On the ecology and evolution of microorganisms associated with fungus-growing termites

Anna A. Visser

Propositions

1 Showing the occurrence of a certain organism in a mutualistic symbiosis does not prove a specific role of this organism for that mutualism, as is illustrated by Actinobacteria species occurring in fungus-growing termite nests.

<div align="right">(this thesis)</div>

2 Instead of playing a role as mutualistic symbiont, *Pseudoxylaria* behaves like a weed, competing for the fungus-comb substrate and forcing termites to do regular gardening lest it overgrows their *Termitomyces* monoculture.

<div align="right">(this thesis)</div>

3 Citing colleagues who are no longer active must be considered as an act of true altruism.

4 The overload of literature on recent 'discoveries' blinds us from old literature, causing researchers to neglect what was already known and possibly duplicate investigations.

5 Keys to evolution of knowledge lie in recognizing the truth of one's intuition, and extending the limits of one's imagination.

6 The presumed creative superiority of left-handed people (Newland 1981), said to be the result of more communication between both sides of the brain, might rather be the result of lifelong selection on finding creative solutions to survive in a right-hand biased environment.

7 An understanding of 'why good ideas usually come by the time *time* is running out and how to manage this', would greatly improve people's intellectual output and the condition in which they perform.

Propositions accompanying the thesis

<div align="center">

"On the ecology and evolution of microorganisms
associated with fungus-growing termites"

</div>

Anna A. Visser
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Reference
Newland GA, 1981. Differences between left and right-handers on a measure of creativity. *Perceptual and Motor Skills* **53**: 787-792.Stellingen

Stellingen

1. Het aantonen van de aanwezigheid van een bepaald organisme in een mutualistische symbiose bewijst niet dat dit organisme een specifieke rol speelt in die mutualistische symbiose, zoals wordt geïllustreerd door Actinobacteriën die in nesten van schimmelkwekende termieten voorkomen.
<div align="right">(dit proefschrift)</div>

2. *Pseudoxylaria* gedraagt zich niet als een mutualistische symbiont maar als een onkruid, concurrerend om het substraat van de schimmeltuin en de termieten dwingend tot regelmatig tuinieren – zo niet dan overwoekert ze de *Termitomyces* monocultuur.
<div align="right">(dit proefschrift)</div>

3. Het citeren van collegae die niet meer actief zijn moet worden opgevat als een daad van werkelijk altruïsme.

4. De overmaat aan literatuur over recente 'ontdekkingen' maakt ons blind voor oude literatuur, wat als gevolg heeft dat onderzoekers negeren wat al bekend was en mogelijk onderzoek herhalen.

5. De sleutel tot evolutie van kennis ligt in het herkennen van de waarheid in iemands intuïtie, en het verleggen van de grenzen van iemands voorstellingsvermogen.

6. De veronderstelde creatieve superioriteit van linkshandige mensen (Newland 1981), naar zeggen het resultaat van meer communicatie tussen de beide hersenhelften, zou eerder het resultaat kunnen zijn van levenslange selectie op het vinden van creatieve oplossingen om in een rechtshandig georiënteerde omgeving te overleven.

7. Begrip van 'waarom goede ideeën meestal komen tegen de tijd dat *tijd* schaars wordt en hoe dit te reguleren', zou de intellectuele prestatie van mensen en de staat waarin ze presteren aanzienlijk kunnen verbeteren.

Stellingen behorende bij het proefschrift getiteld
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Contents

Chapter 1 - General Introduction:

Termites, Engineering, and Fungiculture

"The termites resemble the ants also in their provident and diligent labour, but surpass them as well as the bees, wasps, beavers, and all other animals which I have ever heard of, in the arts of building. It [Macrotermes bellicosus] erects immense buildings of well-tempered earth, which are contrived and finished with such art and ingenuity, that we are at a loss to say, whether they are most to be admired on that account, or for their enormous magnitude and solidity."
– Smeathman 1781 –

Termites (Insecta, order Blattodea) dominate and shape the landscape in large parts of the world (Batra & Batra 1979; Abe *et al.* 2009), and in doing so they are often referred to as ecosystem engineers. The tunnels they dig during mound-building and foraging make the soil permeable for water and air (Konaté *et al.* 1999). Additionally, termites are of chief importance for their contribution to organic matter turnover. They play a crucial part in turning dead plant matter into minerals (Lepage 1984; Jones 1990; Mando & Brussaard 1999; Abe *et al.* 2009), breaking down a quarter of the yearly primary production that is not consumed by fire (Kuyper 2004). With the scale on which they affect physical and chemical soil properties (Mando & Miedema 1997; Jouquet *et al.* 2005), termites are crucial for soil quality. Nutrient-rich patches around termite mounds affect the landscape by offering favourable conditions for plant seedlings to survive (Kiepe 1984; Jouquet *et al.* 2005), creating clusters of vegetation that are better known as 'islands of fertility' (Levick *et al.* 2010; Sileshi *et al.* 2010), see also Figure 1-1. Indisputably, termites play a paramount role in a range of ecosystems. For that reason termites are considered 'ecosystem engineers' (Pardeshi & Prusty 2010).

Of an estimated total of four-thousand termite species 2,500 have been described. Nearly 2,000 of these belong to the family Termitidae. This family contains a subfamily that has adopted an exceptional lifestyle: agriculture (or rather fungiculture). The
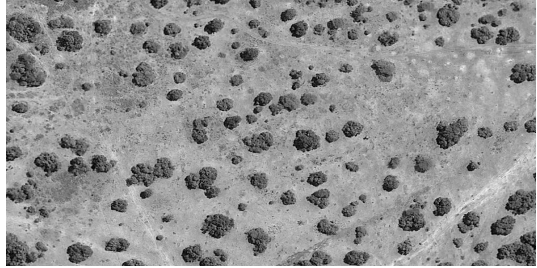
Macrotermitinae (11 genera, about 330 species; Kambhampati & Eggleton 2000) no longer depend solely on the microbiota in their gut for digestion of plant matter, but they cultivate a fungus that digests the substrate outside their body (Sands 1969; Batra & Batra 1979; Wood & Thomas 1989; Darlington 1994).

As illustrated in Figure 1-2, the termites collect and comminute dead plant material that they then deposit in the nest as hardly digested faeces. These faecal deposits are piled up to form a sponge-shaped structure that is overgrown by *Termitomyces* and named 'fungus comb' (Sands 1960). Enhanced by the warm, moist and stable climate of the termite mound, *Termitomyces* degrades the plant material and produces nodules (primordial fruiting bodies). The nodules – nitrogen-rich and high-quality food compared to the original, often woody, plant material – are eaten by the termites and act as inocula of newly added comb substrate (Sands 1960; Batra 1975; Sieber & Leuthold 1981). Finally, also the digested parts of the fungus comb substrate are consumed by the termites, resulting in final faeces which are deposited outside the nest (Darlington 1994). This cooperation between termite and fungus has made them incredibly successful and allowed them to dominate the landscape in sub-Saharan Africa and South Asia (Korb & Aanen 2003).

Figure 1-2 Macrotermitine termites have a fungus-garden inside their nest. Workers collect plant material that is deposited as hardly digested faeces. A fungus grows on this faeces and forms mushroom primordia that are in turn food for the termites.
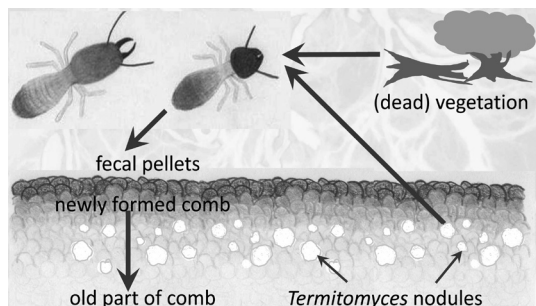
Evolution of mutualistic symbiosis with fungus in termites

While people started plant cultivation and animal husbandry about ten thousand years ago (Kirch 2005), termites started agriculture 24-34 million years ago (Aanen *et al.* 2002; Mueller *et al.* 2005). Termites are not the only organisms that preceded humans in agriculture: dating of phylogenetic trees and fossils show for example that 45-65 my ago ants (Mueller *et al.* 2001) and 20-80 my ago beetles (Farrell *et al.* 2001; Cognato & Grimaldi 2009) also started to cultivate fungi for food. Fungus growing by termites started in the African rainforest, from where it spread across sub-Saharan Africa and into Asia (Aanen & Eggleton 2005).

The fungus-growing practice in termites is thought to have evolved from putting to use the fungus that colonised the carton made of fragmented woody material with which termites built their nest. At present several non-fungus-growers like species of the genus *Nasutitermes* build their nest of undigested residues of plants (Jones 1979). In the case of Macrotermitinae it could be that when the woody carton in their nest became inhabited by fungi, instead of eradicating the termites started to eat the fungus (Sands 1969; Boomsma & Aanen 2009). As non-fungus farming termites prefer to eat wood that is colonised by fungi (Batra & Batra 1979), this is not a far-fetched idea. Termite colonies with particularly beneficial fungi probably gained a slight advantage over their neighbours, harvested more plant substrate, and increased their nest more than otherwise could have happened, providing more substrate for the fungus to grow on. This way, mutualistic behaviour of the fungus was enforced immediately by its effect on the termites, and the other way around: the birth of a mutualistic symbiosis.

Before moving on to the consequences of this mutualism for the evolution and life history of fungus-growing termites, the difference

symbiosis

mutualistic ⟵⟶ *parasitic*
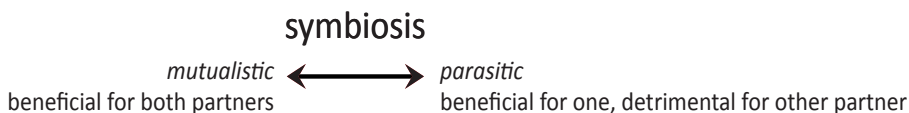beneficial for both partners   beneficial for one, detrimental for other partner

Figure 1-3 Extremes of symbiotic behaviour between which there is a gliding scale of symbiosis with intermediate and other cost-benefit ratios between the partners.

between symbiosis and mutualism needs to be made explicit. All too often these terms are used interchangeably, resulting in confusion. Symbiosis is a general term for organisms living together for the major part of their life, which can occur in several forms along a gliding scale between mutualistic and parasitic symbiosis (Figure 1-3). Mutualism means reciprocal interactions between two organisms with a net beneficial effect for both partners, and does not necessarily involve symbiosis.

Consequences of mutualistic symbiosis for fungus & termites

The initial symbiosis of fungus and termites was enforced by reciprocal mutualistic behaviour from its birth onwards. Benefits for the fungus such as more fragmented plant matter brought to the nest, and better protection against the outside environment; and benefits for the termites such as better decomposed, more nitrogen-rich plant material, mycelium with higher food quality, and more propagules of the fungus to inoculate new parts of the carton; had a positive effect on the fitness of each of the partners and indirectly also on the other partner (Sands 1969; Darlington 1994). Consequently, natural selection promoted traits in the fungus and termites that made these two symbionts more and more adapted to each other, resulting in the fungus-growing termites that we observe today.

Along the evolution of this mutualistic symbiosis, certain traits in the fungus and termites are likely to have become redundant (Szathmáry & Smith 1995). Genes that coded for those traits were prone to get lost or corrupted, either because of a trade-off with newly acquired traits, or because of accumulation of mutations in the absence of selection on gene functionality. For example, the *Termitomyces* species of *Macrotermes bellicosus* and *Microtermes* are transmitted vertically (clonally) by either the founding king or queen to the new termite colony (Johnson 1981). Some of those species may have become unable to form sexual fruiting bodies (mushrooms). As a consequence the fungus and termites became more and more dependent on each other, until at some point in history there was no genetic exchange anymore between the

symbionts and their free-living relatives; the mutualistic symbiosis had become obligatory (Sands 1960; Aanen *et al.* 2002).

It is not hard to imagine that certain combinations between fungus and termite genotypes were more successful than others. As the symbiotic partners evolved into different species, natural selection on mismatches created a certain level of specificity between them. Consequently, fungus and termites evolved together, which can be inferred from their phylogenetic trees (Figure 1-4; Aanen *et al.* 2002). Termites of the genus *Macrotermes* do not share their *Termitomyces* cultivar with any other genus, the closely related genera of *Microtermes* and *Ancistrotermes* share only with *Synacanthotermes*, while several host-switches caused the other termite genera to have less of a monopoly on the clade to which their fungal symbiont belongs (Aanen *et al.* 2002).
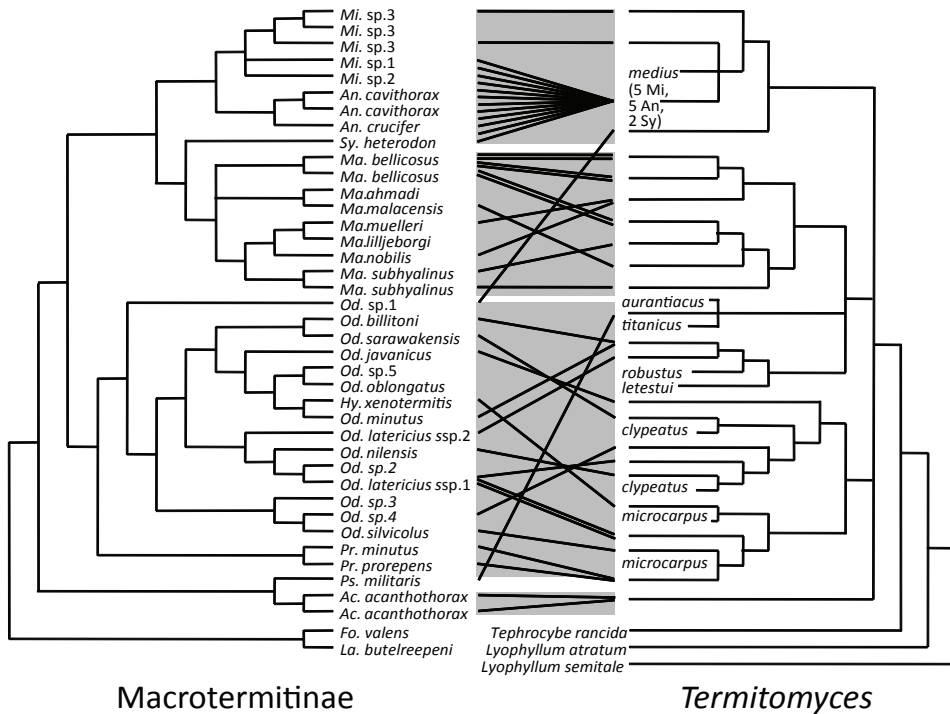

Figure 1-4 Coevolution between Macrotermitinae and *Termitomyces* is reflected in mirror wise resemblance of their phylogenetic trees (adapted from Aanen *et al.* 2002).

The life histories of *Termitomyces* and Macrotermitinae have become closely interlinked. Though the following scenario is not accurate for certain species of fungus-growing termites – *Macrotermes bellicosus* and *Microtermes* species have vertical *Termitomyces* transfer between generations of their colonies (Johnson 1981) and *Termitomyces* mushrooms have not been observed for all fungus-growing termite species that acquire *Termitomyces* horizontally (from the environment) – it will give an idea of the life cycle of both symbionts.

The fungus is generally prevented from making sexual fruiting bodies, as normally all nodules are eaten by the termites before they can grow out to become a mushroom (Aanen 2006). At a certain time of the year workers make holes in the mound to allow termite alates – winged sexual reproductive termites; kings and queens of the next generation of colonies – to fly out of the mound, find a mate, and settle for a new colony (Mitchell 2007, 2008). About one month after the flight of termite alates, *Termitomyces* sexual fruiting bodies pierce through the mound surface to spread their spores. Possibly the lower number of termites in the nest that feed on the nodules allows some of the primordia to develop into sexual fruiting bodies. Around the time that mushrooms appear, the first termite workers that descend from kings and queens of newly founded colonies emerge from the nest (Johnson *et al.* 1981). The single-nucleate *Termitomyces* spores that the first workers collect from the environment during foraging germinate, mate and eventually form a heterokaryon on the termite faecal pellets in the nest (Darlington 1994; De Fine Licht *et al.* 2005). After a while the first nodules are formed on what now has become a real fungus comb, making the circle complete (Darlington 1994).

It has been shown that a single termite colony grows a single strain of *Termitomyces* (Aanen *et al.* 2002, 2009; Katoh *et al.* 2002). How do termites manage to grow such single-strain monoculture starting from a mixture of spores that they collect from the environment, when they start a colony (Sands 1969; Katoh *et al.* 2002; Taprab *et al.* 2002)? Probably two factors play a role. First chance. Every time the termites eat the nodules and comb material, only a proportion of the fungal propagules survive gut passage. Chances of a fungal strain to be present in the new fungus comb are larger if it is more

abundant, which ultimately selects for the presence of one fungus only (Aanen 2006). Second, frequency dependent selection enforces and speeds up the former. Mycelia of *Termitomyces* – like those of other basidiomycetes – fuse if they are genetically identical (i.e. belong to the same genet). Starting mycelia of the same genet that occur very frequently in the new fungus comb can fuse into a larger mycelium that is more efficient in mobilizing resources (Aanen *et al.* 2009). Therefore it can grow faster and gain advantage over small mycelia of genets that occur less frequently, hence: frequency dependent selection (Aanen *et al.* 2009).

The cooperation between fungus and termites has made them very successful. The mutualism allowed them to occupy new niches and expand their territory (Korb & Aanen 2003). Normally a fungus is unable to degrade wood in semi-arid environments, but due to the microclimate that termites create in their nest, *Termitomyces* colonises a hospitable substrate of around 10 kg in *dry* weight in a single termite nest, 2-3 times the dry weight of their host species (averages for *Macrotermes* species, Darlington 1994). And, normally, termites have to cope with far less nutritious food (that is, food with a higher C:N ratio) than what Macrotermitinae encounter in their nest. The success of the termites due their fungiculture finds striking resemblance in the history of humanity. It was agriculture that allowed the development of large cities, and a large increase of the human population in general (Tilman *et al.* 2002; Xie 2008). Furthermore, in humans as well as in termites, agriculture has led to division of labour and inequality among working castes (Sieber & Leuthold 1981; Rodgers 1994).

Use of fungus & termites

Fungus-growing termites are ubiquitous in sub-Saharan Africa and South of Asia where they affect human enterprises. Humans have an ambivalent attitude towards termites. On the one hand, they consider termites a pest. Old termite nomenclature, such as *Termes fatalis* (Linnaeus) and *Termes destructor*, illustrates how termites were perceived by humans (Smeathman 1781). Termites frequently incorporate timber of buildings and human food crops

in their menu, resulting in severe economic losses throughout their distribution range (Rajagopal 2002; Zhong & Liu 2002). Searching the web for 'termite and fungus', one finds more literature on how fungi effectively kill termites than on how termites effectively grow fungi, which gives an idea of the effort spent on eradicating termites. On the other hand, humans recognise termites as useful. Certain crops are especially planted adjacent to or on the termite mounds (Sileshi *et al.* 2009), termites reduce 'the fuel load', and thereby reduce fire intensity, while preserving nutrients beyond the reach of fire (Lepage 1984). Humans use termites, *Termitomyces*, termite mound material, and termite engineering in many ways, of which an overview is presented here.

Termites are considered a delicacy in many countries around the world (Marconi *et al.* 2002; Malaisse 2004; Kagezi *et al.* 2010). Winged reproductive termites, which emerge at a specific time in the rainy season to found new colonies as king and queen, and the large queen from a mature nest are especially appreciated, but also workers and soldiers are eaten. Kagezi *et al.* (2010) describe a number of ways in which termites are collected and that they can be eaten fresh, boiled, fried or dried. Termites are rich in protein and fat and form an important addition to human diets in rural areas (Marconi *et al.* 2002; Kagezi *et al.* 2010). With the current growing interest in insect protein as a replacement for meat, within a decade termites could be part of the human menu world-wide, though they are not the most convenient insect to harvest due to their seasonality and mass-harvesting for export purposes could endanger certain species (Malaisse 2004; Kagezi *et al.* 2010).

Mature *Termitomyces* fruiting bodies, which appear in the rainy season, are a highly appreciated delicacy from South Africa to China (Sands 1969; Kagezi *et al.* 2010). The mushrooms are collected, sometimes dried, and sold at local markets. Though I have not had the privilege of tasting them myself, co-authors from Pretoria have repeatedly informed me about recipes for preparing the mushrooms and how good they taste.

Termite mound material is used in several ways. For example, in certain places people eat it. Especially children and women visit particular mounds regularly to eat the fine clay, a practice that may be explained by its high mineral and iron content (Geissler 2000;

Costa-Neto 2005). Furthermore, termite mounds can be broken apart and used as building material, using large chunks as bricks (Mijinyawa *et al.* 2007) or pulverised and mixed with water for lining of the interior wall of houses (Geissler 2000). Also, because of its high nutritional content the mound material is used for fertilising fields (Siame 2005; Sileshi *et al.* 2009, 2010).

FIGURE 1-5 Termite mounds dominate the landscape.

Termite engineering has an enormous impact on the environment (Darlington 2005), and termite mounds may dominate the landscape (FIGURE 1-5). One termite nest can have up to 6 km of belowground tunnels (Darlington 1982), which increase the water drainage, storage and retention capacity of the soil. This is one of the main qualities – besides termites' other effects on soil structure, mineral contents, and pH – for using termites to remediate degraded and crusted soils (Mando & Miedema 1997; Mando *et al.* 1999; Donovan *et al.* 2001; Dawes 2010a, 2010b). In Africa there is a practice called 'zai' (or depending on the country 'zaï', 'saai', or 'tassa') that involves digging pits in the soil that are filled with organic material such as straw, to attract termites that subsequently improve soil fertility and water retaining capacity by permeating the soil with their tunnels (Mando *et al.* 1999; Fatondji *et al.* 2001; Ouédraogo *et al.* 2004).

In some termites, especially the fungus-growing species, the termite mound architecture ensures ventilation and a constant temperature of close to 30 degrees Celsius in the nest. This is another part of termite engineering that has received a lot of attention. It inspired human engineers to design the Eastgate building in Harare, Zimbabwe; a building that relies on self-regulating air currents, instead of fuel-driven air-conditioning for its interior climate (The Biomimicry Institute 2011).

Finally, termite products can be used for medical purposes. There are records of soldier mandibles being used for suturing wounds (Costa-Neto 2005). In southern India tribes use extracts of termites and termite mound for treating diseases that are deemed associated with microorganisms (Solavan *et al.* 2007). The quest for new medicines as currently used antibiotics meet an increasing resistance in human pathogens, asks us to go beyond locally restricted use of natural antibiotics. Recent discoveries on antimicrobial substances from fungus-growing ants and termites show that they are a promising source for medicinal innovation (Solavan *et al.* 2007; Xu *et al.* 2009; Poulsen 2010).

Challenges for fungus-growing termites

Less studied are the challenges that termites face themselves. What stabilises the fungus-growing termite mutualistic symbiosis? Besides the threat of mound destruction by humans, termite mounds are preyed upon by ants and aardvarks (Lepage 1984). There are also organisms that operate on a less obvious scale. Though they go largely unnoticed, Kistner (1969) shows several insect families with species that occur as commensalist (inquiline) in termite nests. In *Macrotermes* nests they were observed in the fungus garden, as well as in the royal cell, passage ways, and in between the brood (Kistner 1969). Finally, of an even smaller size, micro-organisms probably form the biggest challenge for fungus-growing termites.

Foraging on dead vegetation and living in close contact with the soil, termites encounter many fungi and bacteria. At the same time they grow *Termitomyces* in monoculture (Katoh *et al.* 2002; Moriya *et al.* 2005). In human agriculture, this way of farming is prone to attract weeds and pathogens (Odorfer *et al.* 1994; Piper *et al.* 1996; De Bellaire *et al.* 2010), and no less seems to be the case for fungus-growing termites. Several species of the Xylariaceae typically occur on fungus gardens in nests that are abandoned by termites (Rogers 2000; Rogers *et al.* 2005). Fungus combs that are left without termites become rapidly overgrown by these and other fungi (Darlington 1994). How are these controlled in active termite nests?

Several defence mechanisms have been suggested for termites to suppress unwelcome guests. Defences can be behavioural, immunological, or perhaps involve mutualisms with defensive symbionts – as has been found in fungus-growing ants and other insects (Kaltenpoth 2009). Gut passage of fungus comb material, weeding of the fungus garden, and application of salivary gland secretions are examples of the proposed defences that termites use to manage their *Termitomyces* monoculture (Sieber & Leuthold 1981; Thomas 1987; Wood & Thomas 1989).

