotermes natalensis*	Pretoria3	1.01	1		
2007-02-14	702	E	702.E	*Macrotermes natalensis*	Pretoria3	1.01	1		
2007-02-14	702	F	702.F	*Macrotermes natalensis*	Pretoria3	1.01	1		
2007-02-14	702	H	702.H	*Macrotermes natalensis*	Pretoria3	1.01	1		
2007-02-14	702	K	702.K	*Macrotermes natalensis*	Pretoria3	1.01	1		
2007-02-14	702	L	702.L	*Macrotermes natalensis*	Pretoria3	1.01	1		
2007-02-18	707	H	707.H	*Macrotermes natalensis*	Badplaas	1.01	1		
2007-02-18	708	B	708.B	*Odontotermes*	Badplaas	1.01	1		
2007-02-18	708	H	708.H	*Odontotermes*	Badplaas	1.01	1		
2007-02-25	715	A	715.A	*Macrotermes michaelseni*	Blairbeth	1.01	1		
2007-02-25	715	D	715.D	*Macrotermes michaelseni*	Blairbeth	1.01	1		
2007-02-25	715	F	715.F	*Macrotermes michaelseni*	Blairbeth	1.01	1		
2007-02-25	715	G	715.G	*Macrotermes michaelseni*	Blairbeth	1.01	1		
2007-02-25	715	H	715.H1	*Macrotermes michaelseni*	Blairbeth	1.01	1		
2007-02-25	715	I	715.I	*Macrotermes michaelseni*	Blairbeth	1.01	1		
2007-03-04	716	A	716.A	*Macrotermes natalensis*	Estcourt1	1.01	1		
2007-03-04	716	B	716.B	*Macrotermes natalensis*	Estcourt1	1.01	**1A**	1	FJ425654
2007-03-04	716	E	716.E	*Macrotermes natalensis*	Estcourt1	1.01	1		
2007-03-04	717	A	717.A	*Macrotermes natalensis*	Estcourt2	1.01	1		
2005-12-10	534	1	534.1j	*Odontotermes*	Nabooomspruit	1.02	1		FJ425655
2005-11-24	505	19	505.19	*Macrotermes natalensis*	Nabooomspruit	1.03	1		FJ425656
2003-01-31	323		323	*Odontotermes latericius*	Pretoria2	1.04	1		FJ425657
2007-03-13	721	B	721.B	*Odontotermes badius*	Pretoria2	1.05	1		FJ425658
2005-11-22	501	2	501.2c	*Macrotermes natalensis*	Pretoria2	1.06	1		FJ425659
2005-11-24	505	16	505.16d	*Macrotermes natalensis*	Nabooomspruit	1.07	1		FJ425660
2005-11-24	505	18	505.18a	*Macrotermes natalensis*	Nabooomspruit	1.07	1		
2007-02-14	702	M	702.M	*Macrotermes natalensis*	Pretoria3	1.07	1		
2007-02-25	715	H	715.H2	*Macrotermes michaelseni*	Blairbeth	1.08	1		FJ425661
2005-11-24	502	3	502.3j	*Odontotermes*	Nabooomspruit	1.09	1		FJ425662
2005-11-24	505	12	505.12c	*Macrotermes natalensis*	Nabooomspruit	1.09	1		
2007-03-13	721	C	721.C	*Odontotermes badius*	Pretoria2	1.09	1		
2005-12-08	527	1	527.1d	*Macrotermes natalensis*	Pretoria2	1.10	**1B**	1	FJ425663
2003-01-28	301		301	*Macrotermes natalensis*	Pretoria2	1.11	1		
2003-01-28	307		307	*Odontotermes badius*	Pretoria2	1.11	1		
2003-01-29	313		313	*Macrotermes natalensis*	Pietersburg	1.11	1		
2003-01-31	322		322	*Odontotermes latericius*	Pretoria2	1.11	1		

Table 1 *Continued*

Date	Nest	Comb	Isolate	Termite taxon	Site*	ITS type	ITS OTU	LSU type	GenBank Accession no.
2003-01-31	326		326	*Odontotermes latericius*	Pretoria2	1.11	1		
2003-01-31	332		332	*Odontotermes badius*	Pretoria2	1.11	1		
2003-02-06	350		350	*Odontotermes latericius*	Pienaar's River	1.11	1		
2003-02-06	351		351	*Odontotermes transvaalensis*	Pienaar's River	1.11	1		
2002-02-19	366		366	*Macrotermes*	Pietermaritzburg	1.11	1		
2007-02-17	706	E	706.E1	*Macrotermes natalensis*	Badplaas	1.11	1		
2003-02-02	342	L	342.L	*Macrotermes natalensis*	Pretoria2	1.11	1		
2005-11-22	501	6	501.6b	*Macrotermes natalensis*	Pretoria2	1.11	1		
2005-11-22	501	8	501.8a	*Macrotermes natalensis*	Pretoria2	1.11	1		
2005-11-24	504	5	504.5c	*Odontotermes*	Nabooomspruit	1.11	1		
2005-11-24	505	12	505.12b	*Macrotermes natalensis*	Nabooomspruit	1.11	1		
2005-12-01	518	6	518.6c	*Odontotermes*	Pretoria1	1.11	1		
2005-12-01	518	IO	518.IO5	*Odontotermes*	Pretoria1	1.11	1		
2007-02-14	701	R	701.R	*Macrotermes natalensis*	Pretoria3	1.11	1		
2007-02-14	702	G	702.G	*Macrotermes natalensis*	Pretoria3	1.11	1		
2007-02-14	702	J	702.J	*Macrotermes natalensis*	Pretoria3	1.11	**1C**	1	FJ425664
2007-02-14	704	C	704.C	*Macrotermes natalensis*	Pretoria3	1.11	1		
2007-02-14	704	L	704.L	*Macrotermes natalensis*	Pretoria3	1.11	1		
2007-02-17	705	J	705.J	*Macrotermes natalensis*	Badplaas	1.11	1		
2007-02-17	706	D	706.D	*Macrotermes natalensis*	Badplaas	1.11	1		
2007-02-17	706	J	706.J1	*Macrotermes natalensis*	Badplaas	1.11	1		
2007-02-18	708	E	708.E	*Odontotermes*	Badplaas	1.11	1		
2007-02-18	708	F	708.F	*Odontotermes*	Badplaas	1.11	1		
2007-02-25	715	E	715.E	*Macrotermes michaelseni*	Blairbeth	1.11	1		
2007-03-04	717	C	717.C	*Macrotermes natalensis*	Estcourt2	1.11	1		
2007-02-17	725	G	725.G2	*Macrotermes natalensis*, dead nest	Badplaas	1.11	1		
2005-11-29	509	1	509.1j	*Odontotermes*	Pienaar's River	1.12	1		FJ425665
2005-11-24	502	4	502.4d	*Odontotermes*	Nabooomspruit	1.13	**1D**	1	FJ425666
2005-11-24	504	7	504.7j	*Odontotermes*	Nabooomspruit	2.01	**2**	2	FJ425667
2005-12-01	518	I	518.I9	*Odontotermes*	Pretoria1	2.01	2		
2005-12-01	518	HO	518.HO2	*Odontotermes*	Pretoria1	2.02	2		FJ425668
2005-12-01	518	HO	518.HO1	*Odontotermes*	Pretoria1	2.03	2		FJ425669
2003-01-31	325		325	*Microtermes* I	Pretoria2	3.01	3		
2003-02-02	336		337	*Microtermes* I	Pretoria2	3.01	3		
2005-11-29	517	A	517.A	*Microtermes*	Pienaar's River	3.01	**3A**	3	FJ425670
2003-01-29	309		309	*Microtermes* I	Pietersburg	3.02	**3B**	3	FJ425671
2003-01-29	311		311	*Microtermes* I	Pietersburg	3.02	3		
2003-02-02	335		335	*Macrotermes natalensis*	Pretoria2	4.01	**4**	4	FJ425672
2005-12-01	518	F	518.F8	*Odontotermes*	Pretoria1	5.01	**5**	5	FJ425673
2005-11-29	508	1	508.1j	*Odontotermes*	Pienaar's River	6.01	**6**	6	FJ425674
2003-02-02	341		341	*Microtermes* I	Pretoria2	7.01	**7**	7	FJ425675
2003-02-02	342	D	342.D	*Macrotermes natalensis*	Pretoria2	7.02	7		FJ425676
2003-02-06	352		352	*Odontotermes transvaalensis*	Pienaar's River	8.01	8		FJ425677
2003-01-31	327		327	*Odontotermes latericius*	Pretoria2	9.01	9		
2003-02-02	344		344	*Odontotermes badius*	Pretoria2	9.01	9		
2003-02-02	346		346	*Odontotermes badius*	Pretoria2	9.01	9		
2003-02-06	355		355	*Odontotermes transvaalensis*	Pienaar's River	9.01	9		
2005-11-24	504	4	504.4j	*Odontotermes*	Nabooomspruit	9.01	9		
2007-02-17	706	G	706.G	*Macrotermes natalensis*	Badplaas	9.01	**9A**	9A	FJ425678
2007-02-18	708	D	708.D1	*Odontotermes*	Badplaas	9.01	9		
2007-03-13	720	A	720.A	*Odontotermes badius*	Pretoria2	9.01	9		
2007-03-13	720	B	720.B	*Odontotermes badius*	Pretoria2	9.01	9		
2007-03-13	720	C	720.C	*Odontotermes badius*	Pretoria2	9.01	9		
2007-03-13	720	D	720.D	*Odontotermes badius*	Pretoria2	9.01	9		
2007-03-13	721	A	721.A	*Odontotermes badius*	Pretoria2	9.01	9		