Scope and outline of this thesis

Organisms living in symbiosis fascinate us with their adaptations to live in extreme proximity to, or even inside, a partner that may be from a completely different Class, Phylum or Kingdom. Many combinations of species that live in mutualistic symbiosis seem very exceptional, but when studying an organism more closely – considering for example the multitude of organisms that live in the guts of animals or foliar endophytes in plants – one may find involvement in symbiosis to be the rule rather than an exception. Mutualistic symbioses are actually more likely to occur between members of different kingdoms (Leigh 2010), as they are less likely to compete for the same resources. How are conflicts of interest between symbiotic partners resolved; how does cooperation between species remain stable over evolutionary time scales?

While many studies have addressed these questions, focusing on two mutualistic symbionts only, often the ecology is more complex with multiple organisms present in a mutualistic symbiosis (Little & Currie 2007). When listing some of the threats to the fungus monoculture kept by termites (and to the nest in general), it became clear that also in fungus-growing termite nests there are probably more organisms that play a role. Which other organisms besides macrotermitine termites and *Termitomyces* play a role in the symbiosis? How is the weed and pathogen pressure on the *Termitomyces* monoculture managed in the termite nest? What makes the fungus-growing termites successful to such extent that they dominate semi-arid ecosystems in sub-Saharan Africa and

South Asia? These questions form the foundation of this thesis on the ecology and evolution of microorganisms associated with fungus-growing termites, with particular focus on the role and interactions with associated *Pseudoxylaria*.

Chapter 2 investigates the specificity of *Pseudoxylaria* for fungus-growing termites. We hypothesise that specificity or selectivity for fungus-growing termites would mean that *Pseudoxylaria* is not present coincidentally as opportunist, but truly associated with fungus-growing termites. *Pseudoxylaria* was sampled from hundred-eight South-African fungus-growing termite nests. Partial rDNA sequences of the resulting isolates were compared with those of *Xylaria* isolated from the environment and isolates from other parts of the world. The occurrence, abundance, and specificity of *Pseudoxylaria* in fungus-growing termite nests are discussed.

In Chapter 3, the role of *Pseudoxylaria* in the fungus-growing termite nest is inferred from interactions between mycelia of *Pseudoxylaria*, *Termitomyces* and their free-living relatives. *Pseudoxylaria* and *Termitomyces* were grown independently on different carbon sources, to test if they degrade complementary substrate components as some authors like Batra & Batra (1979) have suggested. Use of the same carbon sources, however, would support our hypothesis that *Pseudoxylaria* is not a beneficial or benign symbiont, but rather competing with *Termitomyces*. Subsequently, to further test this hypothesis, combinations of both fungi were grown on the same plate. From the differences in interaction outcomes – having included free-living relatives in this direct interaction experiment – we infer the role of *Pseudoxylaria* and the evolution of specificity of its interaction with *Termitomyces*.

Chapter 4 tests the hypothesis that termite workers play a crucial role in fungus garden hygiene. The occurrence of microorganisms other than *Termitomyces* was monitored for fungus combs that were incubated with, without, or temporarily without termite workers. The effect of workers on the fungus-comb hygiene, as well as observations on worker cleaning behaviour and their response to mycelium tissue of *Pseudoxylaria* and *Termitomyces* are discussed.

Chapter 5 explores the potential of Actinobacteria for a mutualistic role as protective symbiont in the fungus-growing termite nest. Six fungus-growing termite mounds from two geographically distant sites were sampled for Actinobacteria. Resulting isolates were characterised based on morphology and 16S rRNA sequences and were tested for antibiotic effect on *Termitomyces* and *Pseudoxylaria*. The specificity of Actinobacteria for fungus-growing termite nests and their effects on *Termitomyces* and *Pseudoxylaria* are presented and discussed.

Final Chapter 6 presents a reflection on the previous chapters, focussing on underlying mechanisms. What stabilises the mutualism between termites and *Termitomyces*? What role do *Pseudoxylaria* and other organisms play in the fungus-growing termite symbiosis? What determines whether an organism becomes parasitic or mutualistic, and how does symbiont role affect the level of specificity between symbiotic partners? An analogy is drawn with human agriculture, directions for future research are given, and the discussion ends with the main conclusions of this thesis.

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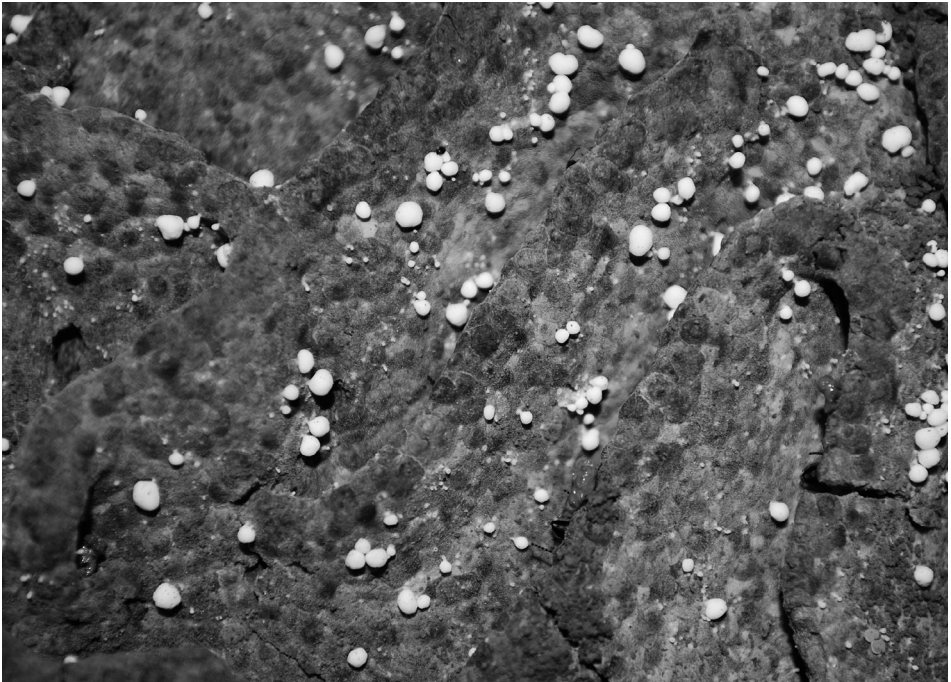
Chapter 2

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.

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 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 coevolution (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 cospeciation 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 twelve different sites across the north-eastern part of South Africa (Table 2-1). Comb samples were taken from nests of eight species of fungus-growing termites belonging to the genera *Macrotermes*, *Micro termes* 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-extract agar plates (20 g/L malt, 2 g/L yeast, 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 2-1 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.

[a] Naboomspruit changed name into Mookgophong

Date	Nest	Comb	Isolate	Termite taxon	Site[a]	ITS type	ITS OTU	LSU type	GenBank accession
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	328		328	*Odontotermes latericius*	Pretoria2	1.01	1		
2003-02-06	353		353	*Odontotermes transvaalenis*	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		

TABLE 2-1 (Continued)

Date	Nest	Comb	Isolate	Termite taxon	Site[a]	ITS type	ITS OTU	LSU type	GenBank accession
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		
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 transvalensis*	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[b]	G	725.G2	*Macrotermes natalensis*	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	337		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

Table 2-1 (Continued)

Date	Nest	Comb	Isolate	Termite taxon	Site[a]	ITS type	ITS OTU	LSU type	GenBank accession
2003-02-02	342	D	342.D	*Macrotermes natalensis*	Pretoria2	7.02	7		FJ425676
2003-02-06	352		352	*Microtermes* IV	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		
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]	B	725.B	*Macrotermes natalensis*	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[b]	C	725.C	*Macrotermes natalensis*	Badplaas	15.04	15		FJ425694
2007-02-17	725[b]	G	725.G	*Macrotermes natalensis*	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[b]	E	725.E	*Macrotermes natalensis*	Badplaas	15.05	15		
2007-02-17	725[b]	F	725.F	*Macrotermes natalensis*	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[b]	D	725.D	*Macrotermes natalensis*	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 2-1 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'-TCCGTAG GTGAACCTGCGG-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 LINS-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 2-2 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

Table 2-2 Overview of all LSU sequences used in this study.

Name	Ecological origin	Geographic origin	GenBank accession
Ingroup		Sequence identity within ingroup: 94.6 to 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 BLAST-hits of BLAST-search on ingroup		Average sequence identity with ingroup: 94.7 to 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 to 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 BLAST-hits of BLAST-search on ingroup		Average sequence identity with ingroup: 93.1 to 93.7 %	
Daldinia concentrica	(unknown)	(unknown)	U47828
Dactylaria fragilis	(unknown)	(unknown)	EU107290
Nemania plumbea	(unknown)	(unknown)	DQ840071
Outgroup			
Sordaria fimicola	(unknown)	(unknown)	AY545728

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 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 2-3), and on 57% of the fungus comb samples (TABLE 2-4). *Xylaria* was significantly more prevalent in *Odontotermes* combs (83%) than in *Macrotermes* and *Microtermes* combs (52% and 45%, respectively, see TABLE 2-4, *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 mm³

Table 2-3 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

Table 2-4 Prevalence of *Xylaria* in comb fragments from nests of South African fungus-growing termites.

Year	2003			2005			2007			
Genus	combs incubated	*Xylaria* emerged	% combs with *Xylaria*	combs incubated	*Xylaria* emerged	% combs with *Xylaria*	combs incubated	*Xylaria* emerged	% combs with *Xylaria*	Weighed mean % 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

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).

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 2-1). 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 (Figure 2-1).
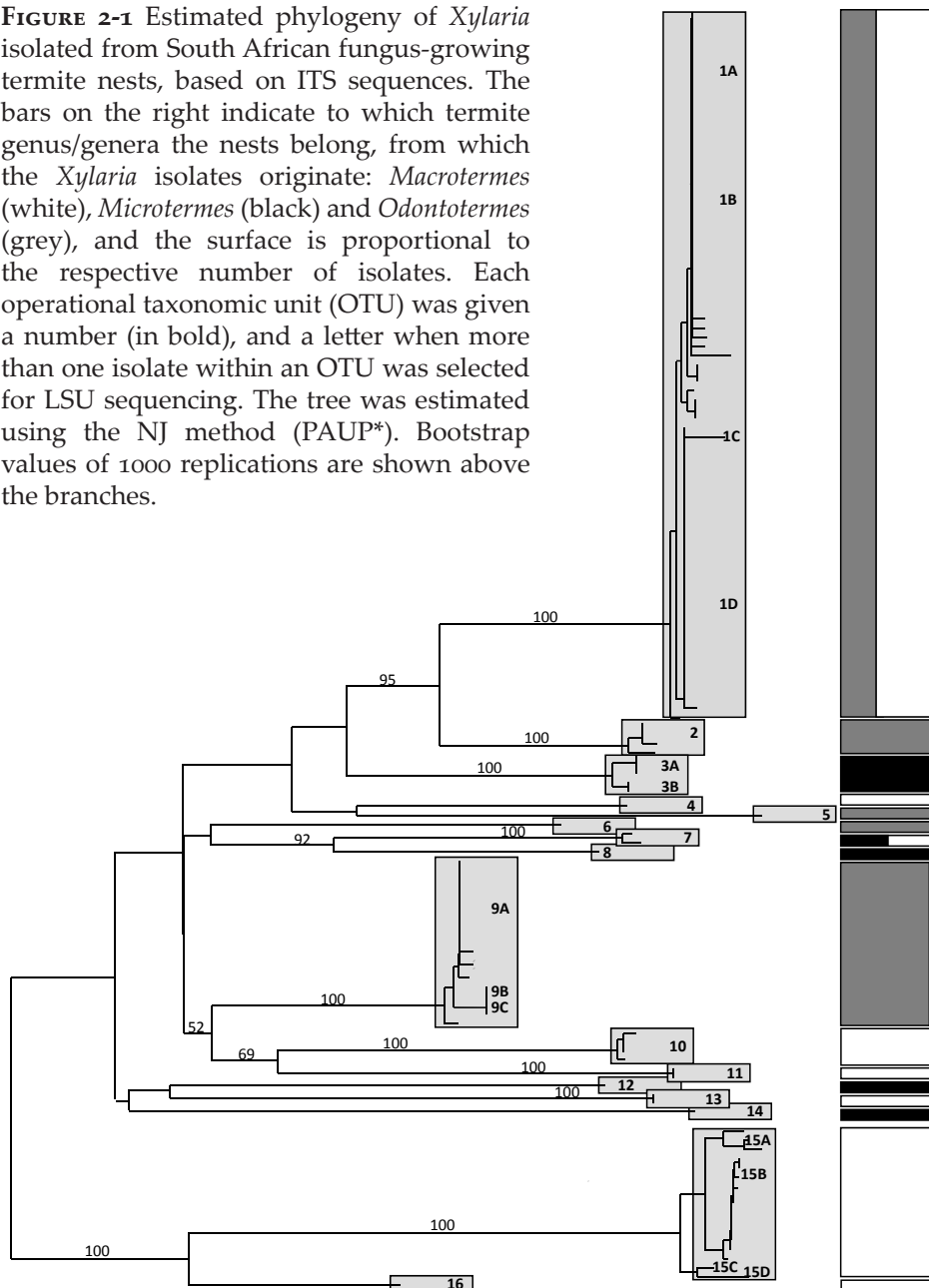
Specificity of *Xylaria* on levels 2-4 was generally low. First, identical ITS types occurred on fungus combs from different termite genera and species. For example, ITS type 1.11 was found in nests of *Macrotermes michaelseni*, *M. natalensis*, *O. badius*, *O. latericius*, and *O. transvaalensis* (Table 2-1). 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 2-1). 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 2-1). 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 Figure 2-2. The phylogenetic tree 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 2-5). 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 2-1).

Figure 2-1 Estimated phylogeny of *Xylaria* isolated from South African fungus-growing termite nests, based on ITS sequences. The bars on the right indicate to which termite genus/genera the nests belong, from which the *Xylaria* isolates originate: *Macrotermes* (white), *Microtermes* (black) and *Odontotermes* (grey), 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.
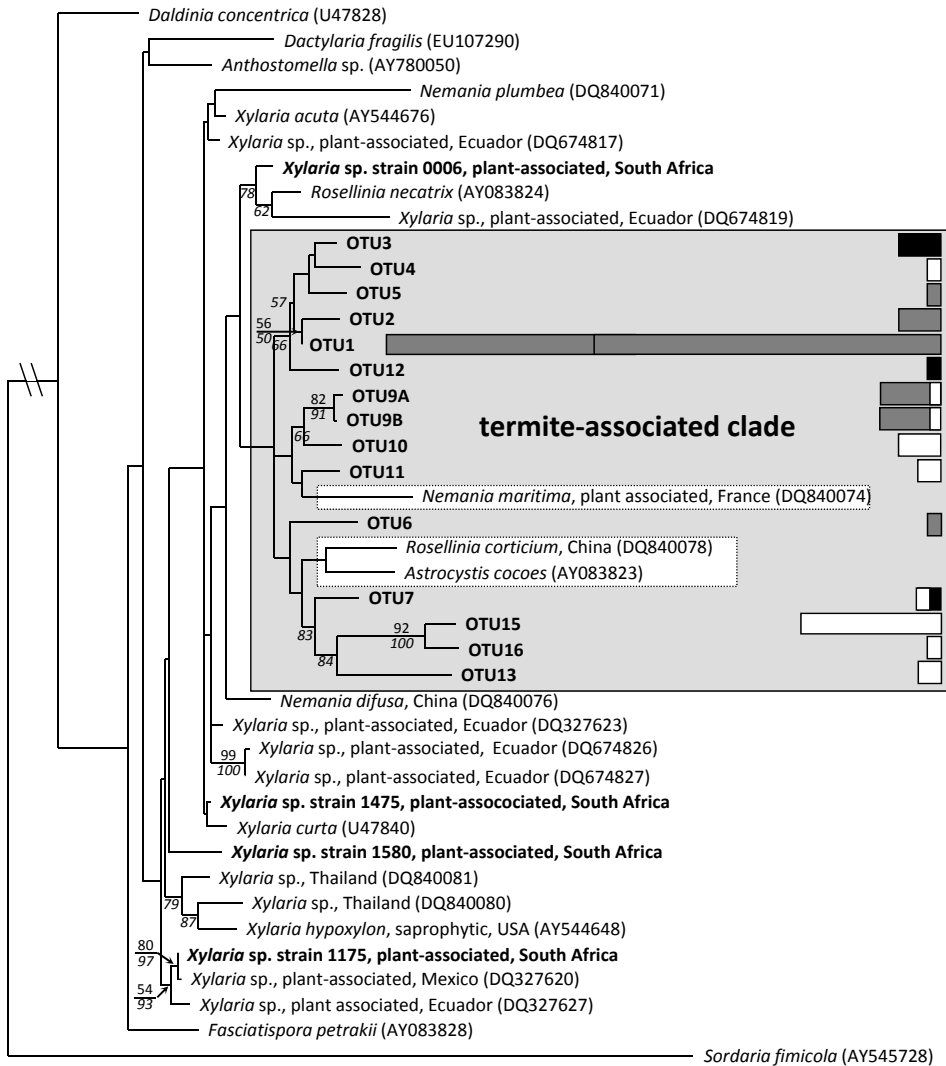
FIGURE 2-2 Estimated phylogeny of *Xylaria* isolated from fungus-growing termite nests (grey area) and non-termite-associated *Xylaria*/Xylariaceae (white area), based on LSU sequences. The width of the coloured bars is proportional to the number of isolates that is represented by each isolate of which the LSU region was sequenced. The colours of the bars on the right indicate to which termite genus/ genera the nests belong, from which the Xylaria isolates originate: *Macrotermes* (white), *Microtermes* (black) and *Odontotermes* (grey). 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.

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

TABLE 2-5 (Continued)

OTU	BLAST hit 1	BLAST hit 2	BLAST hit 3
9	AB217793	EU164400	EU164408
	uncultured xylariaceous fungus	*Xylaria* sp.	*Xylaria* sp.
	termite-associated	termite-associated	termite-associated
	Japan	Central Africa	Central Africa
	100/94%	86/92%	98/89%
	Shinzato *et al.* 2005	unpublished	unpublished
10	AB217793	EU164408	EU164400
	uncultured xylariaceous fungus	*Xylaria* sp.	*Xylaria* sp.
	termite-associated	termite-associated	termite-associated
	Japan	Central Africa	Central Africa
	99/88%	98/87%	90/89%
	Shinzato *et al.* 2005	unpublished	unpublished
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

Discussion

Specificity of Xylaria

Our data show that *Xylaria* has specificity for fungus-growing termites (level 1), as all termite-associated *Xylaria* cluster together (Figure 2-2). 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 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 2-1; Figure 2-1). 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 2-1; Figure 2-1). 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 2-2).

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 dispersal 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 consider 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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Chapter 3

Pseudoxylaria as stowaway of the fungus-growing termite nest: interaction asymmetry between *Pseudoxylaria*, *Termitomyces* and free-living relatives*

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Abstract

Though invisible in healthy nests, *Pseudoxylaria* species are almost always present and regularly overgrowing fungus-growing termite gardens. Whether these fungi are detrimental to the fungus garden, benign, or even beneficial is unclear. We hypothesise that *Pseudoxylaria* is a stowaway that practices a sit-and-wait strategy to survive in the termite nest. Using isolates from three different termite genera to test our hypothesis, we compared *Pseudoxylaria*'s growth on 40 carbon sources with that of *Termitomyces* and tested its interaction with *Termitomyces*. The C-source use of both fungi largely overlapped, indicating competition. One-to-one interactions between *Pseudoxylaria*, *Termitomyces* and free-living relatives showed that *Pseudoxylaria* and *Termitomyces* strains interacted differently with each other than with each other's free-living relatives. *Pseudoxylaria* was more strongly inhibited by *Termitomyces* than free-living Xylariaceae. These results suggest that the symbiotic lifestyle adopted by *Pseudoxylaria* went together with adaptations that changed the interaction between both fungi, consistent with *Pseudoxylaria* being a stowaway.

Keywords

antagonism, carbon source competition, fungal symbionts, fungus-growing termites, interaction, mutualism, *Pseudoxylaria*, symbiosis, *Termitomyces*, *Xylaria*

Introduction

Mutualisms, reciprocal beneficial interactions between species, are ubiquitous and often ecologically dominant in many ecosystems (Korb & Aanen 2003; Mueller *et al.* 2005; Hulcr & Cognato 2010; Leigh 2010). Mutualistic symbioses can be threatened by parasites, but the symbionts can also engage in secondary mutualistic interactions, so that mutualisms often involve more than two species (Little & Currie 2007). Therefore, the evolutionary and ecological aspects of additional players should be considered when studying mutualisms.

Termites of the subfamily Macrotermitinae and fungi of the genus *Termitomyces* (Basidiomycota: Lyophyllaceae) live in an obligate mutualistic symbiosis (Wood & Thomas 1989; Darlington 1994). This mutualistic symbiosis is the result of long-term coevolution (reciprocal genetic adaptation), during which no reversal to free-living state of either of the partners has occurred (Aanen *et al.* 2002). The termites collect and fragment dead plant material, have it pass through the gut where many components remain undigested, and deposit the faeces in their nest (Sands 1960; Sieber & Leuthold 1981; Wood & Thomas 1989). These faecal deposits are piled up to form the sponge-shaped structure that is overgrown by *Termitomyces* and named 'fungus comb' (Sands 1960). Enhanced by the warm, moist and stable climate of the termite mound, *Termitomyces* degrades the plant material and produces primordial fruiting bodies known as nodules. The nodules serve as termite food due to their high nitrogen content, and as somatic inocula of newly added fungus comb substrate (Sands 1969; Wood & Thomas 1989; Leuthold *et al.* 2004).

While under normal circumstances *Termitomyces* grows in monoculture (Katoh *et al.* 2002; Moriya *et al.* 2005; Shinzato *et al.* 2005; Aanen 2006), species of the genus *Xylaria* (Ascomycota: Xylariaceae) are frequently found in and on inactive termite nests (Thomas 1987; Darlington 1994; Rogers *et al.* 2005). Several *Xylaria* species are known for their capacity to degrade lignin and cellulose (Rogers 2000) and occur in a wide range of habitats, as long as there is plant material available (Rogers *et al.* 2005; Hsieh *et al.* 2010). Although many *Xylaria* species do have fairly broad niches, a phylogenetically

distinct group of species within *Xylaria*, classified as subgenus *Pseudoxylaria* (Hsieh *et al*. 2010), specifically occur in fungus-growing termite nests. In a previous study, sixteen operational taxonomic units or 'species' of *Pseudoxylaria* were found in the north-east of South Africa (Visser *et al*. 2009 – Chapter 2). However, within this group no or very little evidence was found for coevolution between *Pseudoxylaria* and either species of *Termitomyces* or groups of fungus-growing termites (Visser *et al*. 2009). Other research groups have confirmed that these *Pseudoxylaria* species do not occur in fungus-growing termite gardens accidentally, and should be considered an additional symbiont (Guedegbe *et al*. 2009; Hsieh *et al*. 2010).

The roles suggested for *Pseudoxylaria* are diverse, ranging from mutualistic to parasitic or as competitor for substrate. For example, it has been stated that in nests of *Odontotermes redemanni*, *Xylaria nigripes* grows as an exclusive mutualistic cultivar (Sannasi 1969). Others suggest that *Xylaria* inside the comb and *Termitomyces* on the surface of the comb are coexisting cultivars; *Xylaria* may prepare the comb substrate for *Termitomyces*, and therefore is an additional mutualistic cultivar (Batra & Batra 1979). The suggestion that *Pseudoxylaria* is the exclusive mutualistic cultivar (Sannasi 1969), however, was reported only once. We presume this notion results from the fact that *Pseudoxylaria* often rapidly overgrows *Termitomyces* when isolates from comb material are used. Under such conditions *Termitomyces* may not be detected, especially when no nodules are present. Though there are no indications for a parasitic role (Visser *et al*. 2009), neither is there conclusive evidence against it; therefore, the hypothesis cannot be rejected. Based on the observation that *Termitomyces* mycelium dominates healthy fungus comb completely (Shinzato *et al*. 2005), that *Pseudoxylaria* could not be retrieved from all incubated combs, and patterns of host specificity, we have previously suggested that *Pseudoxylaria* is a latent weed, a stowaway, meaning that it is a substrate specialist that subsists in the comb until it gets the chance to grow and feast on the comb substrate (Visser *et al*. 2009; Guedegbe *et al*. 2009).

Assuming that *Pseudoxylaria* is an unwelcome guest in the fungus-growing termite nest, it faces a big challenge to survive in the highly dynamic fungus-combs, to which termites constantly add new material on one side and eat parts on the other side.

While *Pseudoxylaria* has to be present and ready to overtake the fungus-garden at any time, how does *Pseudoxylaria* interact with *Termitomyces* while avoiding sanctions? Interacting fungi produce a range of volatiles that change during the course of interaction (Hynes *et al.* 2007; Evans *et al.* 2008), and zones of antagonistic interaction between wood-decaying basidiomycetes and ascomycetes attract mycetophilid fungus gnats of the genus *Bradysia* (Diptera: Sciaridae), for example Boddy *et al.* (1983). The volatiles produced when *Pseudoxylaria* is interacting with *Termitomyces* might attract the attention of grooming worker termites (Batra & Batra 1979). To remain undetected, *Pseudoxylaria* may have evolved towards behaving less competitive towards *Termitomyces* to provoke less antagonism in *Termitomyces*. However, from the perspective of *Termitomyces* fitness, evolution should be towards increased antagonism towards *Pseudoxylaria* to increase the likelihood that termite workers will detect and suppress it. In order to survive, *Pseudoxylaria* may have increased levels of resistance or tolerance to *Termitomyces'* antagonism, in addition to acting inconspicuously. These hypotheses can best be addressed in an explicit evolutionary-empirical framework, using the symbionts *Pseudoxylaria* and *Termitomyces* as well as their free-living sister groups.

To test the role of *Pseudoxylaria* in the fungus-growing termite nest we designed two experiments. First, we measured to what degree these two fungi overlap in diet, by growing *Pseudoxylaria* and *Termitomyces* independently on forty different C-sources. If *Pseudoxylaria* is indeed a competitor for resources from the substrate, we predict substantial overlap in the C-sources used. Second, we studied the interaction between *Pseudoxylaria*, *Termitomyces* and with each other's free-living sister groups (*Nemania diffusa* and *Xylaria* sp. of Xylariaceae, and *Calocybe constricta* of Lyophyllaceae respectively) directly by growing them in different combinations on the same plate. Strains of *Pseudoxylaria* and *Termitomyces* from nests of the same three fungus-growing termite genera (*Macrotermes*, *Microtermes*, and *Odontotermes*) were used to test whether there is interaction specificity between fungi from the same termite genus. Figure 3-1 shows which phylogenetic groups are represented by the test strains chosen.

If *Pseudoxylaria* has indeed evolved as a symbiont, the interactions between *Pseudoxylaria* and *Termitomyces* are expected to differ from the interactions among combinations that include free-living relatives. *Pseudoxylaria*'s adoption of a symbiotic lifestyle is expected to have required certain adaptations that changed the mode of interaction with *Termitomyces*. Including free-living relatives for comparison in the interaction experiment is essential, because it is the only way to determine whether *Pseudoxylaria* has adapted its way of interacting, such as behaving like a stowaway in order to escape termites' attention.
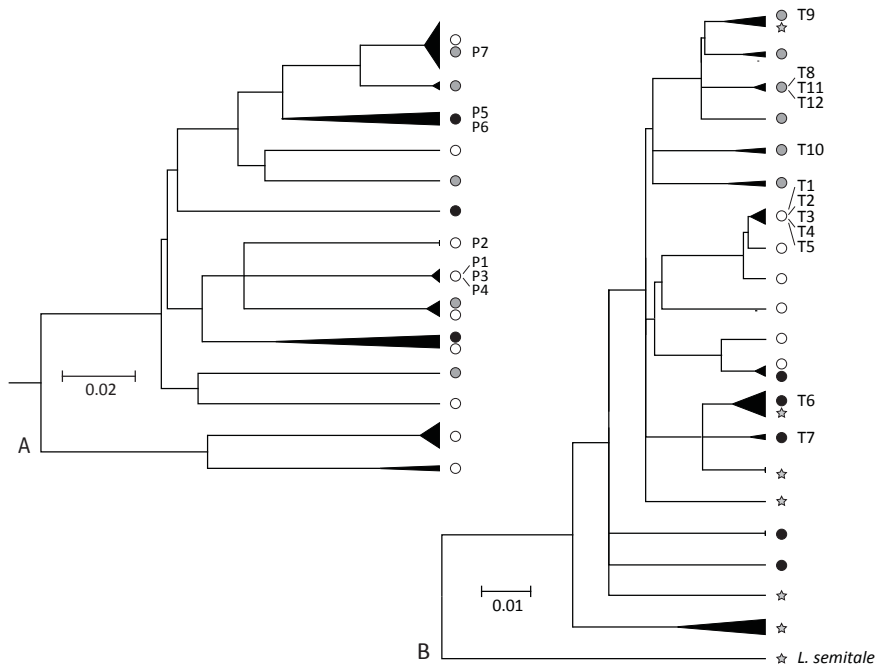
FIGURE 3-1 Simplified phylogenetic trees of *Pseudoxylaria* and *Termitomyces* showing the position of strains used in the experiments. A. Midpoint rooted neighbour-joining (NJ) tree of *Pseudoxylaria* based on 70 non-identical ITS sequences (Visser *et al*. 2009). B. NJ tree of *Termitomyces* based on 53 non-identical ITS sequences with *Lyophyllum semitale* as outgroup (adapted from Nobre *et al*. 2011). The vertical dimension of the triangles is proportional to the number of sequences grouped in that branch; the horizontal dimension indicates the variation within those groups. The termite genera from which the sequenced fungi originate are indicated: *Macrotermes* (white circle), *Microtermes* (black circle), *Odontotermes* (grey circle), and other genera (grey stars). See TABLE 3-1 for strain identification.

Materials and methods

Table 3-1 gives an overview of all Xylariaceae and Lyophyllaceae strains used in the experiments. Strains were grown on malt-yeast-extract agar (MYA, see Visser *et al.* 2009) in the dark at 25 °C, using Petri dishes with diameter 85 mm unless stated otherwise.

Table 3-1 Overview of strains used in the experiments.

	Code	Strain	Origin	C-source exp.	Interaction exp.
Xylariaceae	X1	*Nemania diffusa* (CBS 120711)	free-living, China		x
	X2	*Xylaria* sp. 0006	free-living, South-Africa (SA) 2008		x
	P1	*Pseudoxylaria* 501.11c (CBS 124048)	*Macrotermes natalensis*, SA 2005	x	
	P2	*P.* 711.C (CBS 124047)	*M. natalensis*, SA 2007	x	
	P3	*P.* 801.A3	*M. natalensis*, SA 2008		x
	P4	*P.* 807.30	*M. natalensis*, SA 2008		x
	P5	*P.* 517.A (CBS 124050)	*Microtermes* sp., SA 2005	x	x
	P6	*P.* 806.1	*Microtermes* sp., SA 2008		x
	P7	*P.* 509.1j (CBS 124049)	*Odontotermes* sp., SA 2005	x	x
Lyophyllaceae	L1	*Calocybe constricta* (CBS 320.85)	free-living, Czech Republic		x
	T1	*Termitomyces* 57.A	*M. natalensis*, SA 2005	x	
	T2	*T.* 59.A	*M. natalensis*, SA 2005	x	x
	T3	*T.* 62	*M. natalensis*, SA 2005	x	
	T4	*T.* 77	*M. natalensis*, SA 2005	x	
	T5	*T.* 78	*M. natalensis*, SA 2005	x	
	T6	*T.* 40.B	*Microtermes* sp., SA 2005	x	x
	T7	*T.* 48.A	*Microtermes* sp., SA 2005	x	
	T8	*T.* 67.C	*O. transvaalensis*, SA 2005	x	
	T9	*T.* 68.C	*O. transvaalensis*, SA 2005	x	
	T10	*T.* 69.sscA	*O. transvaalensis*, SA 2005	x	
	T11	*T.* 73	*O. badius*, SA 2005	x	x
	T12	*T.* 74.sscA	*O. badius*, SA 2005	x	

[a]For strains without ITS sequence on GenBank, the LSU accession number or the first BLAST-result (with sequence identity in %) is given.

Preparation of inocula and incubation plates

Termitomyces tissue with nodules, harvested from a culture that had grown 3-5 weeks, was placed in an Eppendorf tube with 0.5 ml saline solution (0.8% NaCl w/v). Mycelial tissue and nodules were nodules were macerated with a small sterile plastic pestle. These mycelium suspensions were used on the same day to inoculate experimental plates.

Pseudoxylaria inoculum for the C-source experiment was made by cutting ±8 mm³ cubes of five times diluted MYA with mycelium. For the interaction experiment the three free-living strains and *Pseudoxylaria* were grown in Erlenmeyers with ±125 ml of liquid malt-yeast-extract liquid broth (MY; 2%, 0.2% w/v respectively). The MY broth was inoculated with a piece of MYA with mycelium and then macerated with a blender to fragment the inoculum and mix it with the broth. The macerating was repeated on the third and fourth day after inoculation, resulting in mycelium suspensions that were used on that fourth day to inoculate experimental plates.

C-source experiment

Minimal medium [6 g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g KCl, 1 mg $FeSO_4.7H_2O$, 1 mg $ZnSO_4.7H_2O$, 1 mg $CuSO_4$, 1 mg $MnCl_2$, 15 g agar, 1 L demi-water (Pontecorvo *et al.* 1953; van Diepeningen *et al.* 2006)] was used, to which different C-sources were added varying from monosaccharides to complex C-sources. In total there were forty different C-sources (see Table 3-2). All media were adjusted to pH 5.8.

The 12 *Termitomyces* strains were inoculated using 5 μl mycelium suspension per inoculum. Plates inoculated with five inocula of T3 and five of T11 were made in duplicate. The other ten *Termitomyces* strains were tested together on one plate, with one inoculum each and two replicates per medium. *Pseudoxylaria* was inoculated using the 8 mm³ agar cubes. Four strains were tested on the same plate, with four replicates per medium. Ordinal growth scores were given for *Pseudoxylaria*, T3 and T11 after eleven days and for the other *Termitomyces* strains after seventeen days: '0' for no or nihil growth (< 1 mm for *T.*, < 3 mm for *P.*), '1' for little growth (< 3 mm for *T.*, 3-10 mm for *P.*, no thick mycelium), and '2' for vigorous growth (thick mycelium and/or more than 3 or 10 mm of outgrowth for *T.* and *P.* respectively).

Interaction experiment

There were two main treatments in the interaction experiment: one in which *Termitomyces* and *Pseudoxylaria* were in contact

immediately after inoculating the latter (treatment A), and a second in which both fungi were inoculated at some distance from each other (treatment B).

In both treatments, the day on which *Pseudoxylaria* and its free-living relatives were inoculated was defined as day 0, the starting point of the interaction experiment. As *Pseudoxylaria* colonises MYA plates much faster than *Termitomyces*, the latter was given a head start by inoculating it three days earlier. The fact that in nature *Termitomyces* propagules are present in the termite faeces from which the fungus comb is constructed (Leuthold *et al.* 1989), where *Termitomyces* thus has a well-established mycelium before *Pseudoxylaria* appears, justifies giving *Termitomyces* a head start.

In treatment A, plates were first inoculated with 20 µl of mycelium suspension of *Termitomyces* strains or its free-living relative (Lyophyllaceae). The inoculum was spread by shaking with 5-15 glass beads (diameter 3 mm) per plate. After three days, on day 0, *Pseudoxylaria* strains or its free-living relatives (Xylariaceae) were inoculated by placing mycelium suspension drops in a triangular position, each drop at about 2 cm from the rim of the plate and at regular distance from each other. In treatment B, three 20 µl drops of mycelium suspension of Lyophyllaceae were placed in a triangular position on the plates, and after three days 20 µl of mycelium suspension of the Xylariaceae was placed in the centre of these plates (see also Figure 3-3). For combinations of *Pseudoxylaria* strains P3, P5 and P7 with Lyophyllaceae, five replicates were made. For all other combinations and blanks, three replicates were made. For the blanks of treatment A and B, strains were inoculated as in treatment A and B respectively, but without the other fungus.

To qualify the type of interaction between the fungi, scores were given after seven days: 'D' for 'Deadlock at distance' in case of a clear zone between mycelia with no growth, 'O' for 'Overgrowing', 'R' for 'Replacement' and 'T' for 'Touching' mycelia (adapted from Dowson *et al.* 1988; see Figure 3-3). To quantify the effect of interaction on the growth of the fungi, mycelium radius was measured on days 3, 6, 9, and 12.

Statistics

A Principal Component Analysis (PCA) ordination plot was made (CANOCO version 4.5) to depict differences between *Pseudoxylaria* and *Termitomyces* growth on the forty different C-sources. For the interaction experiment, 4512 mycelium radius measurements were used in the quantitative analysis. Averages of within-plate pseudo-replicates were used as mycelium radius size (M). Mycelium radius size relative to blank ($M_{relative}$) was used to test if interacting Xylariaceae grew less than their blank ($M_{relative} < 1$, Student's t-test, Microsoft Office Excel 2007, SPSS Inc PASW Statistics version 17). A Wilcoxon signed-rank test was done on the differences in relative radius size of Xylariaceae between treatment A and B, to test if Xylariaceae grew significantly less in treatment A. The ratio (R) between the relative size of both interacting strains was calculated for each interaction combination ($R_{relative\ ij} = M_{relative\ i} / M_{relative\ j}$). Averaging R-values of X1 and X2 gave $R_{average\ free-living\ Xylariaceae}$ and averaging R-values of P3-P7 gave $R_{average\ Pseudoxylaria}$.

Results

The results of the growth experiment on different C-sources are given in Table 3-2. Only few differences between *Pseudoxylaria* and *Termitomyces* were observed. There were very few substrates on which only one genus grew well, while the other genus displayed little or no growth. Both genera grew best on the more complex C-sources, with the exception of lignin and tannin. *Termitomyces* grew better than *Pseudoxylaria* on the two cellulose media. The overall similarity in growth pattern is also reflected in the PCA ordination plot (Figure 3-2). The horizontal axis explains 95% of variation and arranges C-sources and fungal strains according to the value of their total growth score, with no growth (average score 0) on the left, to most fungal growth (average score 2) on the right hand side. The simple C-sources occur mainly on the left and the complex C-sources more on the right side of the graph. *Termitomyces* and *Pseudoxylaria* only show a difference along the vertical axis that explains only 1.4% of additional variation.

TABLE 3-2 Growth scores for *Pseudoxylaria* and *Termitomyces* strains on forty C-sources; '0' for no or nihil growth (< 1 mm for *Termitomyces*, < 3 mm for *Pseudoxylaria*), '1' for little growth (< 3 mm for *Termitomyces*, 3-10 mm for *Pseudoxylaria*, no thick mycelium), and '2' for vigorous growth (thick mycelium and/or more than 3 or 10 mm of outgrowth for *Termitomyces* and *Pseudoxylaria*, respectively). *Not measured. See TABLE 3-1 for strain identification.

C-source	C-source type	Pseudoxylaria				Termitomyces											
		P1	P2	P5	P7	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12
no C-source	none	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
25 mM D-glucose	monosacch.	1	1	1	1	1	1	1	1	1	2	2	1	2	1	2	2
25 mM D-fructose	monosacch.	1	1	1	1	1	1	1	1	1	1	1	1	2	1	2	2
25 mM D-galactose	monosacch.	1	1	0	0	0	0	0	1	0	1	0	0	0	0	0	1
25 mM D-mannose	monosacch.	1	1	1	1	1	1	1	1	1	1	1	0	2	1	1	1
25 mM D-ribose	monosacch.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
25 mM D-xylose	monosacch.	0	0	0	1	1	1	1	1	1	2	2	1	1	1	1	1
25 mM L-arabinose	monosacch.	0	0	1	0	1	1	0	1	1		0	0	1	0	0	0
25 mM L-rhamnose	monosacch.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
25 mM D-galacturonic acid	monosacch.	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0
25 mM D-glucuronic acid	monosacch.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
25 mM cellobiose	disacch.	1	1	0	1	1	1	1	1	1	2	2	1	2	2	1	2
25 mM maltose	disacch.	1	1	1	1	0	0	0	0	0	0	0	0	0	0	1	0
25 mM lactose	disacch.	0	0	0	0	1	1	0	1	1	1	1	0	1	0	0	1
25 mM sucrose	disacch.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
25 mM raffinose	trisacch.	1	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0
1% arabinogalactan	polysacch.	0	0	1	1	1	1	1	1	1	1	1	0	1	0	0	1
1% beechwood xylan	polysacch.	0	0	0	1	1	1	0	1	1	1	1	1	2	1	0	1
1% birchwood xylan	polysacch.	0	0	0	1	1	1	1	1	1	1	1	1	2	1	1	1
1% oat spelt xylan	polysacch.	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	1
1% Arabic gum	polysacch.	0	0	2	1	0	0	0	0	0	0	0	0	0	0	1	0
1% Guar gum	polysacch.	0	0	2	2	2	2	1	2	2	1	0	0	0	0	0	1
1% soluble starch	polysacch.	1	1	1	1	1	1	0	0	0	1	2	1	1	1	1	1
1% apple pectin	polysacch.	1	2	1	2	2	2	0	1	1	1	0	1	1	1	0	1
1% citrus pectin	polysacch.	2	2	2	2	1	2	1	1	1	2	1	1	2	1	1	2
1% inulin	polysacch.	0	1	1	1	1	1	1	1	1	1	0	1	1	0	1	0
1% lignin hydrolytic	polysacch.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1% cellulose	polysacch.	0	0	1	1	1	1	1	1	1	2	1	1	2	1	1	2
1% carboxymethyl cellulose	polysacch.	0	0	0	0	1	1	*	1	1	1	1	1	1	1	*	1
3% wheat bran	complex	1	1	1	2	2	2	2	2	2	2	2	2	2	2	2	2
3% sugar beet pulp	complex	1	0	1	0	2	2	1	2	2	2	0	1	2	2	0	2
3% citrus pulp	complex	2	0	2	2	2	2	2	2	2	2	2	2	2	2	1	2
3% soybean hulls	complex	2	0	2	2	2	2	2	2	2	2	2	2	2	2	2	2
3% rice bran	complex	2	2	2	2	2	1	2	1	1	2	0	0	0	2	1	2
3% cotton seed pulp	complex	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
3% alfalfa meal	complex	2	1	2	2	2	2	2	2	2	2	2	2	2	2	2	2
3% oat hulls	complex	2	2	2	2	2	2	2	2	2	2	2	1	2	2	1	2
3% corn gluten	protein	2	1	1	2	2	2	1	2	2	1	1	0	1	1	1	1
1% casein	protein	2	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1
1% tannin	polyphenol	0	0	0	0	0	0	*	0	0	0	0	0	0	0	*	0

Qualitative scores for the interactions between Xylariaceae and Lyophyllaceae are given in TABLE 3-3. Four broad categories of outcomes can be recognised (FIGURE 3-3). Overgrowing and sometimes replacement occurred in combinations where free-living Xylariaceae interacted with *Termitomyces* and its free-living relative

Calocybe constricta (L1); deadlock at a distance occurred in interactions between *Pseudoxylaria* and L1; and interactions where both mycelia touched each other occurred in interactions between *Pseudoxylaria* and *Termitomyces*. Overgrowth also occurred with *Pseudoxylaria*, mainly by the fast-growing P4, in interaction with *Termitomyces* (see also Figure 3-3). There were no indications of any specificity within *Pseudoxylaria* strains interacting with *Termitomyces*; the outcome of interactions was not dependent on host origin (Table 3-3).

Relative radius sizes show that *Pseudoxylaria* strains grew significantly less in the presence of *Termitomyces* than when growing alone in both treatment A and B (Table 3-4). Xylariaceae were significantly more reduced in treatment A than in treatment B (n = 27, S = 50; P < 0.001). Likewise, *Termitomyces* grew less in the presence of *Pseudoxylaria* (Table 3-5). For each combination the ratio (R) of Xylariaceae relative radius size divided by Lyophyllaceae relative radius size is shown in Figure 3-4. Average R-values reveal
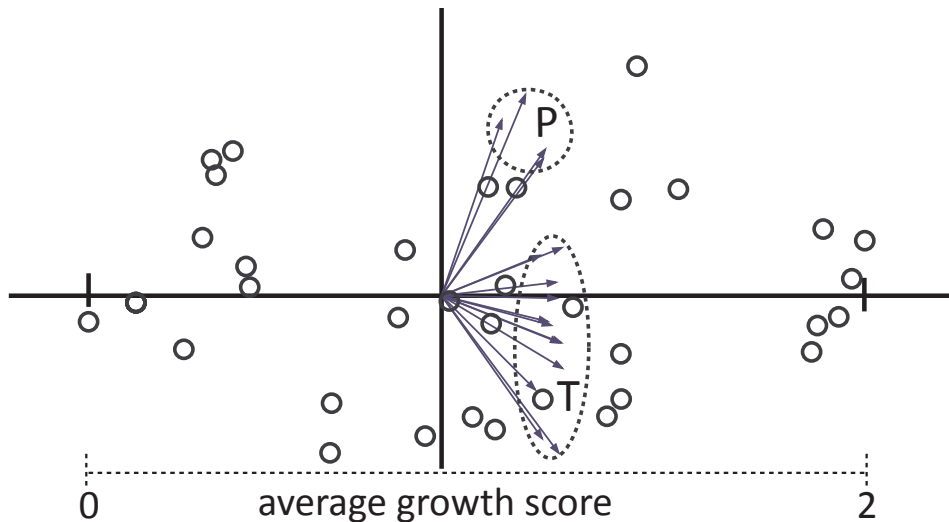
Figure 3-2 Position of *Pseudoxylaria* strains (pointed at by 4 arrows in circle P), *Termitomyces* (pointed at by 12 arrows in circle T – some overlap) and the 40 different C-sources (40 circles, 35 visible as some overlap) in a space determined by the growth scores, using Principal Component Analysis (PCA). The horizontal ordination axis explains 95% of the variation in growth scores, ranging from average score '0' on the far left, to average score '2' on the far right side of the graph. The vertical axis explains 1.4% of the variation in growth scores. See Table 3-1 for strain identification.

that *Pseudoxylaria* was more reduced in size, relative to *Termitomyces*, than free-living Xylariaceae; $R_{average\ Pseudoxylaria}$ = 0.85 while $R_{average\ free-living\ Xylariaceae}$ = 1.24, which is a significant difference (df = 26, t = 3.24; P < 0.005). Apart from this difference between free-living Xylariaceae and *Pseudoxylaria*, there was no pattern of specificity in the *Pseudoxylaria-Termitomyces* interaction; it made no difference if the fungi came from the same termite genus or not.

Absolute radius sizes across time also show a clear difference in interaction effect between free-living Xylariaceae and *Pseudoxylaria*

TABLE 3-3 Qualitative scores for interactions between *Pseudoxylaria*, *Termitomyces* and their free-living relatives for treatment A (Lyophyllaceae spread across the plate and Xylariaceae in 3 spots) and treatment B (Lyophyllaceae in 3 spots and Xylariaceae in center). See TABLE 3-1 for strain identification.

	L1		T2		T6		T11	
	A	B	A	B	A	B	A	B
X1	O	R	O	O	O	O	O	O
X2	O	R	O	O	O	O	O	O
P3	D	D/o	T	-	T	-	T/D	-
P4	O	T	O	O	O	T/O	O	T/O
P5	D	D	T	T	T/R	T	T	T
P6	D	D	T	T	T	T/D	T/O	T
P7	o	T/o	T	T	T	T/o	T	T/O

Legend:	D	=	Deadlock, defense at distance; clear zone between mycelia
	O, o	=	Overgrowing; 'O' if X. overgrows L., 'o' if L. overgrows X.
	R	=	Replacement; X. replaces L.
	T	=	Touching mycelia
	-	=	not scored

Deadlock (P3 vs. L1)   Overgrowing (P4 vs. T2)   Touching (P7 vs. T6)   Replacement (X1 vs. L1)
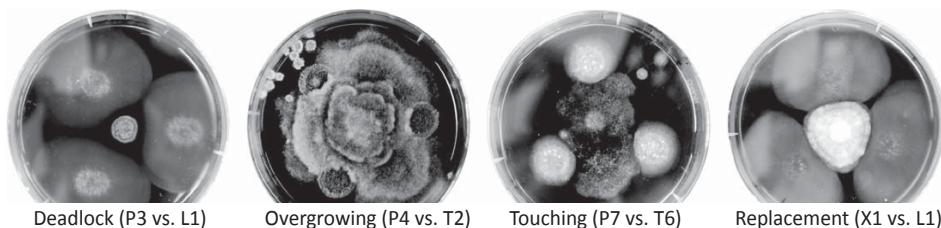
FIGURE 3-3 Representative examples of interactions observed in treatment B (Lyophyllaceae in 3 spots and Xylariaceae in center) on day 7. Pictures show from left to right 'Deadlock (at distance)' with *Pseudoxylaria* and *Calocybe constricta*; *Pseudoxylaria* 'Overgrowing' *Termitomyces*; *Pseudoxylaria* 'Touching' *Termitomyces*; and 'Replacement' of *Calocybe constricta* by *Nemania diffusa*. The small dots on the plates of pictures shown in the middle are accidental *Termitomyces* colonies. See TABLE 3-1 for strain identification.