Table 1 *Continued*

Date	Nest	Comb	Isolate	Termite taxon	Site*	ITS type	ITS OTU	LSU type	GenBank Accession no.
2005-11-29	511	1	511.1j	*Odontotermes*	Pienaar's River	9.02	9		FJ425679
2005-11-05	504	5	504.5j	*Odontotermes*	Nabooomspruit	9.03	9		FJ425680
2005-12-01	518	2	518.2c	*Odontotermes*	Pretoria1	9.04	9		FJ425681
2003-01-31	321		321	*Odontotermes latericius*	Pretoria2	9.05	9		
2005-12-01	518	1	518.1c	*Odontotermes*	Pretoria1	9.05	9		
2007-02-18	708	G	708.G	*Odontotermes*	Badplaas	9.05	**9B**	9B	FJ425682
2007-02-18	708	B	708.B1	*Odontotermes*	Badplaas	9.05	**9C**	9B	
2005-11-24	504	8	504.8a	*Odontotermes*	Nabooomspruit	9.06	9		FJ425683
2007-02-14	702	I	702.I	*Macrotermes natalensis*	Pretoria3	10.01	10		FJ425684
2005-11-22	501	11	501.11c	*Macrotermes natalensis*	Pretoria2	10.02	10		
2007-02-18	707	F	707.F1	*Macrotermes natalensis*	Badplaas	10.02	**10**	10	FJ425685
2007-02-18	707	G	707.G2	*Macrotermes natalensis*	Badplaas	10.03	10		FJ425686
2007-02-24	711	C	711.C	*Macrotermes natalensis*	Matlhase	11.01	**11**	11	FJ425687
2007-02-25	715	C	715.C	*Macrotermes michaelseni*	Blairbeth	11.01	11		
2003-01-29	310		310	*Microtermes* III	Pietersburg	12.01	12	12	FJ425688
2003-02-02	338		338	*Macrotermes natalensis*	Pretoria2	13.01	**13**	13	FJ425689
2003-02-02	343		343	*Macrotermes natalensis*	Pretoria2	13.01	13		
2003-02-06	349		349	*Microtermes* IV	Pienaar's River	14.01	14		FJ425690
2007-02-17	725	B	725.B	*Macrotermes natalensis*, dead nest	Badplaas	15.01	15		FJ425691
2007-03-08	718	B	718.B	*Macrotermes natalensis*	Nabooomspruit	15.02	**15A**	15	FJ425692
2007-02-17	706	A	706.A2	*Macrotermes natalensis*	Badplaas	15.03	15		FJ425693
2007-02-17	725	C	725.C	*Macrotermes natalensis*, dead nest	Badplaas	15.04	15		FJ425694
2007-02-17	725	G	725.G	*Macrotermes natalensis*, dead nest	Badplaas	15.04	15		
2007-02-18	707	E	707.E2	*Macrotermes natalensis*	Badplaas	15.05	15		
2007-02-14	701	P	701.P	*Macrotermes natalensis*	Pretoria3	15.05	**15B**	15	FJ425695
2007-02-17	706	J	706.J2	*Macrotermes natalensis*	Badplaas	15.05	15		
2007-02-17	725	E	725.E	*Macrotermes natalensis*, dead nest	Badplaas	15.05	15		
2007-02-17	725	F	725.F	*Macrotermes natalensis*, dead nest	Badplaas	15.05	15		
2007-02-17	706	L	706.L	*Macrotermes natalensis*	Badplaas	15.06	15		FJ425696
2007-02-18	707	I	707.I2	*Macrotermes natalensis*	Badplaas	15.06	15		
2007-02-17	725	D	725.D	*Macrotermes natalensis*, dead nest	Badplaas	15.06	15		
2007-02-17	706	C	706.C	*Macrotermes natalensis*	Badplaas	15.07	15		FJ425697
2007-02-14	703	B	703.B1	*Macrotermes natalensis*	Pretoria3	15.08	15		FJ425698
2007-02-18	707	C	707.C2	*Macrotermes natalensis*	Badplaas	15.09	**15C**	15	FJ425699
2007-02-18	707	D	707.D2	*Macrotermes natalensis*	Badplaas	15.10	**15D**	15	FJ425700
2007-02-17	705	H	705.H	*Macrotermes natalensis*	Badplaas	16.01	**16**	16	FJ425701

Isolating Xylaria *from fungus combs*

A fragment of each fungus comb (±100 cm^3, except for comb fragments of *Microtermes*, which were ±15 cm^3) was incubated in a sealed cup, to which a paper tissue soaked in sterile demineralised water (DEMI) was added to make a moist chamber. *Xylaria* that developed was transferred to plates. Additionally, to ensure having material for DNA extraction, fungal tissue was taken directly from the comb, put in 96% EtOH and stored at –20 °C.

Some fungus combs were also sampled on a finer scale. They were divided in three sections: young, medium and old, based on colour and structure (Thomas 1987c). Five samples of ±5 mm^3 for each of the three sections per comb were taken and put on plates. Appearing fungi were serially transferred to fresh plates until pure.

Pure cultures were grown on cellophane plates. After three or more days, the mycelium was harvested from the cellophane and stored at –80 °C until further processing.

Isolating Xylaria *from adjacent vegetation*

Three vegetation samples were taken within a 5-m radius around the termite nest. Material that showed marks of termite foraging, mostly wood, was preferred for sampling. On one occasion, dry cow dung with prominent termite feeding corridors was sampled.

Grass, dead wood (including woody herbs), and fresh wood samples were processed in different ways. Grass samples were cut in 1–2 cm pieces, washed by shaking for 20 s in 10 mL DEMI and put on plates. Dead wood samples were cut to core pieces of 0.5 to 2 cm^3, swiftly moved through a Bunsen burner flame, and put on plates. Fresh wood samples were surface sterilised by washing for 1 min in 70% EtOH, 2 min in sodium hypochlorite, 1 min in 96% EtOH, and 30 s shaking in sterile tap-water. They were then dried in brown paper bags for two weeks and finally processed as described for the dead wood samples.

Ten subsamples per vegetation sample were put on plates and incubated. Appearing fungi were transferred serially to fresh plates until pure and further treated as described above.