Table 3-4 Xylariaceae (X and P) in interaction with Lyophyllaceae (L and T) grew less than their blank (growth without Lyophyllaceae or Xylariaceae, respectively). Relative mycelium radius size of Xylariaceae and coded P-values of Student's t-test of Mrelative < 1 were calculated, using radius measurements of day 9 from treatment A (Lyophyllaceae spread across the plate and Xylariaceae in 3 spots) and treatment B (Lyophyllaceae in 3 spots and Xylariaceae in center). See Table 3-1 for strain identification.

		L1		T2		T6		T11	
treatment A	X1	0.46	++++	0.71	+++	0.72	++++	0.76	+++
	X2	0.41	+++	0.81	++++	0.85	+++	0.80	+++
	P3	0.16	++++	0.29	++++	0.37	++++	0.38[b]	++++
	P4	-[a]	-	0.42	++	0.63	++	0.71	+
	P5	0.22	++++	0.36	++++	0.40	++++	0.26	++++
	P6	0.26	++++	0.41	+++	0.47	++++	0.40	+++
	P7	0.15	++++	0.21	++++	0.78[b]	+	0.18[b]	++++
treatment B	X1	0.53	++++	0.66	++++	0.78	+++	0.72	+++
	X2	0.76	++++	0.73	+++	0.86	++	0.81	+++
	P3	0.36	++++	0.75	++++	0.93	n.s.	0.83	++
	P4	0.28	++	0.50	++	0.58	+	0.51	++
	P5	0.29	++++	0.64	++++	0.65	++++	0.75	++++
	P6	0.39	++++	0.82	++++	0.70	++++	0.90	++++
	P7	0.31	+	0.55	+	0.55	+	0.64	++

Legend:

code	P-value
-	= no value
+	= P<0.05
++	= P<0.01
+++	= P<0.001
++++	= P<0.0001

[a]The combination P4 vs. L1 could not be measured as the boundaries between the fungi were unclear.
[b]For these values measurements of day 6 were used.
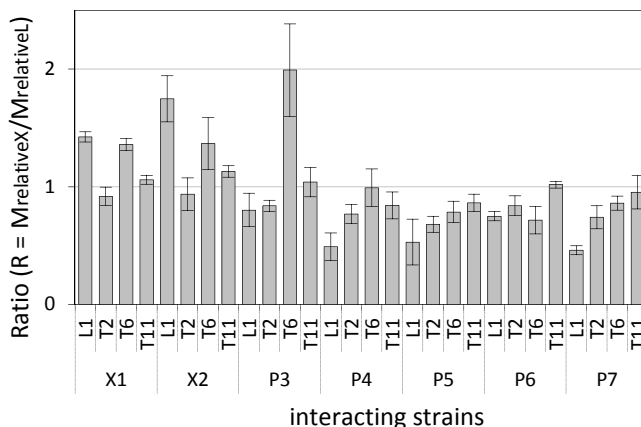
Figure 3-4 Ratio (R) between the relative size of Xylariaceae and Lyophyllaceae in treatment B (Lyophyllaceae in 3 spots and Xylariaceae in center). Bars show the 95% confidence interval. When R < 1 for a combination, the interaction was relatively more negative for the Xylariaceae than for the Lyophyllaceae. See Table 3-1 for strain identification.

TABLE 3-5 Lyophyllaceae (L and T) in interaction with Xylariaceae (X and P) grew less than their blank (growth without Lyophyllaceae or Xylariaceae, respectively). Relative radius size of Lyophyllaceae was calculated using mycelium measurements from treatment B (Lyophyllaceae in 3 spots and Xylariaceae in center) on day 9. See TABLE 1 for strain identification.

	X1	X2	P3	P4	P5	P6	P7
L1[a]	0.39	0.35	0.83	0.64	0.77	0.80	0.73
T2	0.76	0.82	0.86	0.65	0.84	0.84	0.75
T6	0.65	0.73	0.54	0.59	0.79	0.89	0.70
T11	0.74	0.76	0.82	0.60	0.79	0.76	0.70

[a]For L1 the blank measurements of day 6 were used to calculate relative radius size, causing an underestimation of the effect of Xylariaceae on L1.

(see Supplements FIGURE S3-1, S3-2, S3-3). In the blank situation all Xylariaceae strains showed more or less the same growth. When growing with Lyophyllaceae, *Pseudoxylaria* strains showed reduced mycelium size, and often ceased to grow after day 6 (FIGURE S3-1, S3-2). In contrast, free-living Xylariaceae strains had a steady growth increment between all days; they overgrew *Termitomyces* and replaced free-living *Calocybe constricta* (L1), causing a decrease in mycelium size of the latter (FIGURE S3-1, S3-2).

Concerning Lyophyllaceae, L1 colonised the plates much faster than *Termitomyces* (FIGURE S3-3), accompanied by a larger reduction in growth of all Xylariaceae than was caused by *Termitomyces*. Furthermore, L1 was replaced by free-living Xylariaceae and not by *Pseudoxylaria*, while *Termitomyces* strains grew the same in all combinations with Xylariaceae: *Termitomyces* was never replaced but stopped growing after day 3 or 6 (FIGURE S3-3). Absolute radius sizes – like qualitative scores and relative radius sizes – show that combinations of *Pseudoxylaria* and *Termitomyces* had similar outcomes, while there were differences between free-living and fungus-growing termite associated strains.

Discussion

Various hypotheses have been put forward about the role of *Pseudoxylaria* in fungus-growing termite nests. We propose that *Pseudoxylaria* has evolved towards inconspicuousness like a stowaway in the *Termitomyces*-dominated termite fungus garden, until a possibility for abundant outgrowth and reproduction occurs,

e.g. after a major disturbance such as colony collapse after death of the queen, entomopathogenic disease, nematode infestation, or an aardvark or ant attack on the nest.

<p style="text-align:center">C-source experiment indicates competition between
Pseudoxylaria and Termitomyces</p>

The C-source experiment demonstrated that *Pseudoxylaria* and *Termitomyces* occupy essentially the same niche. *Termitomyces* and *Pseudoxylaria* growth on the forty C-sources was almost the same. The PCA ordination diagram confirmed that *Pseudoxylaria* and *Termitomyces* hardly differed from each other in C-sources use: the horizontal ordination axis explaining 95% of the variation in scores separated the C-sources, but did not separate *Termitomyces* from *Pseudoxylaria*. The vertical axis did separate the fungi, however this axis explained only 1.4% of variation. We therefore reject the hypothesis that *Pseudoxylaria* plays a role as complementary degrader as was suggested previously by Batra & Batra (1979). Rather, our results indicate that *Pseudoxylaria* competes with *Termitomyces* for the same substrate.

No growth on lignin and tannin was recorded. This observation seemingly contradicts the notion that both *Xylaria* (subgenus *Pseudoxylaria*) and *Termitomyces* are well known for breakdown of complex compounds that include lignins and tannins. However, in nature the degradation of these compounds takes place in conjunction with degradation of more simple C-compounds, as ligninolysis in itself is considered a process that yields insufficient energy (Kirk & Farrel 1987).

<p style="text-align:center">Interaction experiment reveals differences between free-living strains and fungus-growing termite-associated strains</p>

In all cases, *Pseudoxylaria* and *Termitomyces* strains grew less in combination with each other than when growing alone. This supports the hypothesis that *Pseudoxylaria* does not facilitate *Termitomyces*, but competes for carbon instead.

In combination with *Termitomyces*, *Pseudoxylaria* grew much less than free-living Xylariaceae. Interestingly, *Pseudoxylaria* did not

differ from free-living Xylariaceae when growing in the absence of *Termitomyces*, and *Termitomyces* grew as large with *Pseudoxylaria* as with free-living Xylariaceae. In other words, there is asymmetry in the interaction between Xylariaceae and Lyophyllaceae, with *Pseudoxylaria* growing less than expected in the presence of *Termitomyces*. The interaction asymmetry is consistent with the hypothesised evolutionary scenario that *Pseudoxylaria* has evolved towards an inconspicuous lifestyle, and that it has adaptations to avoid causing strong reactions in *Termitomyces* so as to escape the termites' attention. Furthermore, free-living strains also more often showed antagonistic interactions (deadlock at distance, replacement or overgrowing), than combinations with *Pseudoxylaria* and *Termitomyces*. This reduction in antagonism between the symbiotic strains is also consistent with the hypothesis that *Pseudoxylaria* has evolved towards behaving inconspicuously when it adopted the symbiotic lifestyle.

We observed no pattern of specificity in the *Pseudoxylaria-Termitomyces* interaction. This is in line with our previous study where *Pseudoxylaria* did not show strong specificity for host termite genera (Visser *et al.* 2009), even though termite and *Termitomyces* taxa show a pattern of coevolution (see Figure 1-4; Aanen *et al.* 2002, 2007).

The effect of *Termitomyces* on *Pseudoxylaria* was significantly stronger in treatment A than in B. In treatment A, *Termitomyces* had the advantage of being spread across the whole plate and already germinated when *Pseudoxylaria* was inoculated on top of it. This treatment more closely resembles the situation in nature. There, the material that termites use to construct the fungus comb is vegetation material mixed with a high density of *Termitomyces* (Leuthold *et al.* 2004; Aanen 2006), whereby the fungus comb is instantly completely colonised by *Termitomyces*.

Could the omnipresence of *Termitomyces* in the fungus comb be the factor that restricts the growth of *Pseudoxylaria*? Holmer & Stenlid (1997) wrote that established mycelia may prevent small propagules from colonizing a substrate. The omnipresence of *Termitomyces* in the fungus comb could be one of the factors that restrict the growth of *Pseudoxylaria*. However, when incubating fresh fungus combs, *Pseudoxylaria* generally rapidly overgrows

Termitomyces while the latter is still omnipresent. In the interaction experiment we see only reduction, not prevention of *Pseudoxylaria* growth. Moreover, pilot experiments showed that also plates with a denser and better established mycelium of *Termitomyces* prevented growth of *Pseudoxylaria* only temporarily; after some weeks *Pseudoxylaria* overgrew *Termitomyces* (A.A.V., pers. obs.). Therefore, we think that *Termitomyces* alone cannot prevent *Pseudoxylaria* from overgrowing the fungus garden and suggest for future work to study the extent to which termites play a role in *Pseudoxylaria* control. Additionally, one could imagine that – like in other (fungus-growing) insects – there may also be bacteria that play a role in fungus-growing termite nest hygiene.

Pseudoxylaria*'s role in fungus-growing termite nests*

Like *Pseudoxylaria* species in fungus-growing termite nests, species of the genus *Escovopsis* (anamorphic Ascomycota, Hypocreales) are also restricted to a symbiotic lifestyle and found in fungus-growing ant nests only (Currie *et al.* 1999; Taerum *et al.* 2010). But while *Escovopsis* parasitises the cultivar fungus of fungus-growing ants and shows directed growth towards it (Reynolds & Currie 2004; Gerardo *et al.* 2006), *Pseudoxylaria* grew less in combination with *Termitomyces* than when growing alone and did not show directed growth towards *Termitomyces*. We therefore reject the hypothesis that *Pseudoxylaria* is a mycoparasite.

Our classification of *Pseudoxylaria* as an inconspicuous stowaway is remarkably similar to the sit-and-wait strategy that foliar endophytes apply. *Pseudoxylaria* is not the only member of Xylariaceae with this latent presence while waiting for a chance to devour the substrate. Endophytic xylariaceous fungi apply a similar strategy. *Xylaria* species are an important group among endophytes (Bayman *et al.* 1998; Promputtha *et al.* 2007; Fukasawa *et al.* 2009), and that sit-and-wait potential is also demonstrated by *Xylaria hypoxylon* which was observed to remain in beech logs for more than 4.5 years (Chapela & Boddy 1988).

The comparison may even go beyond sit-and-wait until a resource becomes available. Foliar endophytic fungi can play an important role as protective mutualist by occupying niches in the

plant tissue that otherwise would be filled by pathogens (Arnold *et al.* 2003; Herre *et al.* 2007). Endophytic species of *Xylaria* produce antimicrobial compounds that protect the host plant against more pathogenic endosymbionts (Liu *et al.* 2008), while antimicrobial activity is attributed to Xylariales in general (Vicente *et al.* 2009). *Pseudoxylaria* could fulfill a similar role in the fungus-growing termite nest, which could explain why the termite-*Termitomyces* symbiosis has attracted an additional symbiont.

However, it has been widely observed that the fungus-growing termite garden is a monoculture of *Termitomyces*. Total fungus comb analyses (Moriya *et al.* 2005) show that only five out of 101 sequences were other fungi (among which *Pseudoxylaria*), and T-RFLP could only detect *Termitomyces*. Since *Pseudoxylaria* makes up such a marginal part of the fungal biomass, it remains to be demonstrated that *Pseudoxylaria* has a role in the fungus-growing termite symbiosis as a protective mutualist preventing pathogens from filling the 'gaps' in the fungus garden.

If staying unnoticed in the termite nest is indeed *Pseudoxylaria*'s key to survival, there is selection pressure for *Pseudoxylaria* to avoid antagonistic reactions with *Termitomyces*. *Pseudoxylaria* would need to grow just enough to stay ahead of the fungus-comb replacement, until an occasion occurs that allows it to take over the fungus garden. Assuming that termites play a role in *Pseudoxylaria* control (Shinzato *et al.* 2005) and use interaction volatiles as a cue, *Termitomyces* on the other hand would be selected to react more strongly in order to alarm the termites. This would explain the small size of *Pseudoxylaria* in the nest in terms of biomass (Moriya *et al.* 2005), as long as the termites are actively present.

Symbionts like *Pseudoxylaria* that have closely related free-living sister groups, give unique opportunities to study the effects that adopting a symbiotic lifestyle has on an organism. But also somewhat more distantly related free-living species may provide new insights about the adaptations of a symbiotic organism, including about its interaction with symbiotic partners. For future research we therefore recommend to include more often the free-living relatives of symbionts when addressing evolutionary questions such as symbiont role and interaction specificity.

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Supplements

Figure S3-1 Xylariaceae radius measured on day 3, 6, 9, and 12 in treatment A (Lyophyllaceae spread across the plate and Xylariaceae in 3 spots). Blank shows growth without Lyophyllaceae. Bars show 95% confidence interval. †P4 overgrew L1, but could not be measured because the boundaries between the fungi were unclear; other measurements are lacking in this graph for the same reason. *Only one measurement available as P4 blank reached rim of Petri dish around day 9. See Table 3-1 for strain identification.
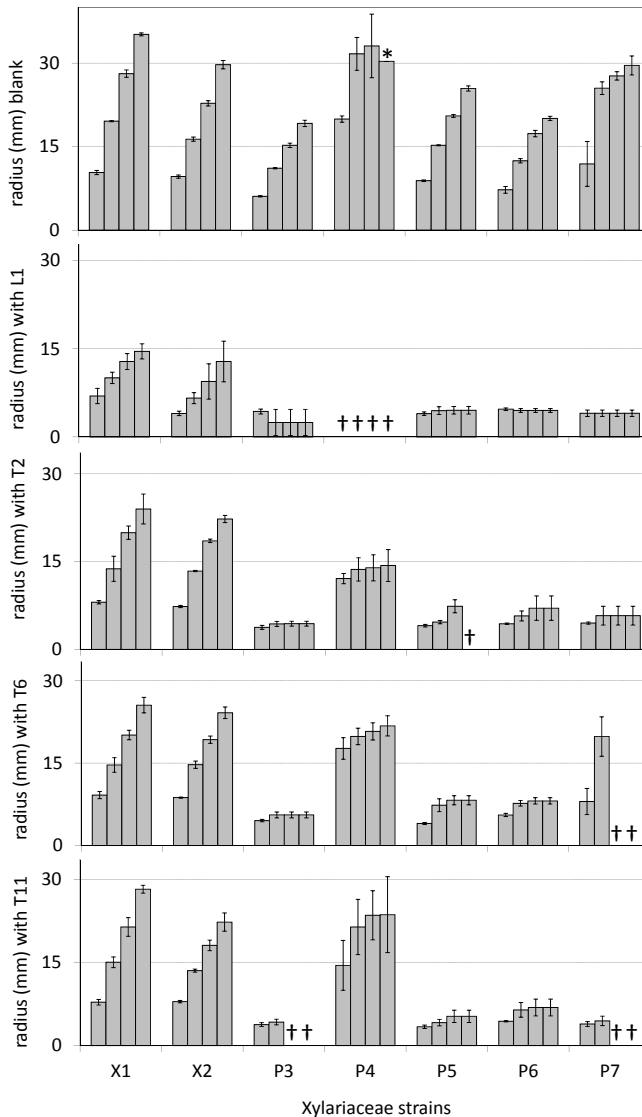
Figure S3-2 *Pseudoxylaria* strains grew less than free-living Xylariaceae in combination with *Termitomyces* and *Calocybe constricta* in treatment B (Lyophyllaceae in 3 spots and Xylariaceae in center). The 'blank' shows growth without Lyophyllaceae. Xylariaceae radius on day 3, 6, 9, and 12; bars show 95% confidence interval. *Only one or no measurements available as P4 reached rim of Petri dish around day 9. See Table 3-1 for strain identification.
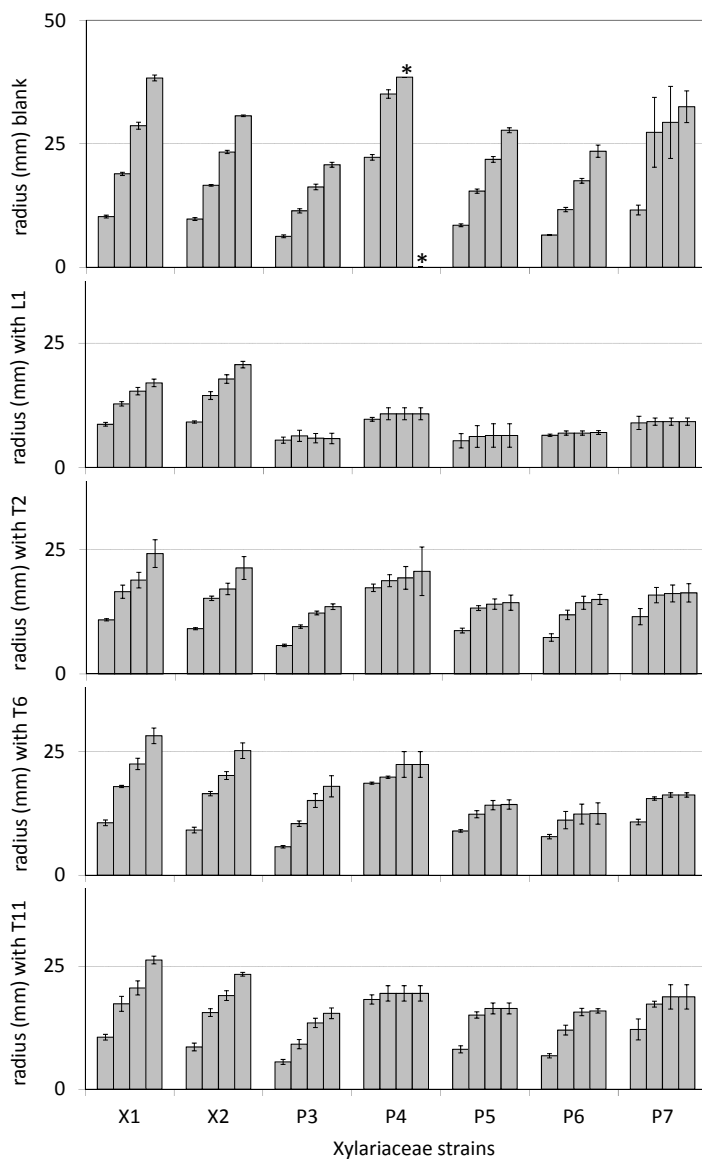
Figure S3-3 *Termitomyces* growth was reduced in all combinations with Xylariaceae, while *Calocybe constricta* mycelium was replaced by free-living Xylariaceae in treatment B (Lyophyllaceae in 3 spots and Xylariaceae in center). The 'blank' shows growth without Xylariaceae. Lyophyllaceae radius measured on day 3, 6, 9, and 12; bars show 95% confidence interval. *No measurements available as the blank of L1 reached rim of Petri dish before day 9. See Table 3-1 for strain identification.
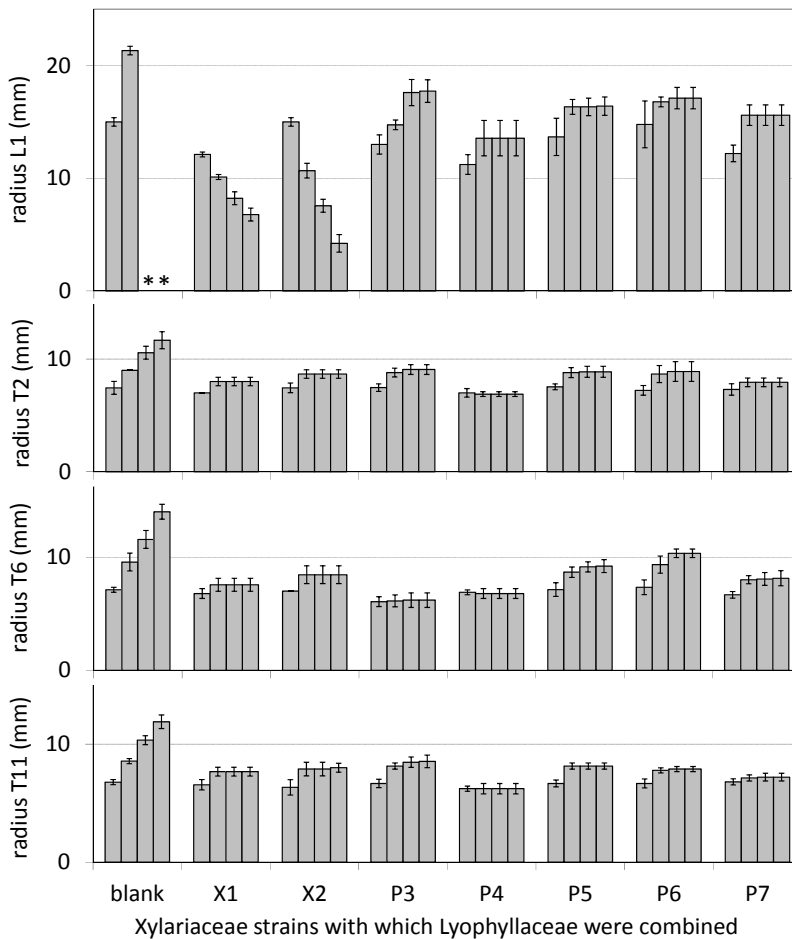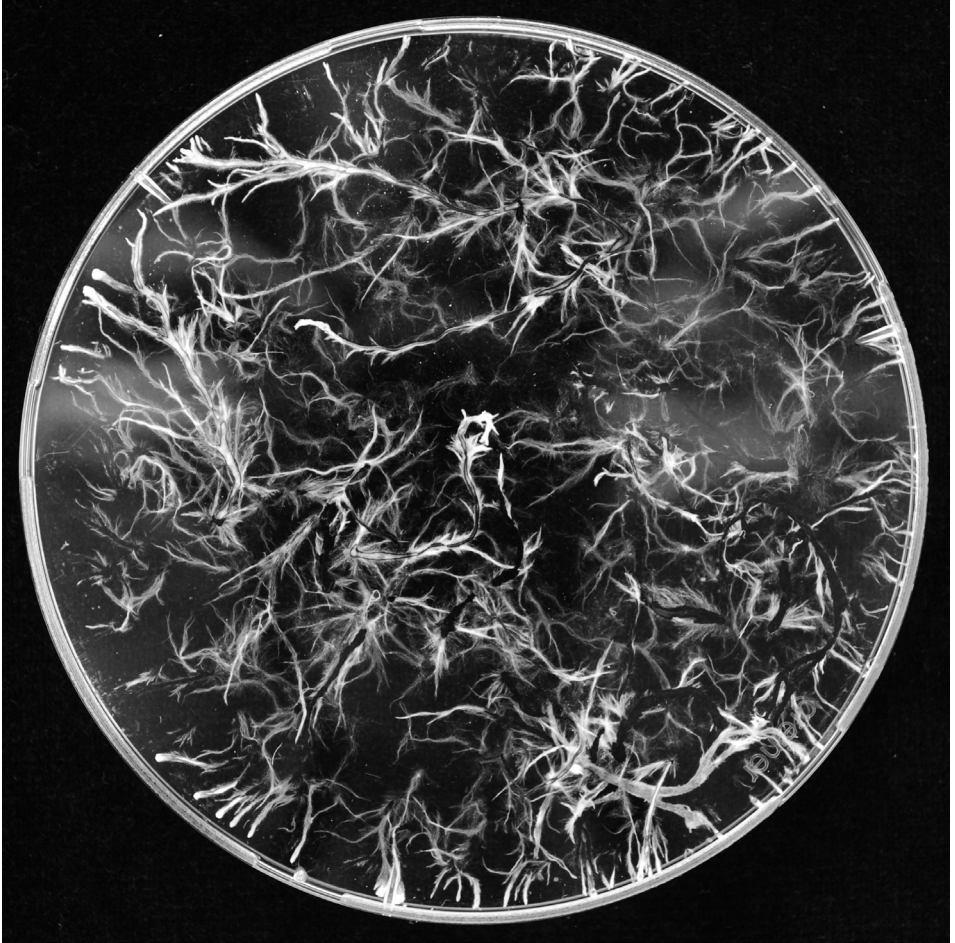
Chapter 4

The role of termite workers in controlling *Pseudoxylaria* and other fungi in their fungus garden

Anna A. Visser, Tânia Nobre & Duur K. Aanen

Abstract

In active colonies of fungus-growing termites *Termitomyces* is reared in monoculture. However, *Pseudoxylaria* is present in most fungus-growing termite gardens, but usually inconspicuously. In the absence of termites, Pseudoxylaria often overgrows the fungus combs and also other fungi appear on the combs. It is unknown how termites control the growth of unwanted fungi. We tested the effect of Macrotermes natalensis workers on the fungus garden hygiene by incubating fungus combs with and without termite workers, and tested whether termite workers can clean up an already infected colony. Combs were less often infected when workers were present, and Pseudoxylaria only developed visible sclerotised tissue on comb fragments kept without workers. Workers added to Pseudoxylaria-infected combs were unable to clean the comb completely. However, workers manipulated Pseudoxylaria stromata by cutting and moving them and manipulated comb fragments that were overgrown by other fungi. We therefore conclude that in Macrotermes natalensis the workers play an important role in the fungus garden hygiene.

Introduction

"Clearly the problem of how insects can control the growth
of a fungus garden remains an intriguing one"
– Batra & Batra 1967 –

Colonies of social insects such as bees, wasps, ants and termites are intriguing manifestations of organisation. Their intricately built nests out of wax, wood carton, soil or other materials can harbour thousands or sometimes millions of individuals, and these

individuals have evolved a complex division of labour (Sands 1960; Sieber & Leuthold 1981; Badertscher *et al.* 1983; Hughes *et al.* 2010){Sands, 1960 #438}. Some nests have designated nurseries for larvae, and in certain termite and ant species also for rearing other organisms either for food or for protection (Sands 1960; Hughes *et al.* 2010).

Threat of weeds and pathogens in social insect colonies

Weeds and pathogens (pests) pose a big challenge to social insects. Two aspects of their social lifestyle make them inherently vulnerable to exploitation by pests (in particular infectious microbial pests). First, the sheer aggregation of thousands of individuals – which have intimate, frequent and shifting contacts with other colony members – represents a large resource to be exploited by pests (Bulmer & Crozier 2004; Stow & Beattie 2008). Once a pest enters the colony, it can potentially sweep through the whole colony without much effort (Rath 2000; Pie *et al.* 2004; Fefferman *et al.* 2007; Guzman-Novoa *et al.* 2010). Second, the intrinsic high relatedness between individuals of most social insect colonies (Hamilton 1964) limits their diversity in immune response (Bulmer & Crozier 2004; Hughes *et al.* 2010) and also their capacity to detect and react to pests (Calleri II *et al.* 2006; Ugelvig *et al.* 2010). Social insects that grow food inside their colony face even an additional challenge: to keep their food free of pests.

Termites of the family Termitidae subfamily Macrotermitinae grow a monoculture of the basidiomycete *Termitomyces* on a plant-derived substrate in their nest (Aanen *et al.* 2002; Katoh *et al.* 2002; Moriya *et al.* 2005; Shinzato *et al.* 2005; Aanen *et al.* 2009). The fungus garden of a single colony may contain several kilograms of fungal biomass and growth substrate together (Darlington 1994). This substrate consists of plant material such as wood and grass that is fragmented by the termites (Sieber & Leuthold 1981). In return, *Termitomyces* assists the termites in the digestion of this material, in general by transforming this lower-quality substrate (with a very high C:N ratio) into a high-quality food source (with a low C:N ratio) for the termites (Sands 1960; Batra & Batra 1979; Wood & Thomas 1989). *Termitomyces* itself can be an additional protein-

rich food source, once consumed by the termites. This mutualistic symbiosis is reciprocally obligatory for both symbiotic partners, as they cannot survive on their own (Sands 1960; Batra & Batra 1979). The accumulation of food in the fungus-growing termite nests is likely to attract organisms that compete for this resource.

There are many examples of fungi, other than *Termitomyces*, that have been isolated from fungus-growing termite nests (Thomas 1987a; 1987b; 1987c; Shinzato *et al.* 2005). Visser *et al.* (2009, Chapter 2) showed that species of *Xylaria* subgenus *Pseudoxylaria* (Hsieh 2010, henceforward *Pseudoxylaria*) were present in almost all nests, although they did never proliferate in active nests. The observation that *Pseudoxylaria* is apparently suppressed in active nests was made before (Batra & Batra 1967; Sands 1969; Wood & Thomas 1989). Competitive exclusion by *Termitomyces* is unlikely (Visser *et al.* submitted, Chapter 3) and is at odds with the rapid overgrowth of the fungus comb when termites are gone (Chapter 2). How, then, do termites keep their fungus garden free of weeds and pathogens?

Weed and pathogen control in fungus-growing termites

With for certain species a few million individuals per colony (Darlington 1994), an early detection and adequate defence is crucial for fungus-growing termites to guard the colony from collapse by weeds and pathogens (Traniello *et al.* 2002; Yanagawa & Shimizu 2007; Ugelvig *et al.* 2010), as is demonstrated by infection transmission and hygienic behaviour models (Pie *et al.* 2004; Fefferman *et al.* 2007). Like apes, birds, cats, and so many other members of the animal kingdom, termites allot time for grooming (Batra & Batra 1966) – a simple but powerful preventive measure against infectious pests. Calleri II *et al.* (2010) wrote that socially mediated immunocompetence may even overrule genetic pathogen resistance. Contrary to fungus-growing ants, termites have no effective self-grooming (Yanagawa & Shimizu 2007; Morelos-Juarez *et al.* 2010), but they have effective mutual grooming (allogrooming). Termites confronted with an entomopathogenic fungus had a significantly lower mortality being with colony members than without colony members (Rosengaus *et al.* 1998b; Traniello *et al.* 2002; Calleri II *et al.* 2006; Fuller 2007). Termite grooming proves so

effective that it is the most plausible explanation for the failure of biological termite eradication programs that use entomopathogens (Chouvenc *et al.* 2008).

Observations on *Ancistrotermes* and *Macrotermes* lab colonies by Sands (1960), Sieber & Leuthold (1981), and Badertscher *et al.* (1983), illustrate how extensively termite workers groom the eggs, king, queen, soldiers, and other workers. Not only the termites, but also the fungus garden is constantly attended. The fungus comb and nodules were palpated continuously with the laciniae by jerking movements of the head (Batra & Batra 1966; Batra & Batra 1967; Sieber & Leuthold 1981). Palpating was alternated with intervals of maxillae chewing movement, but it was unclear whether fungal hyphae were consumed or maxillae were cleaned (Sieber & Leuthold 1981). Batra & Batra (1979), however, observed *Odontotermes obesus* major and minor workers biting *Pseudoxylaria* mycelium, removing it from the nest, and burying it under soil; and that there was *Pseudoxylaria* rind in the gut of soldiers, showing that workers had fed *Pseudoxylaria* to soldiers.

Grooming is facilitated by termite secretions and excretions that possess anti-microbial activity (Lamberty *et al.* 2001; Bulmer & Crozier 2004; Fuller 2007; Sobotnik *et al.* 2010). Cellular encapsulation and gut antifungal activity also form an important part of the pest defence strategy of termites (Rosengaus *et al.* 1998a; Chouvenc *et al.* 2008). But the defence does not necessarily come from the insects themselves. Many attine fungus-growing ants use Actinobacteria, which they rear in and on the cuticle of their body, to suppress the mycoparasitic fungus *Escovopsis* (Currie *et al.* 1999b; Currie *et al.* 2006). It is possible that these Actinobacteria that are readily recruited from soil (Kaltenpoth 2009) also play a role as protective symbiont in the fungus-growing termite symbiosis (Chapter 5). However, since many tests concerning control of microorganisms by termites considered only the hygiene of termites themselves, and mainly in non-fungus-growing species, we were interested in the effect of fungus-growing termite workers on the fungus-comb hygiene.

In this study, we investigated the effect of *Macrotermes natalensis* workers on the fungal community of their fungus comb. In order to test for different mechanisms, combs were incubated under three

treatments: workers present from the beginning, workers absent for the first three days, and workers absent until fungi other than *Termitomyces* were visible on the comb. The effects of *M. natalensis* termite workers under these conditions would demonstrate the role of the workers in keeping the garden free of weeds, and the suppression of *Pseudoxylaria* in particular.

Materials and Methods

Three termite mounds of *Macrotermes natalensis* were sampled in South Africa in January 2010. Fungus comb, termites and termite mound matrix were collected. Material from the field was stored at 5 °C, and processed within one day after collecting.

Termites (juveniles, major and minor workers and soldiers) were kept in plastic boxes with a volume of 0.5-1.5 L. Boxes were filled up with pieces of fungus comb and mound wall material. One or two Whatman® no.1 filter papers (pesticide free paper with no binders) moistened with distilled water were placed on top of the material, were kept moist, and were replaced when eaten to provide the termites with water and a cellulose source. Boxes were kept in the dark at 27 °C and checked daily for moisture content and food availability.

Experimental setup

Fungus comb was placed in plastic cups (diameter 60 mm, height 120 mm). Whatman® no.1 filter paper was folded twice into a quarter with four layers, moistened with distilled water, and placed on the bottom of each cup. Per cup, a fungus-comb fragment of ±3 g was placed on top of the filter paper. Also a piece of clayey inner mound matrix (diameter 5-10 mm) was added, to provide some substrate from their natural environment besides the fungus comb. Cups were closed with a lid made from the bottom of a small Petri dish, to the inside of which another filter paper was glued (with a non-toxic and odourless glue stick, UHU®, Germany), and kept moist with distilled water. Cups were incubated in the dark at 27 °C.

To check for the presence and time of appearance of *Pseudoxylaria* in each termite colony, also larger pieces of fungus comb were incubated. For this control treatment, fragments of ±15 g were put in sealed styrofoam cups with paper tissue soaked in distilled water and incubated in the dark at 25 °C.

For each mound, fifteen cups with 3 g fungus comb were divided over three treatments in groups of five cups: cups to which 50 workers were added at the start of the experiment, right after adding the fungus comb (treatment 1); cups to which 50 workers were added after three days of incubation (treatment 2); and cups to which 50 workers were added after the fungus comb showed fungi other than *Termitomyces* (treatment 3). Treatment 1 tested whether workers can prevent fungi other than *Termitomyces* from growing on the comb or not at all. Under the assumption that three days suffice for *Pseudoxylaria* and other fungi to escape their potential dormancy or inhibition and start growing, treatment 2 tested if workers can eradicate weeds that have developed but are not yet overgrowing the comb – for example by grazing emerging hyphae. Treatment 3 tested if workers can eradicate weeds in a progressed stage of development. In that case, workers would clear combs from all fungi other than *Termitomyces* that appeared, resulting in combs free of weeds in all three treatments by the end of the experiment.

The experimental cups were checked at least every other day. Lids were lifted to let in fresh air and to moisten the attached filter paper if needed. The number of (visible) dead workers was counted. Pictures were taken at regular intervals to register the development of fungi other than *Termitomyces*.

Student's t-tests were used to determine differences between the treatments concerning the incidence of *Pseudoxylaria* and other fungi that appeared on the combs.

Results

Pseudoxylaria was present in all three fungus gardens as it appeared on combs from all three gardens in the control treatment. However, fungus combs where termites were present from the start of the experiment, or added after 3 days, looked healthy in

Table 4-1 Mortality of workers and fungi observed to appear on fungus comb, for each of the three treatments: combs with workers since that start of the experiment (1), combs with workers added after 3 days (2), and combs with workers added after fungi other than *Termitomyces* had emerged (3).

Treatment	Mortality %		Incidence of fungi besides *Termitomyces*		Cups
	Mean	Std Error	*Pseudoxylaria*	other fungi	n
1	63.6	27.7	0	3	15
2	36.7	26.3	0	4	15
3	55.3	35.4	5	10	15

the majority of cases, and in half of those cups the number and size of nodules increased impressively during the first week of the experiment (Figure 4-1, compare 1-E at start and 1-E after 7 days). In treatments 1 and 2, *Pseudoxylaria* was never observed, though eventually some other (sporulating) fungi appeared (Table 4-1). Contrastingly, in cups of treatment 3, many fungi appeared, among which *Pseudoxylaria* (Table 4-1, Figure 4-1). Fungus combs with termite workers (pooling treatment 1 and 2) had a significantly lower incidence of both *Pseudoxylaria* and other fungi than combs without termite workers (treatment 3) (P < 0.05).

Mortality of termite workers did not differ significantly between the three treatments but was generally high (Table 4-1). Mortality was assessed by the absence of movement and the loss of body turgor, which may have overestimated the number of dead workers in some cases. However, the contribution to the maintenance of the colony of these workers that were still alive was probably low. Often these workers were found in the middle of thick fungal mat covered with green spores, a clear indication of their unhealthy state.

Once *Pseudoxylaria* emerged, it rapidly covered the whole comb, rendering workers with a completely covered and sclerotised comb with a stroma (sometimes more than one) on top (Figure 4-2). Stromata of *Pseudoxylaria* were observed in cups A, B, E, N, and O of treatment 3, after 6, 11, 8, 7 and 7 days respectively. Workers were observed to cut off *Pseudoxylaria* stromata (Figure 4-2, comb 3-B). In cups where this did not happen, the stromal surface was manipulated at its base at an increasing height (Figure 4-2, compare 3-E after 11 and 13 days) and covered with clay deposits (Figure 4-2, comb 3-A and 3-E; Figure 4-3, comb 3-E). When a large group of

Figure 4-1 Fungus combs that were constantly tended by workers (left), versus combs that were untended until fungi other than *Termitomyces* had emerged (right).
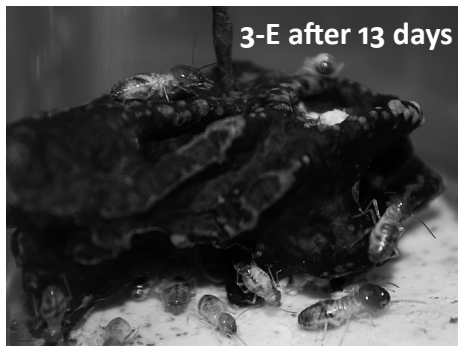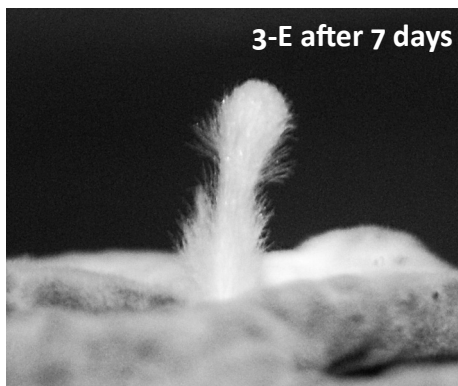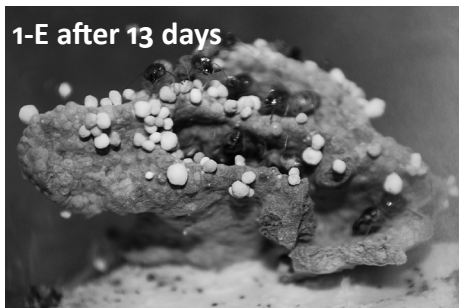
Figure 4-2 Termites tending fungus combs that were untended until fungi other than *Termitomyces* had emerged. In cup 3-B workers cut off the stroma produced by *Pseudoxylaria*. In the cups 3-A and 3-E the stromal surface was manipulated at its base and covered with clay. Black arrows indicate the same part of the stroma in both pictures of cup 3-E show that this progressed over time. White arrows indicate two particularly pronounced clay deposits.
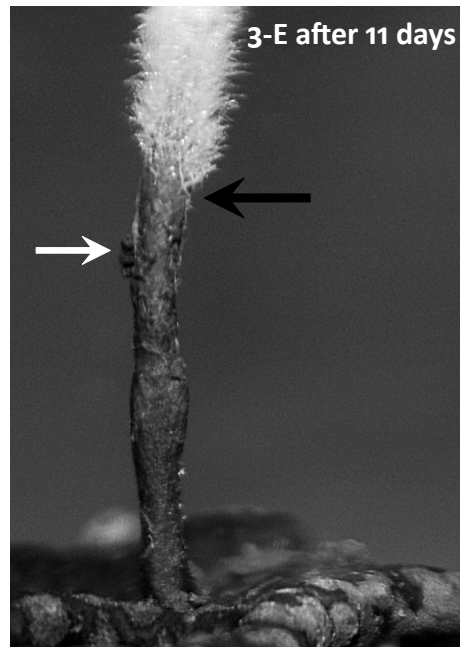
FIGURE 4-3 Workers manipulate the fungi on fungus combs that were untended until fungi other than *Termitomyces* had emerged. In cup 3-D workers removed part of the mycelium; woolly hyphae are still visible in the bottom side of the picture. Arrows point at fragments of filter paper deposits. In cup 3-E workers grazed in 1 day most of the *Pseudoxylaria* hyphae that covered the comb surface and the base of the stroma on the right side of the picture, leaving the sclerotised tissue covering the comb. Arrows point at two clay deposits that have the size of the minor workers head. Pictures were taken when workers had been present for 3 days and 1 day, respectively.
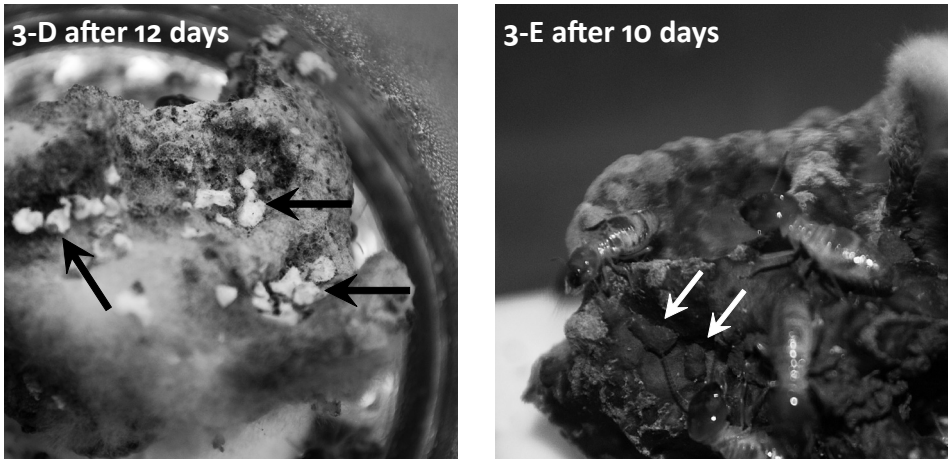
FIGURE 4-4 Termite workers confronted with a piece of *Pseudoxylaria* tissue in the form of a stroma, started manipulating it with their mouthparts. Pieces were cut off (left) and both major workers and minor workers took part in this (right).
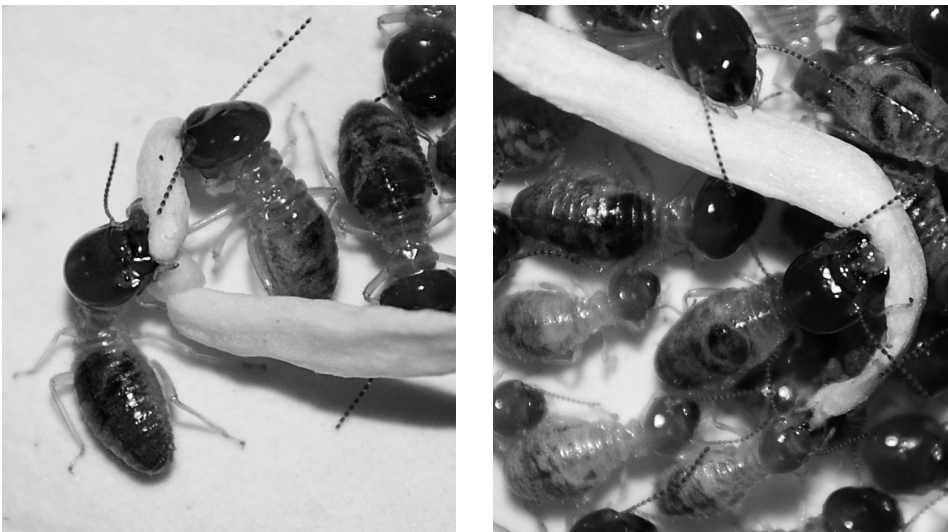
termite workers was confronted with a piece of *Pseudoxylaria* tissue in the form of a stroma, workers started manipulating it with their mouthparts (Figure 4-4), cutting off pieces from the soft white end of the stroma, and displacing those pieces. The sclerotised end of the stroma was not cut, but pushed and pulled. Workers were also observed to remove mycelium of other fungi than *Termitomyces* (4-3), to perform 'grazing' motions when walking on the comb, and occasionally to transport *Termitomyces* nodules that got disconnected from fungus comb to the highest point on the comb.

Towards the end of the experiment small black hair-like structures were discovered on several combs. Upon close inspection, these turned out to be an undescribed *Ophiostoma* species (Z. W. de Beer, FABI, Pretoria, in preparation).

Discussion

Pseudoxylaria did not appear on any of the combs of the first two treatments with termites present from the beginning or added after three days (treatment 1 and 2), whereas on combs with workers added only after fungi other than *Termitomyces* had emerged (treatment 3) *Pseudoxylaria* formed stromata around eight days after incubation in five of the cups. Fungus combs with termite workers present had a significantly lower incidence of *Pseudoxylaria* and other fungi than combs without workers. The results imply that *Macrotermes natalensis* workers play a significant role in keeping the *Termitomyces* fungus garden weed-free. This is in line with the findings described in Chapter 3, where we hypothesised that termites play a role in preventing *Pseudoxylaria* from overgrowing the fungus garden.

The lack of differences between treatment 1 and 2 can be explained in two ways. First, it could mean that termites can remove weeds also after they had three days to develop. Second, it could mean that three days are insufficient for weeds to leave the dormant or inhibited state they might be in and to start growing. However, as soon as six days after incubation, *Pseudoxylaria* had already formed a stroma on the comb. Probably stromata like this one were already developing on day three, when in treatment 2

the termites were added, and then successfully suppressed by those termites. Therefore, we conclude that termites can control or even eradicate weeds at early stages of mycelium development. The experiment did not allow us to assess whether termites prevent spore germination or maintain weed mycelial domains at very small sizes.

So far, the role of termites in controlling the fungi that appear on the fungus comb was tested only once, for *Odontotermes formosanus*, using material of only one nest (Shinzato *et al.* 2005). The termites were found to suppress all other fungi but *Termitomyces*, but the study did not address what would happen if weed fungi were allowed to develop before termite workers were put back on the fungus comb. Besides the fact that the present study deals with a different fungus-growing termite genus and that we use three sympatric nests, our study also makes a valuable contribution to better understanding the role that termite workers play in controlling the *Pseudoxylaria* and other fungi in their fungus garden.

Other fungi on the comb

The experiment revealed that *Pseudoxylaria* sclerotised in a very early stage of development. Soon after forming mycelium the subsurface tissue turned black and sturdy (combs with *Pseudoxylaria* were harder to break in two than those without). The workers removed only the whitish soft top parts, and cut off the stroma only when it was still thin (see pictures). It seems that the sclerotised parts are difficult to remove for the workers. This feature is typical for Xylariaceae, but may give *Pseudoxylaria* a special advantage in the fungus-growing termite nest compared to other weed fungi. That termites are unable to remove an already established mycelium of *Pseudoxylaria* may be one of the reasons why it is a prominent inhabitant of inactive nests. Possibly when for whichever reason a termite colony becomes very weak, *Pseudoxylaria* seizes the reduced surveillance of the fungus garden to rapidly overgrow the fungus comb, leading to the definite end of the fungus-growing termite nest.