Extracting DNA, PCR and sequencing

Table 1 gives an overview of the origin of all sequences. DNA was extracted using three protocols: (i) the QIAGEN DNeasy plant kit for 2003-isolates, (ii) the chloroform-phenol extraction method (Sambrook *et al*. 1989) for 2005-isolates, and (iii) the Chelex extraction method for 2005 and 2007 isolates.

Polymerase chain reaction (PCR) amplification of the ribosomal RNA gene regions ITS1, 5.8S and ITS2 was done using the primers ITS1 and ITS4 (5′-TCCGTAGGTGAACCTGCGG-3′ and 5′-TCCTCCGCTTATTGATATGC-3′, respectively; White *et al*. 1990). PCR amplification of approximately 800 bp of the large subunit (LSU; 28S) ribosomal RNA gene region was done using the primers LR0R and LR5 (5′-ACCCGCTGAACTTAAGC-3′ and 5′-TCCTGAGGGAAACTTCG-3′, respectively; Vilgalys Mycology Lab, Duke University, USA; www.biology.duke.edu/fungi/mycolab/primers.htm).

PCR products were purified with the QIAGEN PCR purification kit or with the Gen Elute PCR Clean-Up kit (Sigma). PCR products were sent to Eurofins MWG Operon Sequencing Department (Martinsried, Germany), where they were sequenced using the primer ITS1 for the ITS region, and LR0R and LR5 for the LSU region.

Estimating phylogeny of Xylaria

Sequences were manually checked and cut to same length in ChromasPro version 1.41 (Technelysium Pty Ltd). The alignments were made in MAFFT version 6 using the L-INS-i method with standard settings (Katoh *et al*. 2005).

ITS sequences were used to test *Xylaria* specificity for termite genus, species and colony (levels 2–4). The phylogenetic tree was estimated using the neighbour-joining (NJ) method and uncorrected distances (Saitou & Nei 1987) in PAUP* version 4.0b10 (Swofford 2002). The NJ tree was midpoint-rooted and branch support values were estimated with 1000 bootstrap samples. Groups of sequences that shared over 97.5% sequence identity were considered as an operational taxonomic unit (OTU). From the ITS tree, *Xylaria* specificity for termite genus, species and nest could be inferred only in a qualitative way.

To quantify *Xylaria* specificity at these levels, an AMOVA in Arlequin version 3.1 (Excoffier & Schneider 2005) was performed with the ITS sequences as input. Differences between *Xylaria* occurrences in nest of termites belonging to different genera were tested with the likelihood ratio test (*G*-test in Sokal & Rohlf 1995), which is approximately distributed as chi-square. Furthermore, BLAST searches were done on the ITS sequences. The origins of the top three BLAST hits were evaluated to check if geographic factors could explain the reconstructed phylogenetic patterns.

Sequences of the more conservative LSU region were used to estimate higher-level phylogenetic relationships between the termite-associated and non-termite-associated *Xylaria*. This way, the specificity of *Xylaria* for fungus-growing termites as a whole (level 1 specificity) could be assessed. Table 2 gives an overview of all LSU sequences that were included in the analysis.

Different groups of isolates were included in the phylogenetic analysis based on the LSU region. First, one up to four isolates of each OTU in the ITS tree (except OTU 8 and OTU 14) were selected for sequencing of the LSU region. This resulted in 15 different LSU sequences. Next, these termite-associated *Xylaria* sequences were blasted, and the top six BLAST hits were included in the LSU data matrix. As many of these hits were shared between OTUs, this resulted in 19 additional LSU sequences. Third, as the retrieved GenBank sequences did not include any African taxa (which is probably due to an under-representation of Africa in studies of Xylariaceae), we obtained 10 South African plant-associated *Xylaria* isolates of which the LSU region was sequenced. This resulted in an additional four different non-termite-associated *Xylaria* LSU sequences. Fourth, to break up the possibly long branch separating the outgroup from the ingroup, we also included three sequences that occurred repeatedly as lower-score BLAST hits. Finally, the LSU phylogeny was rooted with *Sordaria fimicola*, which belongs to the sister group of Xylariales (Sordariales; James *et al*. 2006), as outgroup.

A phylogenetic tree based on the LSU region was estimated using Maximum Likelihood (ML) in PAUP*. Using ModelTest version 3.7 (Posada & Crandall 1998), the optimal nucleotide substitution model for the ML method was calculated; Likelihood settings from best-fit model (TIM + I + G) selected by Akaike information criterion (AIC): Lset Base = (0.2410 0.2203 0.3133); Nst = 6; Rmat = (0.8149 1.8768 0.6456 0.1964 6.5287); Rates = gamma; Shape = 0.4689; Pinvar = 0.6158.

Two different support values for the branches of the ML tree were estimated. First, ML branch support values were estimated, using the Heuristic Search option 'fast step-wise addition' (PAUP*) with 1000 bootstrap samples. Second, the posterior probability of branches was estimated with Bayesian Markov chain Monte Carlo (MCMC) analysis in MrBayes version 3.1.2 (Ronquist & Huelsenbeck 2003). Using MrModeltest version 2.2 (Nylander 2004), the optimal

Table 2 Overview of all LSU sequences used in this study

Name	Ecological origin	Geographic origin	GenBank Accession no.
Ingroup		Sequence identity within ingroup: 94.6–99.6%	
OTU 1	fungus-gr. termite nest	South Africa	FJ425706
OTU 2	fungus-gr. termite nest	South Africa	FJ425707
OTU 3	fungus-gr. termite nest	South Africa	FJ425708
OTU 4	fungus-gr. termite nest	South Africa	FJ425709
OTU 5	fungus-gr. termite nest	South Africa	FJ425710
OTU 6	fungus-gr. termite nest	South Africa	FJ425711
OTU 7	fungus-gr. termite nest	South Africa	FJ425712
OTU 9A	fungus-gr. termite nest	South Africa	FJ425713
OTU 9B	fungus-gr. termite nest	South Africa	FJ425714
OTU 10	fungus-gr. termite nest	South Africa	FJ425715
OTU 11	fungus-gr. termite nest	South Africa	FJ425716
OTU 12	fungus-gr. termite nest	South Africa	FJ425717
OTU 13	fungus-gr. termite nest	South Africa	FJ425718
OTU 15	fungus-gr. termite nest	South Africa	FJ425719
OTU 16	fungus-gr. termite nest	South Africa	FJ425720
Top six hits of BLAST search on ingroup		Average sequence identity with ingroup: 94.7–96.7%	
Anthostomella sp.	(unknown)	Puerto Rico	AY780050
Astrocystis cocoes	(unknown)	(unknown)	AY083823
Fasciatispora petrakii	(unknown)	(unknown)	AY083828
Nemania difusa	(unknown)	China	DQ840076
Nemania maritima	(unknown)	France	DQ840074
Rosellinia corticium	(unknown)	China	DQ840078
Rosellinia necatrix	(unknown)	(unknown)	AY083824
Xylaria acuta	(unknown)	(unknown)	AY544676
Xylaria curta	(unknown)	(unknown)	U47840
Xylaria hypoxylon	rotting wood	USA	AY544648
Xylaria sp.	(unknown)	Thailand	DQ840080
Xylaria sp.	(unknown)	Thailand	DQ840081
Xylaria sp.	tree, *Theobroma cacao*	Ecuador	DQ327623
Xylaria sp.	tree, *Theobroma cacao*	Mexico	DQ327620
Xylaria sp.	tree, *Theobroma cacao*	Ecuador	DQ327627
Xylaria sp.	tree, *Theobroma gileri*	(unknown)	DQ674817
Xylaria sp.	tree, *Theobroma gileri*	Ecuador	DQ674826
Xylaria sp.	tree, *Theobroma gileri*	Ecuador	DQ674827
Xylaria sp.	tree, *Theobroma gileri*	Ecuador	DQ674819
Non-termite-asssociated African Xylaria isolates		Average sequence identity with ingroup: 94.5–96.7%	
strain 0006	tree, *Syzygium* sp.	South Africa	FJ425702
strain 1175	tree, *Syzygium cordatum*	South Africa	FJ425703
strain 1474	tree, *Syzygium legatti*	South Africa	FJ425704
strain 1580	tree, *Syzygium legatti*	South Africa	FJ425705
Lower-score hits of BLAST-search on ingroup		Average sequence identity with ingroup: 93.1–93.7%	
Daldinia concentrica	(unknown)	(unknown)	U47828
Dactylaria fragilis	(unknown)	(unknown)	EU107290
Nemania plumbea	(unknown)	(unknown)	DQ840071
Outgroup			
Sordaria fimicola	(unknown)	(unknown)	AY545728

nucleotide substitution model for the Bayesian analysis was calculated; MrBayes settings for the best-fit model (GTR + I + G) selected by AIC: Prset statefreqpr = Dirichlet (1,1,1,1); Lset Nst = 6; Rates = invgamma. The Bayesian MCMC analysis was run for 20 million generations and every 1000th generation was sampled. The posterior probability values were calculated from these samples with burn-in = 5000.