Batra & Batra (1979) observed major and minor workers of *Odontotermes obesus* to bite *Pseudoxylaria* mycelium, remove it from

the fungus garden, and bury it under soil. During our experiment, workers also bit mycelium, were unsuccessful in removing an established *Pseudoxylaria* mycelium, but still made attempts of covering it with clay. Workers repeatedly deposited clay on the surface of contaminated parts of the comb and on weed fungi, but the amount of clay they were given was insufficient. Furthermore, even if the available clay would suffice to cover the weed fungi, the set up did not allow termites to move mycelium of *Pseudoxylaria*, other pests, or infected comb pieces to a place away from the fungus garden, a behaviour described for dead termites and deemed crucial for nest hygiene (Batra & Batra 1979; Pie *et al.* 2004). The occurrence of *Ophiostoma* species was of particular interest for the authors. This fungus had been isolated from termite fungus comb before (unpublished data Z. W. de Beer, H. H. de Fine Licht & D. K. Aanen), and had been considered as an additional associate of the fungus-growing termites. In the previous two fieldwork seasons, however, we failed to observe *Ophiostoma*. Scott *et al.* (2008) have shown that pine beetles use antibiotics to prevent *Ophiostoma* from overgrowing their cultivar, implying that *Ophiostoma* is a weed that is constantly suppressed by this practice. This fungus is perhaps an irregular inhabitant of fungus-growing termite nests, as in many studies it is not mentioned (for example Thomas 1987a,b,c; Moriya *et al.* 2005). Interestingly, while in two of the three colonies workers were seen with small white arthropods clinging to their exoskeleton before they were added to the combs, it is known that mites clinging to the back of bark beetles carry the *Ophiostoma* spores (Klepzig *et al.* 2001). Could it be that inquiline insects or parasites of the termites act as vector by which in this case *Ophiostoma*, but perhaps also *Pseudoxylaria* and other weeds, enter the termite fungus garden?

Constraints concerning in vitro *experimentation*

Though the following constraints by no means biased our results – it can only have caused an underestimation of the importance of *Macrotermes natalensis* workers for weed control in their fungus garden – we would like to point out four issues that when taken care of could refine any future experiments.

First the assumption that using 50 workers for 3 g of comb should be more than enough workers, since Shinzato *et al.* (2005) used 20 per 10 g comb. However, the mortality rate of termites in small groups is higher and their ability to withstand starvation is less than in bigger groups (Becker 1970). That is why normative European bioassays with subterranean termites require 250 workers and at least 3 soldiers per sub-colony for a jar volume of 500 to maximum 1000 cm^3 (EN117 2005). In an experiment on division of labour in *Macrotermes subhyalinus*, observation nests of 0.45 L, divided in 15 interconnected chambers of 45x45x15 mm, contained 500 termites; and experimental nests of 1 L contained 2000 termites besides earth and fungus comb (Badertscher *et al.* 1983). Container shape, volume (size), and amount of matrix are important to ensure a vigorous performance of termite groups during a bioassay (Lenz *et al.* 1987; Delaplane & LaFage 1990; Lenz *et al.* 1991). Combining this with the importance of allogrooming pointed out in the introduction, having small numbers of workers could be one of the factors that caused a high mortality during the experiment. The small number of workers can also limit their ability to effectively remove fast-sporulating fungi; when workers have insufficient grooming amongst them they easily contaminate clean parts of the comb after cleaning contaminated parts. This could explain why in treatments 1 and 2 a few fungus combs became contaminated with fungi while workers were present.

Second, termites are used to confined spaces and the level of filling of an experimental unit has proven to influence termite worker performance; 70% filling was the minimum for subterranean termites (Nobre *et al.* 2007a). Hence, the workers in our experiment were probably experiencing suboptimal climatic and physical conditions, as the cups were filled for less than a quarter with only a small fragment of clay from the nest. This could be one of the factors that caused the high mortality among the workers. To reduce mortality in future experiments, sterile sand could be used to fill up the experimental containers – a common method with subterranean termites. Fontainebleau sand with particle sizes of 150-210 µm is assumed to work best for subterranean termites (Nobre *et al.* 2007b), and for Mastotermitidae (Howick & Creffield 1975). For a more

realistic substrate, a mixture of Fontainebleau sand with sterilised soil could be used as in Nobre *et al*. (2007b).

Third, we are unsure if we used the right workers. Termites of the genus *Macrotermes* have a strong age-related division of labour (Badertscher *et al.* 1983). Even salivary gland morphology is related to task in *M. bellicosus* (Billen *et al.* 1987), and more recently this was also discovered in two species of *Acromyrmex* fungus-growing ants (Hughes *et al.* 2010). Hence, the ability to control weeds in the fungus garden may vary among workers of different task divisions. Opening the termite mounds for sampling the nest is disruptive and many individuals retreat, while soldiers defending and workers performing nest restoration activities come forward. Workers that tend the fungus garden are normally active in safe and clean parts of the nest and are likely retreat when nests are sampled. Foraging workers, on the other hand, are normally active in hazardous environments and possibly perform most of the nest restoration activities. Therefore, the majority of collected workers used in the experiment may have been foraging instead of nest-tending workers.

Fourth, our experiment lacked sequestration of space. This may have constrained the behaviour of separating contaminated workers and comb pieces from the healthy parts of the colony and enclosing them in chambers (so-called cemeteries) to avoid spreading of an undesired fungus – a behaviour that termites display naturally (Batra & Batra 1979; Sieber & Leuthold 1981). Perhaps the observation of sick (considered dead) termites in the middle of a thick mat of green spores in one of the cups was a cemetery in the making. In the absence of clay or soil, the workers used fragments of filter paper and chewed comb to close certain holes and to cover parts of the comb that were weedy, implying that the workers tried to separate healthy from unhealthy.

Depending on the type and the duration of intended experiments, the experimental setup of Badertscher *et al*. (1983) could prove a useful starting point to avoid part of the discussed constraints.

Additional mechanisms in weed control

In this study we focussed on the effect of termite workers on the fungus comb. The mechanisms by which the workers achieved that effect were monitored at a macroscopic scale, and not at a microscopic or molecular scale. Besides mechanical manipulation, the termites may have used secretions and excretions for weed control. Termites are well-known for using antimicrobial substances to prevent the growth of alien fungi (Batra & Batra 1967; Lamberty *et al.* 2001; Solavan *et al.* 2007). Termite saliva is mixed with the clay and other nest building material to inhibit fungal growth (Batra & Batra 1966; Rosengaus *et al.* 2004), nodules are 'licked', eggs are moistened and other nest components – including the termites themselves – are continuously groomed (Batra & Batra 1966; Sieber & Leuthold 1981). These antimicrobial substances play an important role in these hygienic activities, but how are they produced?

Fungus-growing ants rely on secretions from their metapleural glands in some species, and on Actinobacteria that are grown on their cuticle in other species (Fernandez-Marin *et al.* 2009; Hughes *et al.* 2010). Similarly, in fungus-growing termites, the antimicrobial substances may originate from termites and/or from bacteria associated with the termites. Fernández-Marín *et al.* (2009) asked what the correlation between a more elaborate metapleural gland (and less reliance on Actinobacteria) and larger colony size means – perhaps pest control with chemical cocktails is more effective than bacterial antimicrobial metabolites in large societies?

Many studies have shown the potency of termite glandular secretions (for example Lamberty *et al.* 2001; Sobotnik *et al.* 2010) but, unlike in fungus-growing ants, there are no records of effective Actinobacteria in fungus-growing termites. Hence, the next thing we want to investigate is whether there are Actinobacteria present and, if so, what role they play in fungus-growing termite nests.

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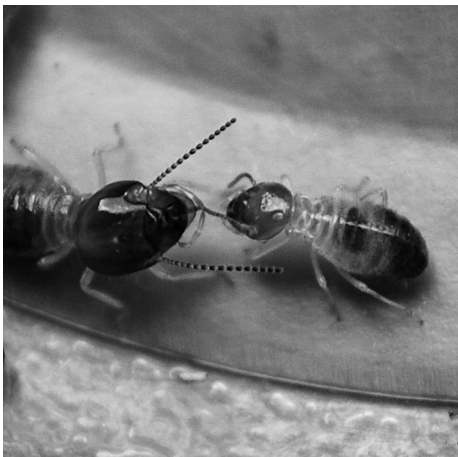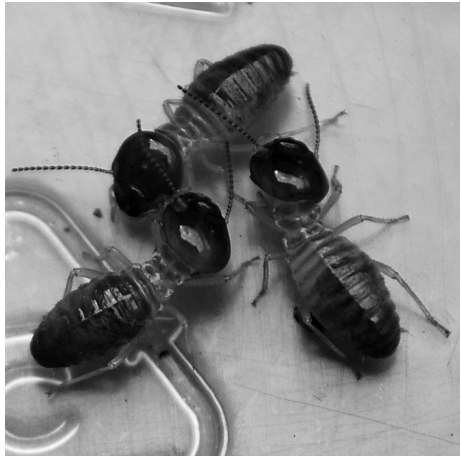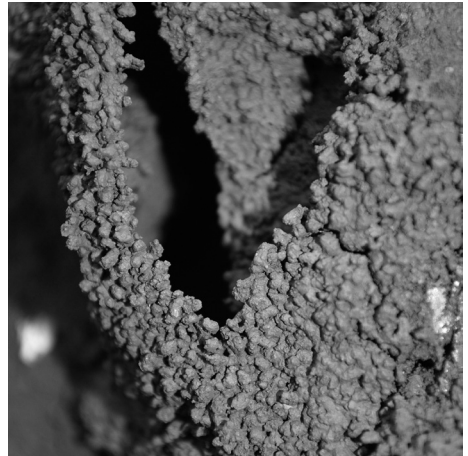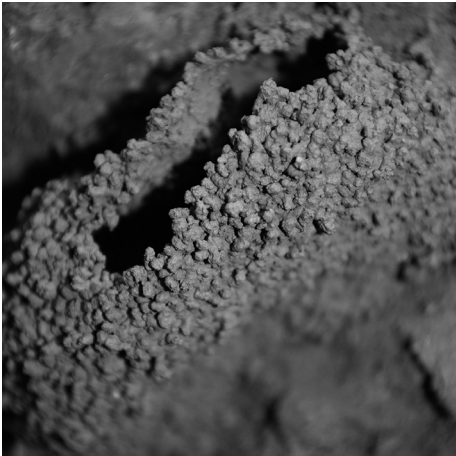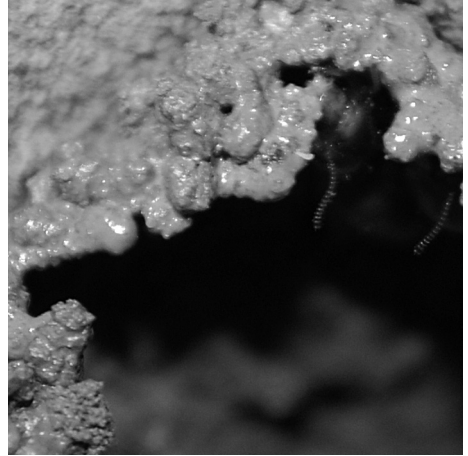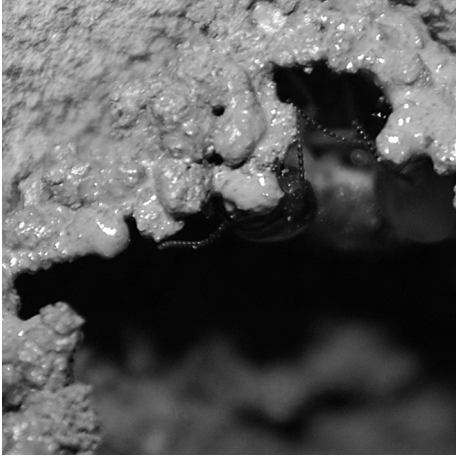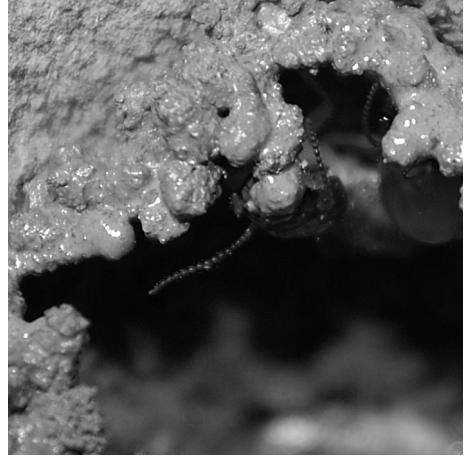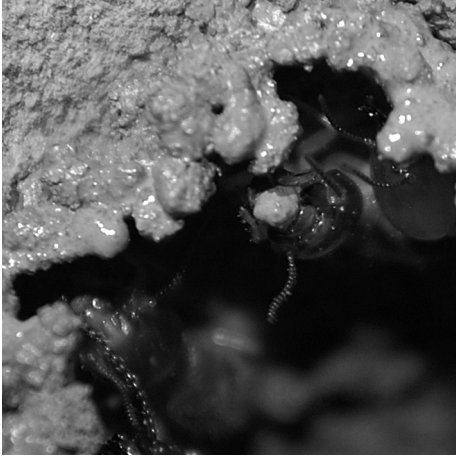
Actinobacteria from fungus-growing termites lack specificity for host and target: an unlikely defence against *Pseudoxylaria* despite antibiotic potential

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Abstract

In fungus-growing termites, fungi of the subgenus *Pseudoxylaria* may
threaten colony health through substrate competition with the termite fungus
(*Termitomyces*). The potential mechanisms with which the termites suppress
Pseudoxylaria has remained unknown. Here we explore if Actinobacteria play a
mutualistic role as defensive symbiont against *Pseudoxylaria* in fungus-growing
termites. Thirty fungus-growing termite colonies, spanning three termite genera
and two geographically distant sites, were sampled for Actinobacteria. A subset
of the resulting 360 isolates was characterised based on morphology and 16S
rRNA sequences. The majority of the Actinobacteria isolates (288) was screened
for selective antibiotic effect on *Pseudoxylaria* versus *Termitomyces*, and a more
detailed bioassay of the specificity in antibiotic effect was performed on a subset
(53) of the Actinobacteria against diverse *Pseudoxylaria* and *Termitomyces* strains.
We describe the first discovery of an assembly of Actinobacteria occurring
in fungus-growing termite nests. Actinobacteria were found throughout
all sampled nests and materials, and in the phylogenetic tree their 16S rRNA
sequences were interspersed with those of Actinobacteria from origins other
than fungus-growing termites. The bioassays for antibiotic properties showed
that many Actinobacteria inhibited both *Pseudoxylaria* and *Termitomyces*. The lack
of specificity of the Actinobacteria for fungus-growing termites, and the lack of
specificity in antibiotics against *Pseudoxylaria*, make it unlikely that these bacteria
play a major role as defensive symbionts against *Pseudoxylaria* in fungus-growing
termites.

Introduction

Symbioses are omnipresent and shape the ecology and evolution of all organisms. Almost every organism faces parasitic symbionts, and parasites play an important role in driving adaptive processes and even species diversification (Berngruber *et al.* 2010; Richards *et al.* 2010; Rohr *et al.* 2010; Triapitsyn *et al.* 2010). As a consequence of the virulence imposed by parasites, defences in hosts are crucial and these can be behavioural, immunological or involve mutualisms with defensive symbionts. In the latter case, symbionts provide a benefit for their partner in the form of defence against parasites (White & Torres 2009). For example, *Spiroplasma* bacteria defend *Drosophila* sp. against nematodes (*Howardula* sp.) in return for obtaining nematodes as food (Hurst & Hutchence 2010); certain species of ants (*Crematogaster mimosae*, *C. nigriceps*, *C. sjostedti*, and *Tetraponera penzigi*) defend whistling thorn (*Acacia drepanolobium*) against herbivores (Goheen & Palmer 2010) in return for receiving housing and food; and certain sea anemones (Stichodactylidae) defend anemone fishes (genera *Amphiprion* and *Premnas*) against predators in return for food and defence against parasitic fishes (Fautin 1991).

Actinobacteria occur as defensive symbionts in certain insect species. For example, fungus-growing ants use antibiotics from Actinobacteria harboured in special structures on the ant cuticle for defending their fungal cultivar against mycoparasitic *Escovopsis* spp. fungi (Currie *et al.* 1999; Currie *et al.* 2006; Oh *et al.* 2009; Cafaro *et al.* 2011). Similarly, European beewolves (*Philanthus* species) harbour Actinobacteria in their antennae, where the bacteria produce antibiotics that help protect the wasp larvae from fungal infections (Kaltenpoth *et al.* 2005; Kroiss *et al.* 2010). Similarly, a defensive mutualism with Actinobacteria appears to be present in Southern Pine Beetles (*Dendroctonus frontalis*), where the bacteria selectively inhibit a competitor fungus of the mutualistic fungus of the beetles (Scott *et al.* 2008; Oh *et al.* 2009).

Fungus-growing termites (Blattodea – previously Isoptera: Termitidae, subfamily Macrotermitinae) live in mutualistic symbiosis with *Termitomyces* (Basidiomycota: Agaricales: Lyophyllaceae). This association is responsible for a major part of

the breakdown of plant material in Sub-Saharan Africa and South-East Asia (Jones 1990; Mando & Brussaard 1999). Enhanced by the warm, moist and stable climate of the termite mound, *Termitomyces* degrades the plant material of faecal deposits, shaped into a comb by the termites, and produces nodules (primordial fruiting bodies). The nodules and digested parts of this fungus comb – nitrogen-rich food compared to the original, often woody, plant material – are eaten by the termites. Cells from nodules survive gut passage and act as inocula for newly added comb substrate (Sands 1969; Leuthold *et al.* 1989; Wood & Thomas 1989). Individual nests harbour *Termitomyces* in monoculture (Katoh *et al.* 2002; Moriya *et al.* 2005; Shinzato *et al.* 2005; Aanen *et al.* 2009), but species of *Xylaria* subgenus *Pseudoxylaria* (Ascomycota: Xylariales: Xylariaceae) are latently present in fungus-growing termite nests (Visser *et al.* 2009 – Chapter 2; Guedegbe *et al.* 2009; Hsieh *et al.* 2010). Fruiting bodies of *Pseudoxylaria* frequently occur in abandoned termite nests (Rogers 2000; Rogers *et al.* 2005), and fungus gardens without termites are rapidly overgrown by species of *Pseudoxylaria* (Sands 1960; Thomas 1987; Visser *et al.* 2009). Previous experiments have shown niche overlap and reduced growth of *Termitomyces* when interacting with *Pseudoxylaria* (Visser *et al.* submitted – Chapter 3). Thus, *Pseudoxylaria* can compete with *Termitomyces* for the substrate provided by the termites, and can thereby negatively impact termite fungus garden productivity. Hence, fungus-growing termites are predicted to have evolved strategies to suppress *Pseudoxylaria*.

The presence of termite workers indeed affects the incidence of *Pseudoxylaria* on the fungus comb, with *Pseudoxylaria* only appearing when workers are absent, suggesting active suppression of *Pseudoxylaria* by the termites (Shinzato *et al.* 2005; Chapter 4). Chemical secretions from the termites (e.g., antimicrobial peptides) may be used for this purpose (Lamberty *et al.* 2001; Fuller 2007); however, their effects have not yet been tested on *Pseudoxylaria*. Consequently, although termite workers suppress *Pseudoxylaria*, the underlying mechanism by which they achieve this – weeding/grazing by the termites, termite secretions like anti-microbial peptides, compounds produced by additional symbionts, or a combination of these – has remained unresolved.

Kaltenpoth (2009) noted that especially fungus-growing insects are expected to employ symbionts as defence against parasites (weeds and pathogens), as fungus gardens likely attract exploiters (see also Chapter 4). Actinobacteria are well-known antibiotic producers and occur as defensive symbionts in many insect-fungus symbioses (Currie *et al.* 1999; Kaltenpoth *et al.* 2005; Scott *et al.* 2008). Actinobacteria are consequently good candidate defensive symbionts in fungus-growing termites.

We address this hypothesis by exploring the presence of Actinobacteria in three genera of fungus-growing termites from two sites in South Africa. The majority (288 isolates) of the Actinobacteria that could be isolated (360) was screened for selective antibiotic effect against *Pseudoxylaria*, using a single *Pseudoxylaria* and *Termitomyces* strain. In order to explore the specificity of antibiotic effect in more detail, we then tested a selected subset (53) of the Actinobacteria against four *Pseudoxylaria* and six *Termitomyces* strains. We discuss the presence, distribution, specificity, and potential of Actinobacteria from fungus-growing termite nests as defensive symbionts in the fungus-growing termite mutualism.

Materials and Methods

Colonies sampled

Termite colonies of *Macrotermes natalensis* (9), *Microtermes* sp. (16), and *Odontotermes* sp. (5) were sampled from two locations in South Africa: Pretoria (S25°43′47.1″ E28°14′07.2″, elevation 1345 m) and Mookgophong (previously Naboomspruit, S24°40′30.5″ E28°47′50.4″, elevation 1045 m) in January 2010. *Microtermes* colonies were all collected from the walls of *Macrotermes* mounds. Fungus comb and termites were collected in clean plastic bags, stored at 5 °C, and processed within one day after collecting. Bacterial and fungal strains were grown on malt-yeast-extract agar (MYA, see Visser *et al.* 2009) in the dark at 25 °C, unless stated otherwise. See Supplements Table S5-1 for an overview of the sampled colonies and isolated strains.

Bacterial isolations

Isolations for Actinobacteria were made from termite workers and from fungus comb material. The termites were individually cleansed by washing in demineralised water (DEMI). Workers were subsequently separated into abdomen and head (including pronotum). Each termite sample was processed separately and mixed with 700 µl of DEMI. The same procedure was used for fungus comb samples (using about 0.1 cm^3 per sample). Bacteria were isolated by plating 350 µl of the mixtures described above on two different selective low-nutrient media: chitin [per litre: 4 g chitin, 0.7 g K_2HPO_4, 0.3 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 5H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.001 g $ZnSO_4$, 0.001 g $MnCl_2$, and 20 g of agar (Hsu & Lockwood 1975)] and microcrystalline (per litre: 5 g microcrystalline and 20 g of agar) medium. Suspensions resulting from the initial washing, one per worker, were plated in the same way, representing bacteria present on the exoskeleton.

Isolates with Actinobacteria-like morphology on these low-nutrient media were transferred to a richer medium (MYA, see Visser *et al*. 2009), and were sub-cultured until pure. This resulted in a total of 360 Actinobacteria isolates, which were subdivided into 44 morphotypes based on their morphology (Table S5-2). In order to determine whether strains within each morphotype belonged indeed to the same phylogenetic group, we set out to sequence 16S rDNA for two randomly chosen isolates per morphotype (hence 88 strains in total) using general primers [8F and 1540R or 27F and 1492R (Lane 1991; Fields *et al.* 2005)] and previously published DNA extraction and PCR protocols (Poulsen *et al.* 2007; Cafaro *et al.* 2011). We obtained positive PCR products for 35 of the strains, and these were subsequently direct-sequenced at the University of Wisconsin Biotechnology Center (http://www.biotech.wisc.edu/). Each sequence was BLASTed in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi). For a more balanced sample, not only the first but also the tenth nBLAST hits were used for phylogeny estimation, together with additional Actinobacteria strains of both fungus-growing ants (12) and fungus-growing beetles (5, plus 2 outgroup sequences). The Neighbour Joining (NJ) tree was estimated using the software Mega5 (Tamura *et al.* 2011), after alignment of the

sequences using a slow iterative refinement method (FFT-NS-i) as implemented in the program MAFFT (Katoh & Toh 2008) (Table S5-3).

Fungal isolations

Pseudoxylaria was isolated from hyphal cords or stroma appearing on fragments of ±15 g of fungus comb that had been incubated for 7-14 days, without termites, in the dark at 25 °C in sealed styrofoam cups with paper tissue soaked in DEMI to preserve humidity. *Termitomyces* strains were obtained by placing nodules from fresh fungus comb directly onto MYA. In some cases, one or more transfers to new plates were needed to obtain a pure culture.

Screening bioassay

To explore the selective antifungal effects of the obtained Actinobacteria strains, we screened 288 Actinobacteria strains (selected based on having growth on MYA) for their effect against one *Pseudoxylaria* (P2) and one *Termitomyces* strain (T1), both isolated from a *Macrotermes natalensis* nest. These fungal strains belong to the largest clades in their representative phylogenetic trees (Aanen *et al.* 2007; Visser *et al.* 2009; Nobre *et al.* 2010). If the selection of Actinobacteria created a bias, it would be an underestimation rather than an overestimation of the antibiotic effect, as high levels of antibiotic production are likely to be traded off with slow growth of the bacterial colony.