To test the specific phylogenetic hypothesis that termite-associated *Xylaria* form a monophyletic group, we used the Bayes factor test (Kass & Raftery 1995). In this test, the marginal likelihood of the constrained tree topology is compared with the marginal likelihood of the unconstrained topology and the ratio of these likelihoods is defined as the Bayes factor (B_{10}). The Bayes factor values were interpreted according to recommendations developed by Kass & Raftery (1995): values of 2 $\log_e(B_{10})$ (two times the difference between the harmonic means of the two models) above 10 are considered as strong evidence to support the unconstrained model over the other.

Results

Distribution of Xylaria

Xylaria appeared on samples from 69% of the fungus-growing termite nests (Table 3), and on 57% of the fungus comb samples (Table 4). *Xylaria* was significantly more prevalent in *Odontotermes* combs (83%) than in *Macrotermes* and *Microtermes* combs (52% and 45%, respectively, see Table 4, *G*-test: $G = 12.52$, d.f. = 2; $P < 0.005$).

Although *Xylaria* was present in the majority of nests and fungus combs, it appeared only twice on plates with the ±5 mm^3 fine-scale samples (two out of 360 samples). Thus, when the sample size is small, the chance that *Xylaria* emerges is small. This suggests that *Xylaria* is distributed in the fungus comb in distinct patches. Many other fungi, as well as yeast and bacteria did emerge from the fine-scale samples. Plates with fine-scale samples of young and medium sections showed a range of microorganisms (1–5 different microorganisms per sample such as *Alternaria* sp., *Penicillium* sp., *Trichoderma* sp., *Rhizopus* sp.), while plates with samples from the old section regularly only showed growth of *Termitomyces*. This finding that fresh fungus comb contains more microorganisms than old comb, is in accordance with observations by Thomas (1987b).

Table 3 Prevalence of *Xylaria* in South African fungus-growing termite nests

Year	Nests sampled	Nests with *Xylaria* on incubated combs	% nests with *Xylaria*
2003	54	37	69
2005	37	20	54
2007	17	17	100
Total	108	74	69

No *Xylaria* species emerged from any of the vegetation samples. Isolates with *Xylaria*-like culture morphology were sequenced, but BLAST results showed that none of the sequenced strains belonged to the genus *Xylaria*. On plates with these samples, mainly fast-sporulating fungi (i.e. *Alternaria* sp., *Aspergillus* sp., *Fusarium* sp., *Penicillium* sp., *Rhizopus* sp.), yeasts and bacteria were observed.

Specificity of Xylaria

The ITS region was successfully sequenced for 142 *Xylaria* isolates from fungus comb material (Table 1). The phylogenetic tree based on *Xylaria* ITS sequences shows 16 well-defined clades, which each have over 97.5% sequence similarity and therefore were treated as OTUs (Fig. 1).

Specificity of *Xylaria* on levels 2–4 was generally low. First, identical ITS types occurred on fungus combs from different termite genera and species. For example, ITS type 1.11 was found in nests of *Macrotermes michaelseni*, *M. natalensis*, *O. badius*, *O. latericius*, and *O. transvaalensis* (Table 1). Second, different ITS types occurred on fungus combs from the same termite nest. For example, ITS types from OTUs 1, 9, and 15 were all found in nest 706 (Table 1). However, there are patterns in the ITS tree that suggest some specificity. First, all five nests of *Microtermes* contained OTU 3 (with ITS type 3), while this OTU 3 was never encountered in nests of the two other termite genera. Second, OTU 1 (with ITS type 1) was never encountered in nests of *Microtermes*, while OTU 1 was the most common taxon in nests of *Macrotermes* and *Odontotermes* (Table 1). The AMOVA test, used to quantify *Xylaria* specificity, showed that 10% of the molecular variation in ITS sequences was explained by genus and 7% by species (AMOVA: $P \ll 0.001$).

Specificity of *Xylaria* on level 1, i.e. for fungus-growing termites, can be inferred from Fig. 2. The phylogenetic tree

Table 4 Prevalence of *Xylaria* in comb fragments from nests of South African fungus-growing termites

	2003			2005			2007			Weighed mean
Genus	Combs incubated	*Xylaria* emerged	% Combs with *Xylaria*	Combs incubated	*Xylaria* emerged	% Combs with *Xylaria*	Combs incubated	*Xylaria* emerged	% combs with *Xylaria*	% combs with *Xylaria*
Macrotermes	14	8	57.14	116	40	34.48	108	75	69.44	51.68
Microtermes	18	9	50	13	5	38.46				45.16
Odontotermes	22	20	90.91	22	20	70.37	7	7	100	83.3
Total	54	37	68.52	151	65	43	115	82	71	57.37

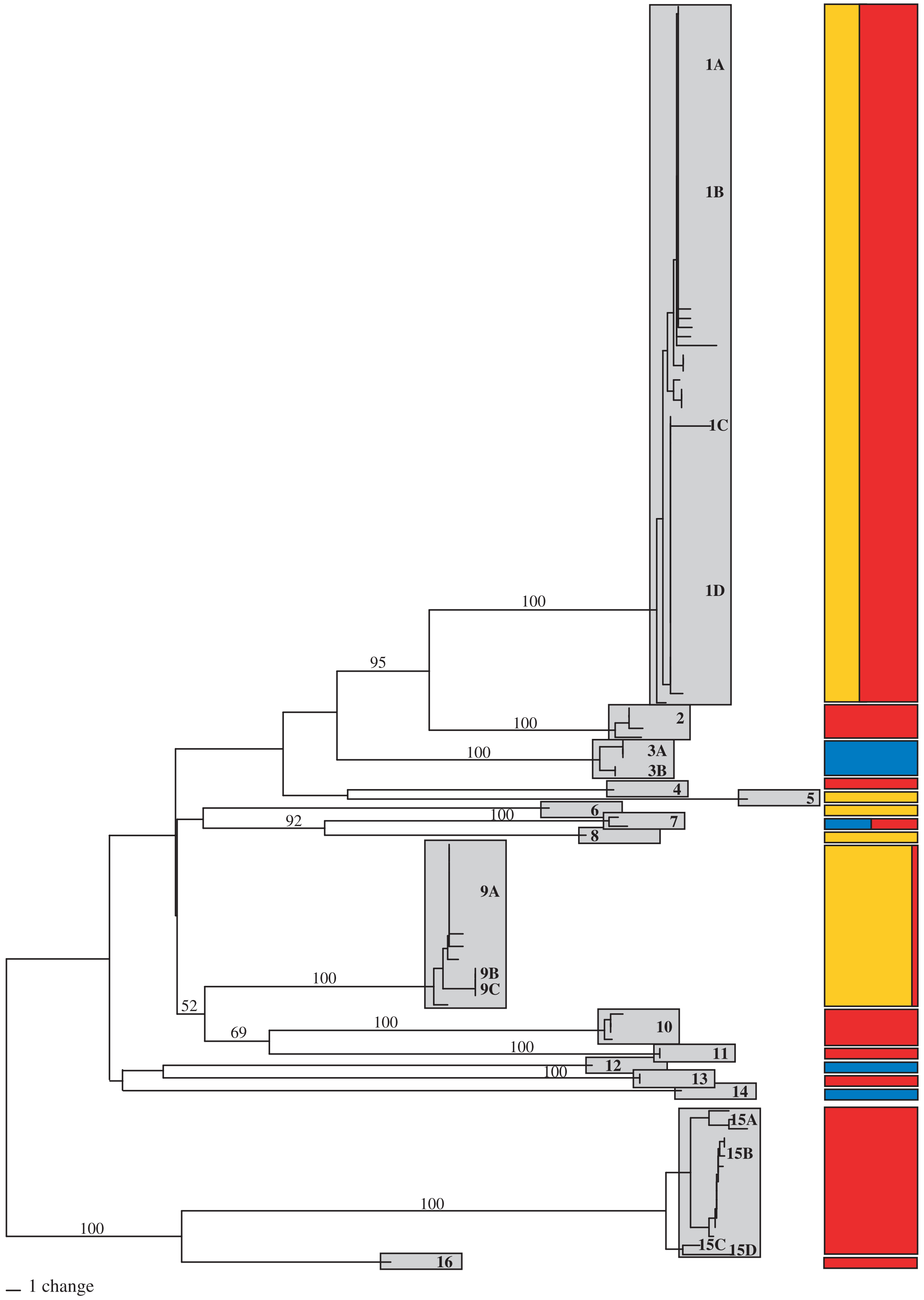

Fig. 1 Estimated phylogeny of *Xylaria* isolated from South African fungus-growing termite nests, based on ITS sequences. The colours of the bars indicate to which termite genus/genera the nests belong, from which the *Xylaria* isolates originate: *Macrotermes* (red), *Microtermes* (blue) and *Odontotermes* (yellow), and the surface is proportional to the respective number of isolates. Each operational taxonomic unit (OTU) was given a number (in bold), and a letter when more than one isolate within an OTU was selected for LSU sequencing. The tree was estimated using the NJ method (PAUP*). Bootstrap values of 1000 replications are shown above the branches.

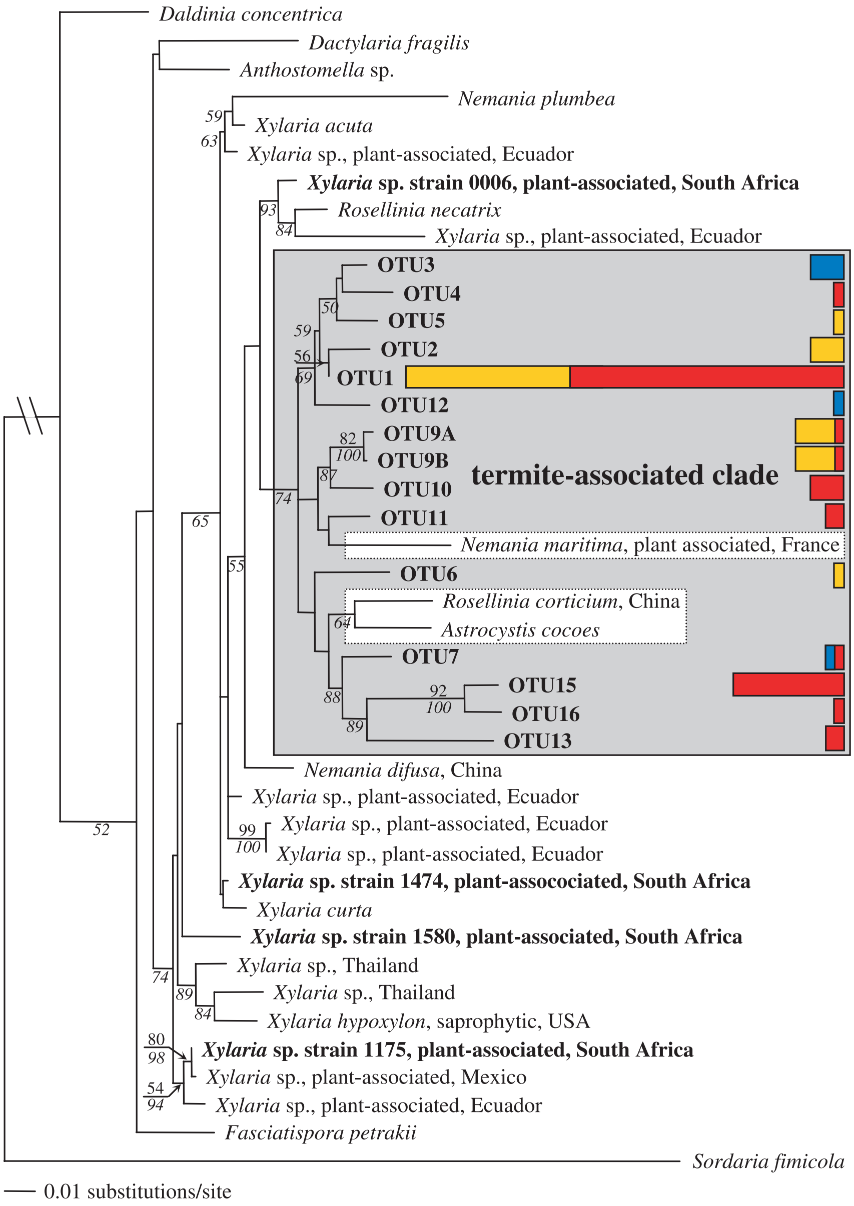

Fig. 2 Estimated phylogeny of *Xylaria* isolated from fungus-growing termite nests (grey area) and non-termite-associated *Xylaria(ceae)* (white area), based on LSU sequences. The width of the coloured bars is proportional to the number of isolates that is represented by each isolate of which the LSU region was sequenced. The colours of the bars indicate to which termite genus/genera the nests belong, from which the *Xylaria* isolates originate: *Macrotermes* (red), *Microtermes* (blue) and *Odontotermes* (yellow). The tree was estimated using maximum likelihood (PAUP*) with *Sordaria fimicola* (Ascomycota, Sordariales) as an outgroup. Branch support was estimated in two ways, and values > 50% are given: 1 (above the line) ML bootstrap values of 1000 replicates (using a heuristic search option), and 2 (below line) Bayesian posterior probability values.

based on LSU sequences shows that all termite-associated *Xylaria* belong to a single clade, together with only three of the 26 non-termite-associated strains. A tree in which the termite-associated *Xylaria* are constrained to form a monophyletic group is strongly rejected using the Bayes factor test 2 $\log_e(B_{10}) = 17.52$ (Kass & Raftery 1995).

To check if geographic or temporal factors could be causing the clustering, a BLAST search on ITS sequences was done (Table 5). This showed that top BLAST hits of 12 of the 16 OTUs were fungus-growing termite-associated isolates, half of which came from Asia. We neither found evidence for temporal factors influencing the structure of our data. For example, multiple identical ITS types were found over all sampling years (e.g. ITS type 1.01 and 9.01; Table 1).