Termitomyces inoculum for the bioassay plates was obtained from plate cultures. *Termitomyces* mycelium and nodules were placed in an Eppendorf tube with 0.5 ml saline solution (0.8% NaCl w/v), after which the material was fragmented and suspended by mashing and twisting with a pestle. *Pseudoxylaria* inoculum was grown in Erlenmeyer flasks with ±125 ml of liquid broth (malt 2% and yeast 0.2% w/v). The broth was inoculated with a piece of MYA with *Pseudoxylaria* mycelium and macerated with a blender to fragment and mix the inoculum. Macerating was repeated on the third and fourth day after inoculation of the flasks. The resulting mycelium suspensions of *Termitomyces* and *Pseudoxylaria* were used

immediately to inoculate bioassay plates (diameter 85 mm). They were inoculated with 50 µl of suspension per plate, and spread by shaking with 5-15 sterile glass beads (diameter 3 mm). Subsequently, the glass beads were removed and the plates were incubated overnight before adding the Actinobacteria. This allowed plates to dry to prevent Actinobacteria from floating across the plate after inoculation.

Actinobacteria were inoculated on day zero by placing a 3x3 mm cube of 2-3 week old MYA cultures upside-down on the *Pseudoxylaria* and *Termitomyces* plates. Groups of five Actinobacteria were tested on each plate, at 10 mm from the edge of the plate and at equal distance from each other (see also Figure 5-3). The effects of Actinobacteria secretions on fungal growth were measured 8 days after starting the bioassay. The zone of effect (ZOE) was the distance between the bacteria and the point where the fungus showed normal growth, and often included a zone where fungus was inhibited completely (ZOI, only shown in supplementary tables). Measurements were done using the edge of the bacterial colony as a point of reference (see also Figure 5-3A).

Detailed bioassay

From the 288 Actinobacteria strains, we selected strains for a more detailed bioassay based on their effects in the screening bioassay (Table S5-4): a group of 19 bacterial strains with a large effect on *Pseudoxylaria* but no (or little) effect on *Termitomyces* (selection P), 21 with a large effect on both *Pseudoxylaria* and *Termitomyces* (selection P & T), and 13 that had an effect on *Termitomyces* but no effect on *Pseudoxylaria* (selection T).

The 53 Actinobacteria were tested against four *Pseudoxylaria* and six *Termitomyces* strains. For both fungi, representative strains from three different termite genera were chosen: *Macrotermes*, *Microtermes*, and *Odontotermes* (Table S5-1). The choice of *Pseudoxylaria* and *Termitomyces* strains was based on their respective phylogenetic placement (Visser *et al.* 2009; Nobre *et al.* 2010). Fungi and Actinobacteria were inoculated as described for the screening bioassay above, and ZOE and ZOI were measured as in the screening bioassay.

Primary antibiotic production assay

To explore antibiotic effects caused by metabolites produced by the Actinobacteria in the absence of another organism (primary antibiotics), we tested agar plugs obtained in close proximity to Actinobacteria colonies growing in pure culture on one *Pseudoxylaria* and one *Termitomyces* strain. This was done simultaneous with the screening bioassay. The nine Actinobacteria strains used were chosen randomly, although only strains with colonies far enough apart to allow plugs being taken without including bacteria could be used. The plugs were placed in the same positions on the fungal plates, and ZOE and ZOI were measured in the same way as described for the screening bioassay.

What distinguishes the above-described bioassays from those published for other fungus-growing insects, is that the target of the candidate defensive symbiont was inoculated on the whole surface of the test plates. With this method, there is guaranteed interaction between the challenged microbes; it circumvents the risk of observing halo's due to nutrient depletion that may occur during the time that the microbes take to grow towards each other if inoculated at a distance.

Statistics

Statistical tests were done in SPSS Inc PASW Statistics version 17. A paired t-test with H_1: $ZOE_{Pseudoxylaria} > ZOE_{Termitomyces}$ was done to test the hypothesis that Actinobacteria selectively suppress *Pseudoxylaria*. To test for differences between Actinobacteria with respect to their origin, ANOVA was done for differences in ZOE between termite genera, between fungus comb and different termite body parts, and between isolation media. Student's t-tests were done to further explore the difference in ZOE between Actinobacteria from *Microtermes* and those isolated from the other two termite genera.

Results

Occurrence and distribution of Actinobacteria with fungus-growing termites

Actinobacteria were obtained from both geographic locations, all three termite genera, all termite colonies, and all types of colony parts that were sampled (see Supplements Table S5-1, S5-2). The 360 Actinobacteria isolates showed no apparent specificity for origins, were frequently isolated from each type of colony part sampled, and showed no bias towards one of the isolation media (Table 5-1).

In the estimated NJ tree, Actinobacteria from fungus-growing termites are interspersed with Actinobacteria from non-fungus-growing origins, and appeared in clades that also contain Actinobacteria from other fungus-growing insects (Table S5-3, Figure 5-1). The 44 assigned morphotypes were not supported by the sequencing data, see Table S5-3 last part, and thus not used in further analyses.

Table 5-1 Overview of the proportional abundance of Actinobacteria per sampling origin and isolation medium.

Site		Termite genus		Colony part		Medium	
Mookgophong (8 colonies)	36.4%	*Macrotermes*	58.6%	Abdomen	38.5%	Chitin	59.1%
Pretoria (22 colonies)	63.6%	*Microtermes*	24.7%	Body	19.5%	Microcrystaline	40.9%
		Odontotermes	16.7%	Comb	24.4%		
				Wash	17.6%		

Antibiotic effect of Actinobacteria on Pseudoxylaria *and* Termitomyces

In the screening bioassay with 288 Actinobacteria, instead of being selectively inhibited, *Pseudoxylaria* was significantly less affected than *Termitomyces* (Figure 5-2, Table S5-4, P = 0.0001).

In the detailed bioassay with 53 Actinobacteria, average ZOE of *Pseudoxylaria* strains was again less than the ZOE of *Termitomyces* strains (t = -4.795, df = 52, P < 0.0001; Table S5-5), and this difference remained apparent even at detailed level when Actinobacteria were grouped according to isolation origin (Figure 5-3). Table 5-2 summarises the effects of Actinobacteria and shows only ZOE values that exceeded 2% of the total ZOE observed with the fungal

Figure 5-1 Estimated Neighbour Joining (NJ) tree showing fungus-growing insect related Actinobacteria (bold) interspersed with strains from other origins. Host genus is indicated for Actinobacteria from fungus-growing termite nests: Ma = *Macrotermes*, Mi = *Microtermes*, Od = *Odontotermes*; and their effects in the screening bioassay are given between brackets (ZOE *Pseudoxylaria* – ZOE *Termitomyces*). See Table S5-3 for stain information.
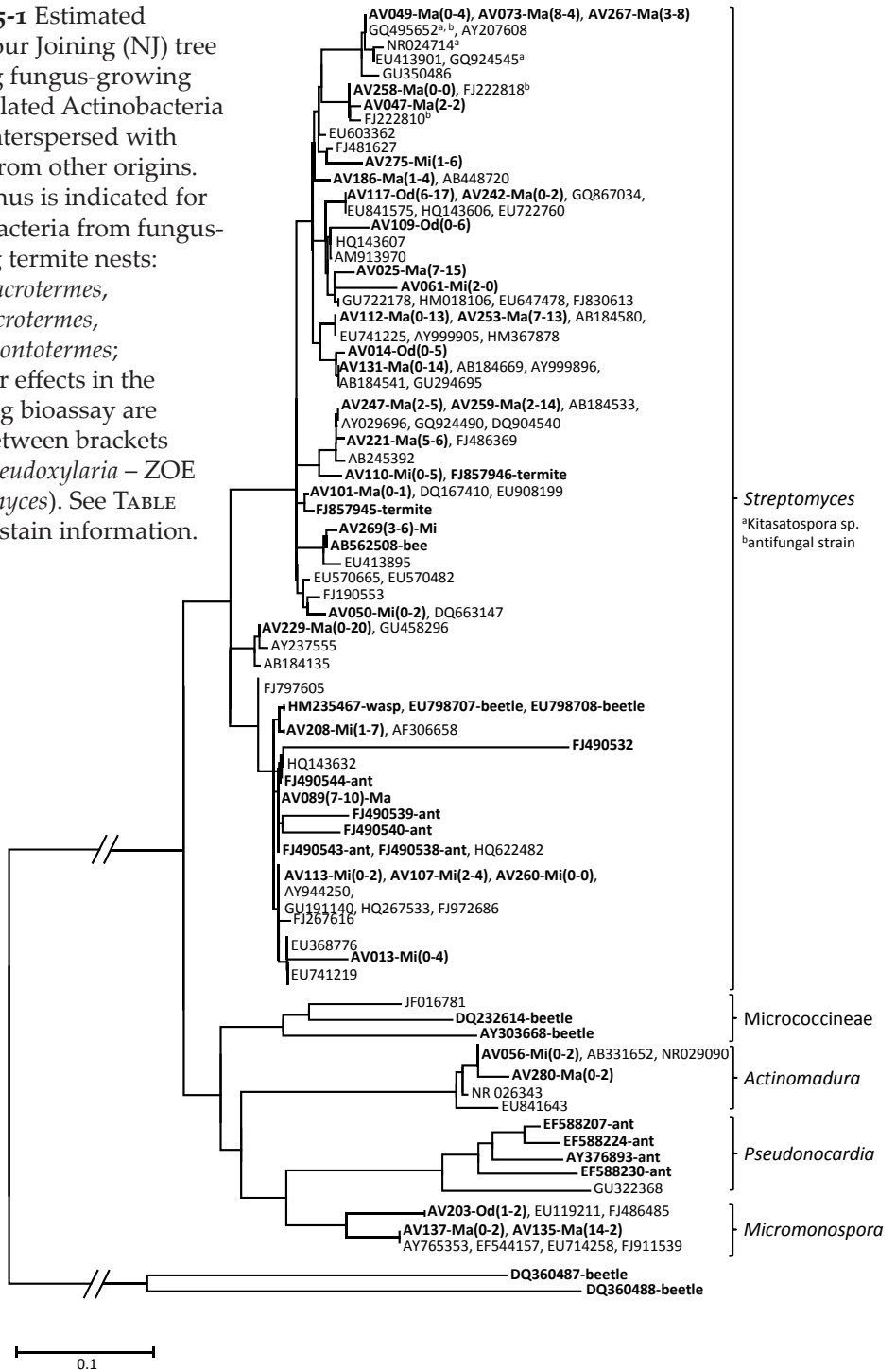
strain concerned. Twelve Actinobacteria that did not exceed this 2% threshold for any of the ten fungal strains tested are thus not shown (see Table S5-5 for the complete data).

Only two Actinobacteria had a pronounced and consistent antibiotic effect exclusively on *Pseudoxylaria* strains, and three had a strong effect exclusively on *Termitomyces* (top and bottom rows of Table 5-2). Single Actinobacteria strains varied considerably in their effect on *Pseudoxylaria* and *Termitomyces*; the categories by which Actinobacteria were selected in the screening bioassay (with one strain for each fungus), did not match the results of the detailed bioassay in half of the cases (Table 5-2). Certain Actinobacteria caused a large ZOE for only a part of the *Pseudoxylaria* strains, not affecting other *Pseudoxylaria* strains, and the same happened for *Termitomyces* strains. Placement of Actinobacteria in the NJ tree was uncorrelated with effect on *Pseudoxylaria* and *Termitomyces* in the screening bioassay (Figure 5-1).

Actinobacteria did not show specificity for fungi isolated from the same host (Figure 5-3 C-F). The only trend observed was that Actinobacteria from *Microtermes* colonies seemed to have a stronger effect on average on all fungal strains (Figure 5-3 C, E). In the detailed bioassay effects of Actinobacteria differed significantly between termite genera indeed (F = 3.338, df = 50, P = 0.044). ZOE
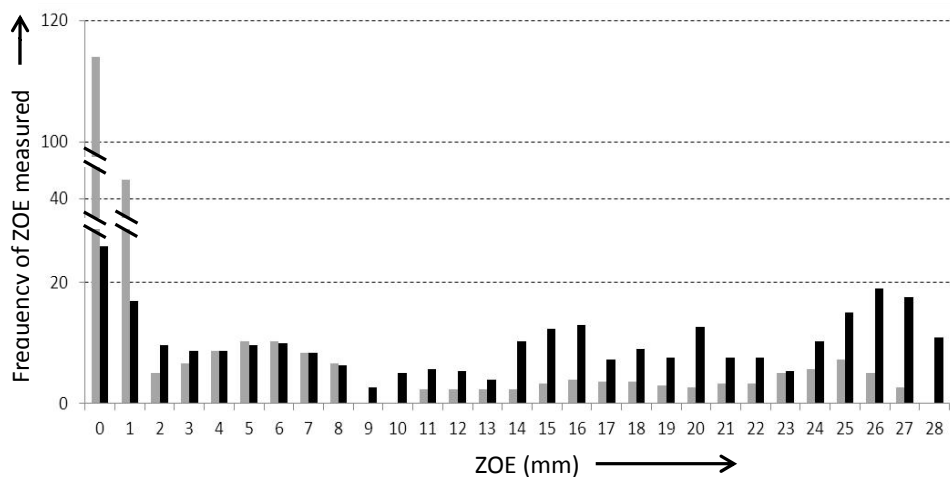
Figure 5-2 Frequency distribution of Actinobacteria effect sizes (ZOE) on *Pseudoxylaria* (black) and *Termitomyces* (grey) in the screening bioassay.

Table 5-2 Summary of the detailed bioassay: Effect of Actinobacteria on *Pseudoxylaria* and *Termitomyces* strains. See Table S5-5 for the complete data for this assay.

Strain	Effect in screening bioassay on:	Effect in detailed (this) bioassay on:	ZOE *Pseudoxylaria* (mm)					ZOE *Termitomyces* (mm)						
			P1	P3	P4	P5	Sum	T1	T2	T3	T4	T5	T6	Sum
AV156	P		11	10	9	7	37							
AV092	P & T			12	10	11	33							
AV040	P	*Pseudoxylaria* only			10	8	18							
AV222	P			11			11							
AV067	P & T					8	8							
AV210	P & T		27	20	15	12	74	19	21	15	19	18	14	106
AV240	T		21	19	17	11	68	15	25	13	17	16	15	101
AV212	P & T		14	20	15	12	61	14	15	12	13	16	15	85
AV057	P & T		17	17	16	10	60	18	20	20	23	17	16	114
AV030	P & T	4/4 *Pseudoxylaria* & *Termitomyces*	15	14	12	17	58	16	18	16	20	20	17	107
AV213	P & T		19	16	9	8	52	20	20	15	17	19	18	109
AV090	P & T		15	14	12	11	52		19	13			13	45
AV255	P & T		20	12	12	8	52	18	20	12	20	15	14	99
AV086	P & T		13	15	12	10	50		17	12			15	44
AV007	P & T		13	12	11	13	49	20	20	15	20	17	17	109
AV080	P & T		23		11	15	49	17	19	15	19	19	17	106
AV001	P & T			13	14	18	45	20	22	19	22	20	20	123
AV266	P	3/4 *Pseudoxylaria* & *Termitomyces*		14	17	14	45	13	21	14	15	17	17	96
AV055	P & T		14		14	7	35	12	13	10	14			49
AV027	T			13	10	7	30	19	17	17	11	16	14	94
AV063	P & T		9		8	8	25	11	15	13	15	17	12	83
AV053	P & T	2/4 *Pseudoxylaria* & *Termitomyces*	13	12			25	20	20	10		15	14	79
AV054	P			12	8		20	15	13	11				39
AV072	P & T		10		8		18	23		10	13	14		60
AV082	P			22			22	12	13	10	11	16	11	73
AV264	T		13				13					10		10
AV215	P & T				12		12	13	14	11	12	14	11	75
AV206	P		11				11			10	10	12	10	42
AV118	P & T	1/4 *Pseudoxylaria* & *Termitomyces*			10		10		18			10		28
AV123	P		9				9				10			10
AV272	T		9				9		15	11	10		10	46
AV044	P				7		7	11						11
AV037	P				7		7					11		11
AV138	T				7		7		20	14	23	15	17	89
AV145	P										12			12
AV081	T								15					15
AV062	T	*Termitomyces* only							10	10				20
AV039	P											12	11	23
AV166	T							12		15	14	10		51
AV035	P & T							11	17	10		12	15	65
AV270	P							14	19	14	15	15	16	92
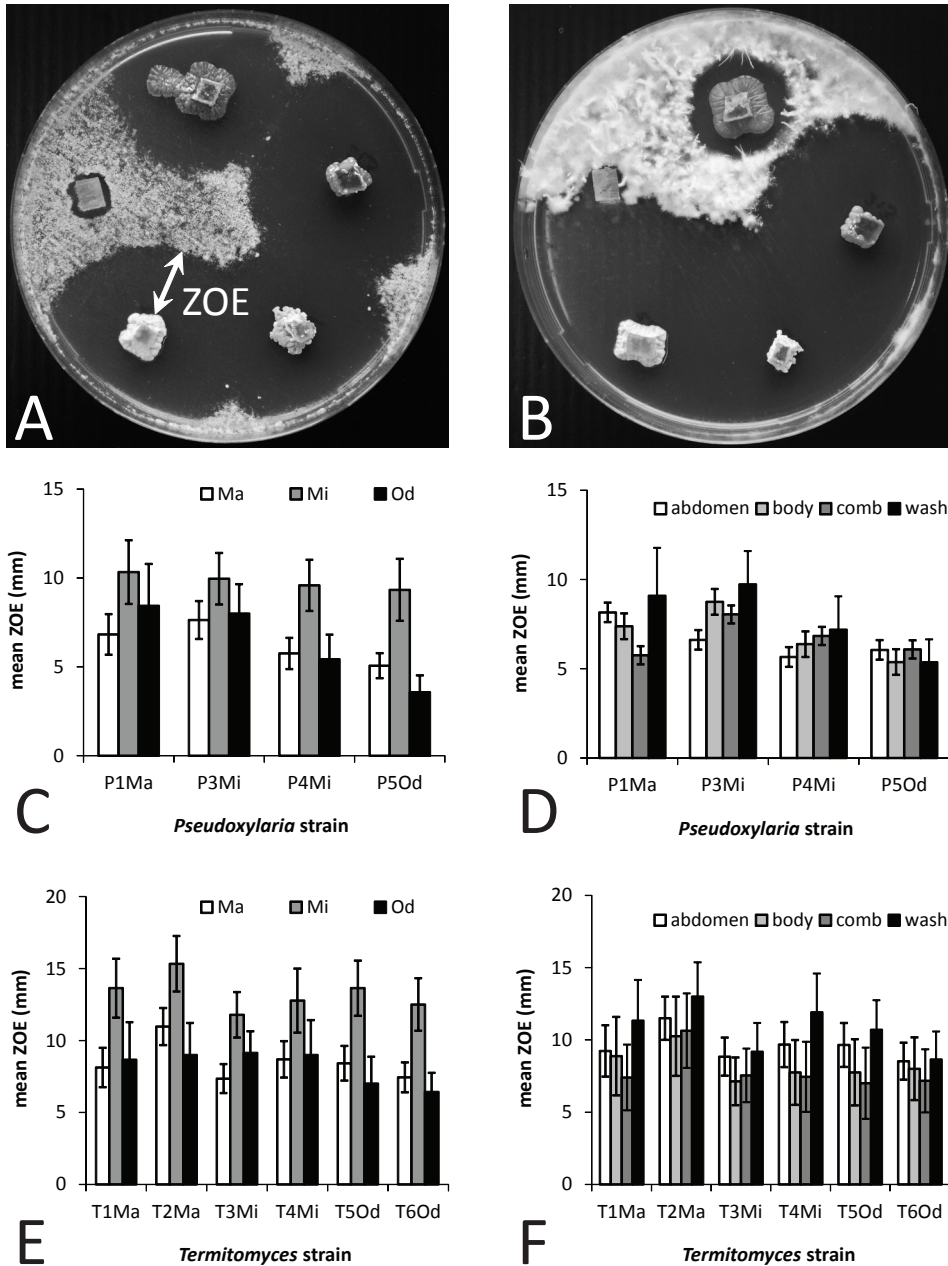
FIGURE 5-3 Results detailed bioassay. Examples of effect of Actinobacteria on *Pseudoxylaria* (A) and *Termitomyces* (B), and zone of effect (ZOE) caused by Actinobacteria averaged for each fungal strain per termite host genus (C, E) and per origin material (D, F) from which the bacteria were isolated.

TABLE 5-3 Effect of primarily produced metabolites (ZOE agar) versus effect of presence of Actinobacteria (ZOE bacteria) on the growth of *Pseudoxylaria* (P2) and *Termitomyces* (T1) in mm.

Strain	*Pseudoxylaria* P2		*Termitomyces* T1	
	ZOE by agar	ZOE by Actino-bacteria	ZOE by agar	ZOE by Actino-bacteria
AV001	12	15	17	18
AV009	0	0	8	1
AV033	1	0	23	2
AV057	1	9	20	15
AV083	9	0	14	2
AV105	0	4	3	6
AV132	0	4	17	10
AV209	6	7	20	15
AV225	3	8	23	22
Total effect	32	47	145	91

of Actinobacteria isolated from *Microtermes* caused a significantly higher ZOE in both *Pseudoxylaria* and *Termitomyces* (t = 2.355, df = 51, P = 0.022; and t = 2.602, df = 51, P = 0.012), but no significant differences were found for *Microtermes* Actinobacteria effects in the screening bioassay. There were no significant differences in the average antibiotic effect between Actinobacteria strains isolated from comb, head, or abdomen; and neither was there a difference in effect concerning the medium from which Actinobacteria were isolated.

In the primary antibiotic production assay agar blocks cut from positions adjacent to pure Actinobacteria colonies had an effect on *Pseudoxylaria* and *Termitomyces* that was similar to the effect of Actinobacteria themselves (TABLE 5-3).

Discussion

Actinobacteria in fungus-growing termite nests

Comparisons of fungus-growing termite symbiosis with other fungus-growing insects are made frequently, in particular with the New World fungus-growing ants (for example Mueller *et al.* 2005). As niches in symbiotic alliances are likely filled by organisms that are ubiquitous or close at hand, similar solutions may be found and similar mechanisms may be used for example to defend the symbiosis against weeds and pathogens. The separate origins of fungus-growing termites and other fungus-farming mutualisms

make comparisons particularly valuable, because it is possible to test if the same 'solutions' to evolutionary problems have independently arisen multiple times or if different solutions arose in different mutualisms.

Actinobacteria, being omnipresent, in the course of evolution have become integrated in microbial defence in symbiotic associations around the globe (Currie *et al.* 1999; Kaltenpoth *et al.* 2005; Scott *et al.* 2008; Kaltenpoth 2009). Here, we investigated whether the fungus-growing termite symbioses involve Actinobacteria for defence against *Pseudoxylaria*, an antagonist of the termite fungus garden. Our results show that Actinobacteria are abundantly present in fungus-growing termite nests. We found Actinobacteria in both geographic locations, in all termite genera, all colonies, and all colony parts sampled, which leaves open the possibility that these bacteria are specifically associated with the termites.

Specificity of Actinobacteria for fungus-growing termite nests

Actinobacteria are ubiquitous in soil and related substrates and almost all bacteria produce antibiotics to secure part of a nutrient substrate from competitors (Dehnad *et al.* 2010). Their omnipresence in the environment may allow these Actinobacteria to enter the nest via workers that perform nest-building and foraging activities. The NJ tree showed that Actinobacteria isolated from termite workers are genetically similar to, and intermingled with, those that occur outside termite nests. Because only a small subset (35 of 360 strains) of the obtained Actinobacteria was sequenced, more work is needed to fully explore the phylogenetic placement and distribution of termite-associated Actinobacteria.

Even if not occurring as specialised defensive symbionts with fungus-growing termites, Actinobacteria could still be beneficial for the termites if useful antibiotics are obtained from the bacteria that are picked up from the environment. As stated by Kaltenpoth (2009), fungus-gardens of insects face a high risk of specialised pathogens and can thus be expected to have defensive symbionts. He also noted that in the ant-*Pseudonocardia* as well as the beewolf-*Steptomyces* associations, there is horizontal transfer and *de novo* uptake of Actinobacteria from the environment. Hence,

Actinobacteria could still be beneficial for fungus-growing termites, despite lacking strong termite host specificity. Alternatively, the risk of specialised pathogens and the risk of more opportunistic weeds could be sufficiently distinct to have led to different control strategies in termites and ants.