Discussion

Specificity of Xylaria

Our data show that *Xylaria* has specificity for fungus-growing termites (level 1), as all termite-associated *Xylaria* cluster together (Fig. 2). We find three (out of the 26) related non-termite-associated isolates in that same clade, although for two of these three, the origin is unclear. There are several possible explanations for this pattern. First, it could mean that there is a clade of *Xylaria* species that have a preference for — but are not restricted to — colonies of fungus-growing termites. Second, the pattern could mean that there have been five independent transitions of

Table 5 First hits of BLAST search on the first ITS type of each OTU. Indicated are (from top to bottom): GenBank Accession number, name, ecology, geographical origin, query coverage/maximum identity, reference

OTU	BLAST hit 1	BLAST hit 2	BLAST hit 3
1	EU203587 *Xylaria* sp. termite-associated Central Africa 98/100% unpublished	EU203585 *Xylaria* sp. termite-associated Central Africa 98/100% unpublished	EU164405 *Xylaria* sp. termite-associated Central Africa 98/100% unpublished
2	EU164401 *Xylaria* sp. termite-associated Central Africa 98/97% unpublished	EU164402 *Xylaria* sp. termite-associated Central Africa 98/97% unpublished	EU203584 *Xylaria* sp. termite-associated Central Africa 98/97% unpublished
3	AY572970 *Podosordaria tulasnei* coprophilous UK 86/96% Ridderbusch *et al.* 2004	AB274817 *Xylaria polymorpha* termite-associated Japan 84/95% Okane & Nakagiri 2007	EF423534 *Xylaria* sp. endophytic Panama 84/95% Gilbert & Webb 2007
4	AY315402 *Xylariaceae* sp. endophytic (unknown) 90/91% Davis *et al.* 2003	EF026121 *Nemania primolutea* (unknown) Asia 88/92% unpublished	EU678666 *Xylaria* sp. endophytic Asia 90/91% unpublished
5	EU164407 *Xylaria* sp. termite-associated Central Africa 98/96% unpublished	AY572970 *Podosordaria tulasnei* coprophilous UK 84/90% Ridderbusch *et al.* 2004	AF163029 *Xylaria arbuscula* (unknown) (unknown) 84/90% Lee *et al.* 2000
6	EU164404 *Xylaria* sp. termite-associated Central Africa 90/90% unpublished	AB217793.1 Uncultured xylariaceous fungus termite-associated Japan 100/88% Shinzato *et al.* 2005	AB274815.1 Xylaria angulosa termite-associated Japan 100/87% Okane & Nakagiri 2007
7	EU164400 *Xylaria* sp. termite-associated Central Africa 98/97% unpublished	EU164408 *Xylaria* sp. termite-associated Central Africa 87/90% unpublished	AB217793 uncultured xylariaceous fungus termite-associated Japan 87/89% Shinzato *et al.* 2005
8	EU164400 *Xylaria* sp. termite-associated Central Africa 91/92% unpublished	EU164408 *Xylaria* sp. termite-associated Central Africa 98/90% unpublished	AB217793 uncultured xylariaceous fungus termite-associated Japan 100/87% Shinzato *et al.* 2005
9	AB217793 uncultured xylariaceous fungus termite-associated Japan 100/94% Shinzato *et al.* 2005	EU164400 *Xylaria* sp. termite-associated Central Africa 86/92% unpublished	EU164408 *Xylaria* sp. termite-associated Central Africa 98/89% unpublished
10	AB217793 uncultured xylariaceous fungus termite-associated Japan 99/88% Shinzato *et al.* 2005	EU164408 *Xylaria* sp. termite-associated Central Africa 98/87% unpublished	EU164400 *Xylaria* sp. termite-associated Central Africa 90/89% unpublished

Table 5 *Continued*

OTU	BLAST hit 1	BLAST hit 2	BLAST hit 3
11	AB217793	EU164406	EU164408
	uncultured xylariaceous fungus	*Xylaria* sp.	*Xylaria* sp.
	termite-associated	termite-associated	termite-associated
	Japan	Central Africa	Central Africa
	100/87%	98/86%	98/85%
	Shinzato *et al.* 2005	unpublished	unpublished
12	DQ491487	AF163029	AY183369
	Xylaria hypoxylon	*Xylaria arbuscula*	*Xylaria arbuscula*
	(unknown)	(unknown)	endophytic
	(unknown)	Asia	(unknown)
	78/91%	78/91%	77/91%
	AFTOL project	Lee *et al.* 2000	unpublished
13	AB274815	EU164408	EU113197
	Xylaria angulosa	*Xylaria* sp.	uncultured fungus
	termite-associated	termite-associated	root endophyte
	Japan	Central Africa	Australia
	100/98%	97/87%	90/87%
	Okane & Nakagiri 2007	unpublished	Chambers *et al.* 2008
14	AY315404	DQ780445	AB041994
	Xylaria sp.	*Xylaria* sp.	*Xylaria* sp.
	Endophyte	Endophyte	Endophyte
	USA	Thailand	Japan
	100/91%	99/91%	100/91%
	Davis *et al.* 2003	Promputtha *et al.* 2007	unpublished
15	AB274813	AB217790	AB217789
	Geniculisynnema termiticola	uncultured xylariaceous fungus	uncultured xylariaceous fungus
	termite-associated	termite-associated	termite-associated
	Japan	Japan	Japan
	76/92%	76/86%	74/87%
	Okane & Nakagiri 2007	Shinzato *et al.* 2005	Shinzato *et al.* 2005
16	AB274813	AB217790	AB217789
	Geniculisynnema termiticola	uncultured xylariaceous fungus	uncultured xylariaceous fungus
	termite-associated	termite-associated	termite-associated
	Japan	Japan	Japan
	100/92%	76/89%	76/89%
	Okane & Nakagiri 2007	Shinzato *et al.* 2005	Shinzato *et al.* 2005

Xylariaceae to an association with fungus-growing termites. However, a more parsimonious explanation than five independent transitions is a single transition to termite nests in the most recent common ancestor of the termite-associated clade, and two reversals to a free-living state afterwards. We have provided evidence that this observed specificity pattern is not a result of geographic origin of our samples or temporal factors.

We found no strong specificity at lower taxonomical levels (levels 2–4). Different ITS types of *Xylaria* appeared on a single fungus comb, whereas single ITS types appeared on combs from different termite genera (Table 1; Fig. 1). Only 10% and 7% of the ITS sequence variation could be explained by termite genus and species, respectively. Thus, there is no congruence between *Xylaria* phylogeny and fungus-growing termite genera, in contrast to what was found earlier for *Termitomyces* and fungus-growing termites (Aanen *et al*. 2002, 2007) or for *Escovopsis* and fungus-growing ants (Currie *et al*. 2003). Despite that result, nests of *Microtermes* harboured different *Xylaria* taxa than nests of the two other termite genera sampled in this study (Table 1; Fig. 1). This pattern could be the result of differences between termite genera in selection pressures that act on *Xylaria*. For example, the comb material, structure or turnover time, or the characteristics of (faecal) excretions could differ between termite genera.

In our study, we observed 16 different OTUs of termite-associated *Xylaria*, indicating a large cryptic species richness of the fungal group involved. Whereas Batra & Batra (1979) mentioned only one *Xylaria* species, viz. *X. nigripes*, as the termite-nest associate, recent studies mention at least four (Okane & Nakagiri 2007) or even 20 different termite-associated *Xylaria* species (Ju & Hsieh 2007). Our study provides further evidence of a large number of unknown

Xylaria species in termite nests, whose evolutionary relationships and ecological roles deserve further study.

It should be noted that there is a need for more representative sampling of species, more ecological information about the sampled species, more taxonomic work and more molecular data on the specimens. As an illustration, when performing a BLAST search on the LSU sequences, none of the BLAST hits were African taxa and none were termite-associated taxa. This could mean that African LSU or termite-associated *Xylaria* LSU sequences are underrepresented in GenBank, or both. Furthermore, information on the origin is often incomplete (Table 2).

Distribution of Xylaria

Xylaria was found in the vast majority of sampled fungus-growing termite nests, but not on all fungus comb samples from nests where *Xylaria* was present. While *Xylaria* emerged from 57% of the 100 cm^3 comb samples, it emerged hardly from the ±5 mm^3 samples. In the (fine-scale) comb samples where *Xylaria* was not observed, it may have been present but suppressed or out-competed by other fungi, as no selective medium was used for plating. However, we consider it likely that absence of *Xylaria* in individual combs is the result of a patchy distribution within nests. In contrast with what Batra & Batra (1979) have reported, our results indicate that *Xylaria* is not present throughout the comb as continuous mycelium, but either as spores or as small mycelial patches.

We have no explanation for the differences between termite genera in *Xylaria* prevalence, although one might hypothesise that this is the result of differences in fungus garden hygiene or structure.

Visible *Xylaria* structures were never observed in living termite colonies, while they occurred frequently and prominently when the fungus combs were incubated without termites. Furthermore, we obtained five genetically different pure *Xylaria* cultures from a dead termite colony, where *Xylaria* was fruiting throughout the nest. These observations match earlier reports that, in the presence of termites, fungi other than the cultivated *Termitomyces* do not develop (Shinzato *et al*. 2005) and that *Xylaria* typically produces fruiting structures in decaying or dead termite nests (Thomas 1987 c; Wood & Thomas 1989; Rogers *et al*. 2005). It has been hypothesised that termites actively control the species composition in their nests, for example by excreting antimicrobial peptides (Lamberty *et al*. 2001; Fuller 2007). Active suppression by termites of spore germination and/or mycelial growth could explain the inferred patchy distribution of *Xylaria* across fungus combs in living termite nests. Considering these observations, we can hypothesise that (i) in living termite colonies *Xylaria* is controlled effectively; (ii) *Xylaria* is not eliminated but controlled only temporarily; and (iii) *Xylaria* is better than other fungi at taking over the comb in the absence of termites.

An unanswered question is how *Xylaria* enters the nest and survives until the nest is decaying. It seems unlikely that *Xylaria* enters the termite nest from the soil, since Thomas (1987a) did not observe *Xylaria* in the surrounding soil. Members of the genus *Xylaria* (Ascomycotina, Xylariales) occur in a wide variety of habitats (Whalley 1996). They are found not only on dead plant material, but also as endophytes in living plants (Petrini & Petrini 1985; Whalley 1996; Davis *et al*. 2003). *Xylaria* species can degrade lignin, causing white rot in wood and plant debris (Whalley 1996; Osono & Takeda 1999). Since termites feed on (dead) wood, they could bring inocula of *Xylaria* into the nest through foraging activities. However, we were not able to isolate *Xylaria* from vegetation adjacent to the nest or dead wood on which termites had been foraging, for comparison with our termite-associated *Xylaria* isolates. Rogers *et al*. (2005) suggest that certain *Xylaria* species (*X. escharoidea*, *X. furcata* and *X. nigripes*) have co-evolved with termites, because they seem to have been selected for smaller spore size. Assuming termites as the dispersion agents, small spores are more easily ingested or otherwise carried by insects and thus increase chances of dispersal (Rogers 2000). As for surviving once inside the termite nests, we may speculate that *Xylaria* is latently present in some less hygienic corners of the nest or in the core regions of the fungus-comb until the termite colony disintegrates. Termite-associated *Xylaria* may behave like 'sit-and-wait saprotrophs', foliar-endophytes that are latently present on the leaf and only start degrading it when the leaf falls from the tree (Herre *et al*. 2007). Having large quantities of the wood-derived substrate, termite nests are certainly worth waiting for.

The nature of termite-associated Xylaria

Since termite-associated *Xylaria* show specificity for fungus-growing termites, a next question is what the nature of *Xylaria* in fungus-growing termite nests is. In fungus-growing ants — an independently evolved symbiosis between social insects and fungi — an ascomycete fungus has also been found, *Escovopsis* (Currie *et al*. 1999). *Escovopsis* is a prevalent mycoparasitic symbiont that is highly specialised on the ant fungus garden and has co-evolved with the ants (Currie *et al*. 2003; Reynolds & Currie 2004). *Xylaria* might be a mycoparasite too. However, no mycoparasitic members of the Xylariales are known. Moreover, *Termitomyces* is not known to suffer from parasites. It can easily be isolated in pure culture from a healthy fungus comb, without a selective medium (Aanen *et al*. 2007). Additionally, when *Termitomyces* and *Xylaria* are grown on one plate, they are both growing in delimited areas, and *Xylaria* does not seem to directly interfere with *Termitomyces* growth (A.A. Visser and D.K. Aanen, unpublished observations). We therefore deem it unlikely that *Xylaria* is a mycoparasite of *Termitomyces*.

Second, one could hypothesise that *Xylaria* has a beneficial role like *Termitomyces* (Batra & Batra 1979). This cannot be excluded based on our data, although the patchy distribution of *Xylaria* within a nest, and the fact that multiple genotypes were obtained from single nests, plead against this idea. Furthermore, Shinzato *et al*. (2005) showed in a quantitative analysis of the fungus comb that about 99% of the fungal tissue was *Termitomyces*, which also pleads against this hypothesis.

Third, the nature of *Xylaria* in fungus-growing termite nests could be analogous to that of weeds in human agriculture (Mueller *et al*. 2005). In human agriculture, most weeds do not specialise on the farmers, nor on the crops, but on the substrate and the favourable growth conditions created by the farmers. Likewise, termite-associated *Xylaria* are a distinct group within the Xylariaceae, without having specificity for fungus-growing termites at lower taxonomic levels. We therefore hypothesise that *Xylaria* is a (latent) weed in the fungus-growing termite colony that has specialised on the fungus comb substrate.

Experimental studies are required to further elucidate the nature of termite-associated *Xylaria*. Important questions include which substrates the various termite-associated *Xylaria* species can degrade and how strongly these *Xylaria* species depend on the substrate provided by fungus-growing termites. Future studies also need to address the question how *Xylaria* is suppressed in living termite nests.

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References

Aanen DK (2006) As you reap, so shall you sow: coupling of harvesting and inoculating stabilizes the mutualism between termites and fungi. *Biology Letters*, **2**, 209–212.

Aanen DK, Eggleton P, Rouland-Lefevre C *et al*. (2002) The evolution of fungus-growing termites and their mutualistic fungal symbionts. *Proceedings of the National Academy of Sciences, USA*, **99**, 14887–14892.

Aanen DK, Ros VID, Licht HHD *et al*. (2007) Patterns of interaction specificity of fungus-growing termites and Termitomyces symbionts in South Africa. *BMC Evolutionary Biology* **7**, 115.

Batra LR, Batra SWT (1979) Termite-fungus mutualism, In: *Insect-Fungus Symbiosis: Mutualism and Commensalism*, pp. 117–163. Allaheld & Osmun, Montclair, New Jersey.

Chambers SM, Curlevski NJA, Cairney JWG (2008) Ericoid mycorrhizal fungi are common root inhabitants of non-Ericaceae plants in a south-eastern Australian sclerophyll forest. *Fems Microbiology Ecology*, **65**, 263–270.

Currie CR, Mueller UG, Malloch D (1999) The agricultural pathology of ant fungus gardens. *Proceedings of the National Academy of Sciences of the United States of America*, **96**, 7998–8002.

Currie CR, Wong B, Stuart AE *et al*. (2003) Ancient Tripartite Coevolution in the Attine Ant-Microbe Symbiosis. *Science*, **299**, 386–388.

Darlington JPEC (1994) Nutrition and evolution in fungus-growing termites. In: *Nourishment and Evolution in Insect Societies* (eds Hunt JH, Nalepa CA), pp. 105–130. Westview Press, Boulder, Colorado.

Davis EC, Franklin JB, Shaw AJ, Vilgalys R (2003) Endophytic *Xylaria* (Xylariaceae) among liverworts and angiosperms: Phylogenetics, distribution, and symbiosis. *American Journal of Botany*, **90**, 1661–1667.

Dunn DW, Segar ST, Ridley J *et al*. (2008) A role for parasites in stabilising the fig-pollinator mutualism. *Public Library of Science, Biology* **6**, e59.

Excoffier LGL, Schneider S (2005) Arlequin version 3.0: an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online* **1**, 47–50.

Fuller CA (2007) Fungistatic activity of freshly killed termite, *Nasutitermes acajutlae*, soldiers in the Caribbean. *Journal of Insect Science* **7**, 14.

Gilbert GS, Webb CO (2007) Phylogenetic signal in plant pathogen-host range. *Proceedings of the National Academy of Sciences of the United States of America*, **104**, 4979–4983.

Gumming MS (1996) Behavioural and ecological aspects of nuptial flights of the termitophilous phorids *Termitophilomyia zimbraunsi* and *Mesopathusa modesta* (Diptera: Phoridae) in Zimbabwe. *Journal of Zoology*, **239**, 675–690.

Herre EA, Knowlton N, Mueller UG, Rehner SA (1999) The evolution of mutualisms: exploring the paths between conflict and cooperation. *Trends in Ecology & Evolution*, **14**, 49–53.

Herre EA, Mejia LC, Kyllo DA *et al*. (2007) Ecological implications of anti-pathogen effects of tropical fungal endophytes and mycorrhizae. *Ecology*, **88**, 550–558.

Hongoh Y, Ekpornprasit L, Inoue T *et al*. (2006) Intracolony variation of bacterial gut microbiota among castes and ages in the fungus-growing termite *Macrotermes gilvus*. *Molecular Ecology*, **15**, 505–516.

James TY, Kauff F, Schoch CL *et al*. (2006) Reconstructing the early evolution of Fungi using a six-gene phylogeny. *Nature*, **443**, 818–822.

Ju YM, Hsieh HM (2007) *Xylaria* species associated with nests of *Odontotermes formosanus* in Taiwan. *Mycologia*, **99**, 936–957.

Kass RE, Raftery AE (1995) Bayes factors. *Journal of the American Statistical Association*, **90**, 773–795.

Katoh K, Kuma K, Toh H, Miyata T (2005) MAFFT version 5: improvement in accuracy of multiple sequence alignment. *Nucleic Acids Research* **33**, 511–518.

Katoh H, Miura T, Maekawa K, Shinzato N, Matsumoto T (2002) Genetic variation of symbiotic fungi cultivated by the macrotermitine termite *Odontotermes formosanus* (Isoptera: Termitidae) in the Ryukyu Archipelago. *Molecular Ecology*, **11**, 1565–1572.

Lamberty M, Zachary D, Lanot R *et al*. (2001) Insect immunity. Constitutive expression of a cysteine-rich antifungal and a linear antibacterial peptide in a termite insect. *Journal of Biological Chemistry*, **276**, 4085–4092.

Lee JS, Ko KS, Jung HS (2000) Phylogenetic analysis of *Xylaria* based on nuclear ribosomal ITS1-5.8S-ITS2 sequences. *Fems Microbiology Letters*, **187**, 89–93.

Little AEF, Currie CR (2007) Symbiotic complexity: discovery of a fifth symbiont in the attine ant–microbe symbiosis. *Biology Letters*, **3**, 501–504.

Moriya S, Inoue T, Ohkuma M *et al*. (2005) Fungal community analysis of fungus gardens in termite nests. *Microbes and Environments*, **20**, 243–252.

Mueller UG, Gerardo NM, Aanen DK, Six DL, Schultz TR (2005) The evolution of agriculture in insects. *Annual Review of Ecology Evolution and Systematics*, **36**, 563–595.

Noda S, Kitade O, Inoue T *et al*. (2007) Cospeciation in the triplex symbiosis of termite gut protists (*Pseudotrichonympha* spp.), their hosts, and their bacterial endosymbionts. *Molecular Ecology*, **16**, 1257–1266.

Nylander JAA (2004) MrModelTest version 2. Program distributed by the author, Evolutionary Biology Centre, Uppsala University, Uppsala, Sweden.

Okane I, Nakagiri A (2007) Taxonomy of an anamorphic xylariaceous fungus from a termite nest found together with *Xylaria angulosa*. *Mycoscience*, **48**, 240–249.

Osono T, Takeda H (1999) Decomposing ability of interior and surface fungal colonizers of beech leaves with reference to lignin decomposition. *European Journal of Soil Biology*, **35**, 51–56.

Petrini L, Petrini O (1985) Xylariaceous fungi as endophytes. *Sydowia*, **38**, 216–234.

Posada D, Crandall KA (1998) ModelTest: testing the model of DNA substitution. *Bioinformatics*, **14**, 817–818.

Promputtha I, Lumyong S, Dhanasekaran V *et al*. (2007) A phylogenetic evaluation of whether endophytes become saprotrophs at host senescence. *Microbial Ecology*, **53**, 579–590.

Reynolds HT, Currie CR (2004) Pathogenicity of *Escovopsis weberi*: the parasite of the attine ant–microbe symbiosis directly consumes the ant-cultivated fungus. *Mycologia*, **96**, 955–959.

Ridderbusch DC, Weber RWS, Anke T, Sterner O (2004) Tulasnein and podospirone from the coprophilous xylariaceous fungus *Podosordaria tulasnei*. *Zeitschrift Fur Naturforschung C – a Journal of Biosciences*, **59**, 379–383.

Rogers JD (2000) Thoughts and musings on tropical Xylariaceae. *Mycological Research*, **104**, 1412–1420.

Rogers JD, Ju YM, Lehmann J (2005) Some *Xylaria* species on termite nests. *Mycologia*, **97**, 914–923.

Ronquist F, Huelsenbeck JP (2003) MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, **19**, 1572–1574.

Rouland-Lefèvre C, Diouf MN, Brauman A, Neyra M (2002) Phylogenetic relationships in *Termitomyces* (Family Agaricaceae) based on the nucleotide sequence of ITS: a first approach to elucidate the evolutionary history of the symbiosis between fungus-growing termites and their fungi. *Molecular Phylogeny and Evolution*, **22**, 423–429.

Sachs JL, Simms EL (2006) Pathways to mutualism breakdown. *Trends in Ecology & Evolution*, **21**, 585–592.

Saitou N, Nei M (1987) The neighbour–joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, **4**, 406–425.

Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning: a Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sands WA (1969) The association of termites and fungi. In: *Biology of Termites* (eds Krishna K, Weesner FM), pp. 495–524. Academic Press, New York.

Sannasi A (1969) Possible factor responsible for specific growth of *Xylaria nigripes* in the 'fungus gardens' of the mounds of the termite *Odontotermes redemanni*. *Entomologia experimentalis et applicata*, **12**, 183–190.

Shinzato N, Muramatsu M, Watanabe Y, Matsui T (2005) Termite-regulated fungal monoculture in fungus combs of a macrotermitine termite *Odontotermes formosanus*. *Zoological Science*, **22**, 917–922.

Sokal RG, Rohlf FJ (1995) *Biometry*. W.H. Freeman, New York.

Swofford DL (2002) *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods)*, Version 4. Sinauer Associates, Sunderland, Massachusetts.

Thomas RJ (1987a) Distribution of *Termitomyces heim* and other fungi in the nests and major workers of *Macrotermes bellicosus* (smeathman) in Nigeria. *Soil Biology and Biochemistry*, **19**, 329–333.

Thomas RJ (1987b) Distribution of *Termitomyces* and other fungi in the nests and major workers of several Nigerian Macrotermitinae. *Soil Biology and Biochemistry*, **19**, 335–341.

Thomas RJ (1987c) Factors affecting the distribution and activity of fungi in the nests of Macrotermitinae (Isoptera). *Soil Biology and Biochemistry*, **19**, 343–349.

Wade MJ (2007) The co-evolutionary genetics of ecological communities. *Nature Reviews Genetics*, **8**, 185–195.

Whalley AJS (1996) The xylariaceous way of life. *Mycological Research*, **100**, 897–922.

White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: A Guide to Methods and Applications* (ed. White TJ), pp. 315–322. Academic Press, New York.

Wood TG, Thomas RJ (1989) The mutualistic association between Macrotermitinae and *Termitomyces*. In: *Insect–Fungus Interactions* (eds Wilding N, Collins NM, Hammond PM, Webber JF), pp. 69–92. Academic Press, London.

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