The role of Actinobacteria in fungus-growing termite nests

Determining if Actinobacteria play a role as defensive symbionts, depends on more than making inferences based on Actinobacteria defensive symbionts in other insects, establishing their presence in termite nests, and showing (lack of) host specificity. Hence, we explored whether fungus-growing termite-associated Actinobacteria inhibit the invasive fungus *Pseudoxylaria*, and whether they affect the cultivar fungus *Termitomyces*. Although an *in vitro* bioassay may not be representative for the dynamics within termite nests, previous work has shown that what is observed in Petri plates (*in vitro*) can match what happens in miniature colonies (*in vivo*) (Poulsen *et al.* 2010). However, *in vitro* antagonism observed outside the natural system (like in Petri plate assays as we employ here) may not reflect natural interactions, because the production of antibiotics in pure cultures of bacteria is much higher than what is expected in bacterial populations in the environment from which they were isolated (Poulsen & Currie 2010).

Both bioassays showed that the obtained Actinobacteria secrete compounds with antibiotic properties, and that some of these compounds inhibit the invasive fungus *Pseudoxylaria*. But in contrast to Haeder (2009), who found that *Streptomyces* bacteria do not affect the ant cultivar but only inhibit the mycoparasite *Escovopsis*, we observed no target specificity. *In vitro*, Actinobacteria inhibited the termite cultivar fungus *Termitomyces* more often and more severely than they inhibited *Pseudoxylaria*. Whether *Termitomyces, in vivo*, is also affected by these secretions remains to be tested. Agar plugs taken adjacent to pure Actinobacteria cultures caused similar inhibition of both fungi, suggesting constitutive production of antibiotics, irrespective of the presence of other microorganisms. Altogether, the bioassays did not establish Actinobacteria as

defensive symbionts against *Pseudoxylaria* as the Actinobacteria showed general antifungal properties.

One might argue that these bacteria can still play a role in the suppression of *Pseudoxylaria* if they are applied in a directed way, in this way suppressing *Pseudoxylaria* without affecting *Termitomyces*. Active directed application has been suggested for Actinobacteria-derived antibiotics in fungus-growing ants (Boomsma & Aanen 2009; Poulsen & Currie 2010), where the bacterial secretions also have inhibitory properties against the ants' cultivar fungus *in vitro* (Sen *et al.* 2009; Poulsen *et al.* 2010), but not *in vivo* (Poulsen & Currie 2010). This, however, seems not to apply to fungus-growing termites as we found Actinobacteria in all sampled parts of the colony: termites and fungus comb alike.

Concluding remarks

This report describes the first discovery of an assembly of Actinobacteria occurring in fungus-growing termite nests. Actinobacteria were found throughout all sampled nests and materials, and the bioassays showed that many affect both the substrate competitor *Pseudoxylaria* and the termite cultivar *Termitomyces*. Lack of specificity in the Actinobacteria for fungus-growing termites as their host, combined with lack of specific defence against *Pseudoxylaria*, makes it unlikely that Actinobacteria play a role as defensive symbiont in fungus-growing termites.

We are aware that exploring the presence and role of microbes in a given environment exclusively with culture-based methods, as we do here, offers limited detail of putative associations. Only a fraction of the bacterial diversity of an environment, in this case termite nests, is recoverable using this approach because many bacteria still remain unculturable (Hongoh 2010; Lewis *et al.* 2010). Consequently, our study does not present an exhaustive search for all bacteria present in the association with fungus-growing termites. Metagenomic community analyses of the different termite colony parts would aid in identifying new Actinobacteria potentially specific for fungus-growing termites. If this proves to be the case, their role within colonies should be more thoroughly explored

before rejecting the hypothesis that Actinobacteria are defensive symbionts in fungus-growing termites.

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Supplements

TABLE S5-1 Overview of sampled termite colonies and isolated strains. *Not sampled for Actinobacteria.

Date	Location	Colony	Termite host	Number of isolates	Pseudo-xylaria	Termito-myces
2010-01-13	Pretoria	101	*Macrotermes natalensis*	28	P1	
2010-01-13	Pretoria	102	*Macrotermes natalensis*	3		
2010-01-13	Pretoria	103	*Macrotermes natalensis*	27		
2010-01-13	Pretoria	104	*Macrotermes natalensis*	28		
2010-01-15	Mookgophong	105	*Macrotermes natalensis*	28		T1
2010-01-15	Mookgophong	106	*Macrotermes natalensis*	42		
2010-01-15	Mookgophong	107	*Macrotermes natalensis*	22		
2010-01-18	Pretoria	109	*Macrotermes natalensis*	13		T2
2010-01-18	Pretoria	110	*Macrotermes natalensis*	20	P2	
2010-01-13	Pretoria	101A	*Microtermes* sp.	5		
2010-01-13	Pretoria	101B	*Microtermes* sp.	2		T3
2010-01-13	Pretoria	101C	*Microtermes* sp.	5		
2010-01-13	Pretoria	101D	*Microtermes* sp.	10		
2010-01-13	Pretoria	104A	*Microtermes* sp.	4	P3	
2010-01-13	Pretoria	104B	*Microtermes* sp.	3		
2010-01-13	Pretoria	104C	*Microtermes* sp.	2		T4
2010-01-13	Pretoria	104D	*Microtermes* sp.	2		
2010-01-13	Pretoria	104E	*Microtermes* sp.	2		
2010-01-13	Pretoria	104F	*Microtermes* sp.	3		
2010-01-13	Pretoria	104G	*Microtermes* sp.	12		
2010-01-15	Mookgophong	106A	*Microtermes* sp.	3		
2010-01-15	Mookgophong	106B	*Microtermes* sp.	3		
2010-01-15	Mookgophong	106C	*Microtermes* sp.	3		
2010-01-15	Mookgophong	106D	*Microtermes* sp.	19		
2010-01-15	Mookgophong	106E	*Microtermes* sp.	11	P4	
2010-01-19	Pretoria	111	*Odontotermes badius*	8		T5
2010-01-22	Pretoria	112	*Odontotermes latericius*	18		
2010-01-22	Pretoria	114	*Odontotermes latericius*	22		
2010-01-22	Pretoria	115	*Odontotermes latericius*	12		
2010-01-25	Pretoria	116[a]	*Odontotermes badius*	-	P5	
2004-02-04	Pretoria	01005[a]	*Odontotermes badius*	-		T6

TABLE S5-2 (page 1/8) Overview of Actinobacteria showing isolation details, assigned morphotype, and strain code used in the bioassays.

Isolate code	Termite genus	Colony	Colony part	Sample	Medium	Morphotype	Strain code
MP001	*Macrotermes*	101	abdomen	2	chi	TA35	
MP002	*Macrotermes*	101	abdomen	1	chi	TA38	AV019
MP003	*Macrotermes*	101	abdomen	1	chi	TA38	AV119
MP004	*Macrotermes*	101	abdomen	1	chi	TA16	AV121
MP005	*Macrotermes*	101	abdomen	2	chi	TA38/TA19	AV065
MP006	*Macrotermes*	101	abdomen	2	chi	TA38/TA19	AV232
MP007	*Macrotermes*	101	abdomen	1	micr	TA5	AV016
MP008	*Macrotermes*	101	abdomen	3	chi	TA19	AV074
MP009	*Macrotermes*	101	abdomen	3	chi	TA19	AV077
MP010	*Macrotermes*	101	abdomen	2	chi	TA28	AV070
MP011	*Macrotermes*	101	abdomen	4	chi	TA5	AV021
MP012	*Macrotermes*	101	body/wash	1 & 2	micr	TA26	AV213
MP013	*Macrotermes*	101	wash	3	chi	TA5	AV020
MP014	*Macrotermes*	101	abdomen	1	chi	TA26/TA29	AV071
MP015	*Macrotermes*	101	abdomen	1	chi	TA26/TA29	AV083
MP016	*Macrotermes*	101	abdomen	1	micr	TA19	AV217
MP017	*Macrotermes*	101	abdomen	2	micr	TA7	AV028
MP018	*Macrotermes*	101	abdomen	2	chi	TA16	
MP019	*Macrotermes*	101	abdomen	1	chi	TA26	AV066
MP020	*Macrotermes*	101	abdomen	3	chi	unknown	AV118
MP021	*Macrotermes*	101	abdomen	1	micr	unknown	AV158
MP022	*Macrotermes*	101	body	1	micr	TA19/TA38	AV187
MP023	*Macrotermes*	101	body	1	micr	TA19/TA38	AV096
MP024	*Macrotermes*	101	comb	2	chi	TA4?	AV196
MP025	*Macrotermes*	101	comb	1	chi	TA26	
MP026	*Macrotermes*	101	wash	2	chi	TA32	AV128
MP027	*Macrotermes*	101	wash	1	chi	TA35	AV120
MP028	*Macrotermes*	101	wash	1	micr	TA35	AV241
MP029	*Macrotermes*	102	comb	1	chi	TA28	AV089
MP030	*Macrotermes*	102	comb	1	micr	TA5	AV188
MP031	*Macrotermes*	102	comb	1 & 2	chi	TA16	AV114
MP032	*Macrotermes*	103	abdomen	1	chi	TA20	AV253
MP033	*Macrotermes*	103	body	1	micr	TA38	
MP034	*Macrotermes*	103	wash	2	micr	TA26	AV091
MP035	*Macrotermes*	103	wash	1	micr	TA19	AV237
MP036	*Macrotermes*	103	abdomen	1	micr	TA26	AV214
MP037	*Macrotermes*	103	wash	1	chi	TA13	AV164
MP038	*Macrotermes*	103	wash	1	micr	TA19/TA26	AV072
MP039	*Macrotermes*	103	wash	1	micr	TA19/TA26	AV093
MP040	*Macrotermes*	103	abdomen	2	chi	TA38	AV174
MP041	*Macrotermes*	103	body	1	micr	TA5	AV046
MP042	*Macrotermes*	103	wash	3	chi	TA17	
MP043	*Macrotermes*	103	wash	1	chi	TA18	AV240
MP044	*Macrotermes*	103	wash	1	micr	TA6	
MP045	*Macrotermes*	103	wash	2	chi	TA6	AV210

TABLE S5-2 (page 2/8) Overview of Actinobacteria showing isolation details, assigned morphotype, and strain code used in the bioassays.

Isolate code	Termite genus	Colony	Colony part	Sample	Medium	Morphotype	Strain code
MP046	*Macrotermes*	103	abdomen/wash	3	micr	TA20	AV136
MP047	*Macrotermes*	103	abdomen	1	micr	TA26	AV017
MP048	*Macrotermes*	103	abdomen	1 & 2	micr	TA26	AV230
MP049	*Macrotermes*	103	abdomen	1	chi	TA38	AV139
MP050	*Macrotermes*	103	body	1	micr	TA38	AV186
MP051	*Macrotermes*	103	wash	1	chi	TA20	AV138
MP052	*Macrotermes*	103	abdomen	2	micr	TA19	AV064
MP053	*Macrotermes*	103	body	1	micr	TA16	
MP054	*Macrotermes*	103	wash	2	chi	TA26	AV229
MP055	*Macrotermes*	103	wash	1	chi	TA38	AV175
MP056	*Macrotermes*	103	comb	1	chi	TA20	AV140
MP057	*Macrotermes*	103	comb	1	micr	TA5	AV044
MP058	*Macrotermes*	103	comb	2	micr	TA9	AV082
MP059	*Macrotermes*	104	abdomen	1	chi	TA19/TA5/TA6	AV043
MP060	*Macrotermes*	104	abdomen	1	chi	TA19/TA5/TA6	AV045
MP061	*Macrotermes*	104	abdomen	1	micr	unknown	AV205
MP062	*Macrotermes*	104	abdomen	2	chi	unknown	AV059
MP063	*Macrotermes*	104	body	1	micr	TA19	AV177
MP064	*Macrotermes*	104	body	1	micr	TA19	AV218
MP065	*Macrotermes*	104	body	2	micr	TA16	AV146
MP066	*Macrotermes*	104	wash	1	micr	TA38	
MP067	*Macrotermes*	104	wash	1	chi	TA38	
MP068	*Macrotermes*	104	abdomen	2	micr	TA3	AV258
MP069	*Macrotermes*	104	abdomen	1 & 2	chi	TA38	AV023
MP070	*Macrotermes*	104	abdomen	1	chi	TA4	AV092
MP071	*Macrotermes*	104	abdomen	2	chi	TA5	AV018
MP072	*Macrotermes*	104	wash	1	micr	TA8	AV216
MP073	*Macrotermes*	104	abdomen	1	chi	TA18	
MP074	*Macrotermes*	104	abdomen	2	chi	TA8	AV040
MP075	*Macrotermes*	104	abdomen	2	micr	TA37	
MP076	*Macrotermes*	104	abdomen	1	chi	TA24/TA18	AV242
MP077	*Macrotermes*	104	abdomen	2	chi	TA5/TA24	AV025
MP078	*Macrotermes*	104	abdomen	2	chi	TA5/TA24	AV047
MP079	*Macrotermes*	104	abdomen	3	chi	TA30	AV276
MP080	*Macrotermes*	104	comb	3	chi	TA10	AV147
MP081	*Macrotermes*	104	comb	2	chi	TA16	AV251
MP082	*Macrotermes*	104	comb	1	micr	TA5	AV190
MP083	*Macrotermes*	104	comb	1	chi	TA6	AV239
MP084	*Macrotermes*	104	comb	1	chi	TA29/TA37	AV098
MP085	*Macrotermes*	104	comb	2	chi	TA6	AV090
MP086	*Macrotermes*	104	comb	3	chi	TA17	AV200
MP087	*Macrotermes*	105	body	1	chi	TA28	AV067
MP088	*Macrotermes*	105	wash	1	chi	TA23	AV100
MP089	*Macrotermes*	105	abdomen	2	chi	TA28	AV069
MP090	*Macrotermes*	105	abdomen	1	micr	TA4	AV097

Table S5-2 (page 3/8) Overview of Actinobacteria showing isolation details, assigned morphotype, and strain code used in the bioassays.

Isolate code	Termite genus	Colony	Colony part	Sample	Medium	Morphotype	Strain code
MP091	*Macrotermes*	105	abdomen	1	micr	TA4	AV123
MP092	*Macrotermes*	105	abdomen	1	chi	TA38	
MP093	*Macrotermes*	105	abdomen	2	chi	TA19	AV084
MP094	*Macrotermes*	105	abdomen	4	chi	TA8	
MP095	*Macrotermes*	105	abdomen	1	chi	TA26	
MP096	*Macrotermes*	105	body	1	chi	TA38	
MP097	*Macrotermes*	105	body	2	chi	TA38	
MP098	*Macrotermes*	105	body	3	chi	TA38	AV068
MP099	*Macrotermes*	105	wash	1	chi	TA5	AV278
MP100	*Macrotermes*	105	abdomen	2	chi	TA35	AV116
MP101	*Macrotermes*	105	abdomen	1	micr	TA16	AV115
MP102	*Macrotermes*	105	wash	1	micr	TA27	AV112
MP103	*Macrotermes*	105	abdomen	1	chi	TA8/TA16	AV078
MP104	*Macrotermes*	105	abdomen	1	chi	TA8/TA16	AV167
MP105	*Macrotermes*	105	abdomen	2	chi	TA13	AV166
MP106	*Macrotermes*	105	body	1	micr	TA32	AV245
MP107	*Macrotermes*	105	wash	2	chi	TA28	
MP108	*Macrotermes*	105	comb	2	chi	TA17	AV252
MP109	*Macrotermes*	105	comb	1	chi	TA37	AV247
MP110	*Macrotermes*	105	comb	1	micr	TA26	AV234
MP111	*Macrotermes*	105	comb	1	chi	TA17	AV133
MP112	*Macrotermes*	105	comb	2	chi	TA18	AV135
MP113	*Macrotermes*	105	comb	1	micr	TA23	AV125
MP114	*Macrotermes*	105	comb	2	chi	TA8	
MP115	*Macrotermes*	106	abdomen	2	chi	TA21	AV101
MP116	*Macrotermes*	106	abdomen	1	micr	TA22	
MP117	*Macrotermes*	106	abdomen	2	micr	TA22	AV182
MP118	*Macrotermes*	106	abdomen	1	chi	TA31	
MP119	*Macrotermes*	106	body	1	chi	TA33	AV280
MP120	*Macrotermes*	106	wash	1	chi	TA31	AV228
MP121	*Macrotermes*	106	wash	2	micr	TA8	
MP122	*Macrotermes*	106	abdomen	2	chi	TA13	AV270
MP123	*Macrotermes*	106	abdomen	1	micr	TA3	AV257
MP124	*Macrotermes*	106	body	1	chi	TA33	AV273
MP125	*Macrotermes*	106	wash	1	chi	TA18	AV256
MP126	*Macrotermes*	106	wash	1	micr	TA22	AV262
MP127	*Macrotermes*	106	wash	2	chi	TA32	AV129
MP128	*Macrotermes*	106	wash	1	chi	TA3	AV154
MP129	*Macrotermes*	106	abdomen	2	chi	TA19	AV076
MP130	*Macrotermes*	106	abdomen	1	chi	TA31	
MP131	*Macrotermes*	106	body	1	chi	TA33	
MP132	*Macrotermes*	106	wash	2	chi	TA13	AV157
MP133	*Macrotermes*	106	wash	2	chi	TA13	AV272
MP134	*Macrotermes*	106	wash	2	micr	TA38	AV005
MP135	*Macrotermes*	106	wash	3	chi	TA4	AV102

Table S5-2 (page 4/8) Overview of Actinobacteria showing isolation details, assigned morphotype, and strain code used in the bioassays.

Isolate code	Termite genus	Colony	Colony part	Sample	Medium	Morphotype	Strain code
MP136	*Macrotermes*	106	wash	1	micr	TA9	AV026
MP137	*Macrotermes*	106	abdomen	1	micr	TA22	AV035
MP138	*Macrotermes*	106	body	1	micr	TA38	AV015
MP139	*Macrotermes*	106	body	1	chi	TA7	AV171
MP140	*Macrotermes*	106	body	3	chi	TA7	
MP141	*Macrotermes*	106	was	1	chi	TA21	AV148
MP142	*Macrotermes*	106	wash	1	chi	TA22	
MP143	*Macrotermes*	106	wash	3	chi	TA38	AV161
MP144	*Macrotermes*	106	abdomen	1	chi	TA19	AV087
MP145	*Macrotermes*	106	abdomen	2	chi	TA6	AV221
MP146	*Macrotermes*	106	abdomen	3	chi	TA17	AV137
MP147	*Macrotermes*	106	body	1	chi	TA8	AV073
MP148	*Macrotermes*	106	body	3	chi	unknown	AV169
MP149	*Macrotermes*	106	wash	2	chi	TA19	AV095
MP150	*Macrotermes*	106	comb	1 & 4	chi	TA1	AV058
MP151	*Macrotermes*	106	comb	5	chi	TA10	AV285
MP152	*Macrotermes*	106	comb	2	chi	TA19	AV094
MP153	*Macrotermes*	106	comb	6	chi	TA35	
MP154	*Macrotermes*	106	comb	4	chi	TA17	AV143
MP155	*Macrotermes*	106	comb	1 & 2	chi	TA21	AV149
MP156	*Macrotermes*	106	comb	3	chi	TA33	
MP157	*Macrotermes*	107	abdomen	4	chi	TA37	AV145
MP158	*Macrotermes*	107	wash	2	micr	TA22	AV124
MP159	*Macrotermes*	107	wash	1	micr	TA41	AV131
MP160	*Macrotermes*	107	abdomen	2	chi	TA25	AV156
MP161	*Macrotermes*	107	abdomen	1	chi	TA42	
MP162	*Macrotermes*	107	abdomen	1	micr	TA19	AV207
MP163	*Macrotermes*	107	wash	1	micr	TA20	AV153
MP164	*Macrotermes*	107	wash	1	chi	TA42	
MP165	*Macrotermes*	107	abdomen	1	chi	TA32	AV126
MP166	*Macrotermes*	107	wash	1	chi	TA7	
MP167	*Macrotermes*	107	abdomen	2	chi	TA10	AV144
MP168	*Macrotermes*	107	abdomen	1	chi	TA38	AV286
MP169	*Macrotermes*	107	abdomen	2	micr	TA26	AV233
MP170	*Macrotermes*	107	abdomen	3	chi	TA32	AV193
MP171	*Macrotermes*	107	abdomen	2	chi	TA25	AV244
MP172	*Macrotermes*	107	abdomen/wash	1	micr/chi	TA42	AV265
MP173	*Macrotermes*	107	body/wash	1	micr/chi	TA7	AV051
MP174	*Macrotermes*	107	wash	1	micr	TA42	AV049
MP175	*Macrotermes*	107	comb	1	chi	TA38	AV010
MP176	*Macrotermes*	107	comb	1	micr	TA20	AV142
MP177	*Macrotermes*	107	comb	2	chi	TA29	AV224
MP178	*Macrotermes*	107	comb	1	chi	TA17	
MP179	*Macrotermes*	109	abdomen	1	micr	TA29	AV219
MP180	*Macrotermes*	109	abdomen	1	micr	TA38	AV108

TABLE S5-2 (page 5/8) Overview of Actinobacteria showing isolation details, assigned morphotype, and strain code used in the bioassays.

Isolate code	Termite genus	Colony	Colony part	Sample	Medium	Morphotype	Strain code
MP181	*Macrotermes*	109	wash	1	micr	TA9	AV179
MP182	*Macrotermes*	109	wash	1	chi	TA4	AV104
MP183	*Macrotermes*	109	abdomen	2	micr	TA13	AV165
MP184	*Macrotermes*	109	abdomen	1	micr	TA5/TA19	AV048
MP185	*Macrotermes*	109	body	1	chi	TA9	AV052
MP186	*Macrotermes*	109	wash/abdomen	2	chi	TA19	AV063
MP187	*Macrotermes*	109	wash	1	micr	TA42	AV267
MP188	*Macrotermes*	109	abdomen	1	micr	TA42/TA1?	
MP189	*Macrotermes*	109	body	1	micr	TA5	AV037
MP190	*Macrotermes*	109	body	1	micr	TA15	AV099
MP191	*Macrotermes*	109	body	1	micr	TA29	AV225
MP192	*Macrotermes*	110	body	1	chi	TA37	AV259
MP193	*Macrotermes*	110	body	1	micr	TA6	AV212
MP194	*Macrotermes*	110	wash	1	micr	TA5	AV039
MP195	*Macrotermes*	110	abdomen	1	micr	TA5	AV199
MP196	*Macrotermes*	110	body	1	chi	TA7	AV281
MP197	*Macrotermes*	110	wash	1	micr	TA1	AV060
MP198	*Macrotermes*	110	wash	1	chi	TA37	AV057
MP199	*Macrotermes*	110	wash	1	chi	TA37	AV178
MP200	*Macrotermes*	110	abdomen/wash	1 & 2	chi/micr	TA26	AV215
MP201	*Macrotermes*	110	body	1	micr	TA5	AV038
MP202	*Macrotermes*	110	wash	1	micr	TA20	AV271
MP203	*Macrotermes*	110	wash	1	chi	TA5	
MP204	*Macrotermes*	110	wash	1	micr	TA5	AV172
MP205	*Macrotermes*	110	wash	2	micr	TA7	AV029
MP206	*Macrotermes*	110	abdomen	2	micr	TA5	AV024
MP207	*Macrotermes*	110	abdomen	1	micr	TA9	AV170
MP208	*Macrotermes*	110	abdomen	1	micr	TA9	AV042
MP209	*Macrotermes*	110	comb	1	micr	TA13	AV204
MP210	*Macrotermes*	110	comb	1	micr	TA13	AV198
MP211	*Macrotermes*	110	comb	2	micr	TA5	AV173
MP212	*Microtermes*	101A	comb	1	chi	TA18	
MP213	*Microtermes*	101A	comb	1	chi	TA28	
MP214	*Microtermes*	101A	comb	2	chi	TA6	AV180
MP215	*Microtermes*	101A	comb	1	micr	TA29	AV079
MP216	*Microtermes*	101A	comb	2	chi	TA6	
MP217	*Microtermes*	101B	comb	1	chi	TA12	AV004
MP218	*Microtermes*	101B	comb	2	chi	TA6	AV003
MP219	*Microtermes*	101C	abdomen	1	micr	TA9	AV027
MP220	*Microtermes*	101C	body	1	micr	TA6	AV209
MP221	*Microtermes*	101C	comb	1	chi	TA6	
MP222	*Microtermes*	101C	comb	1	chi	TA7	
MP223	*Microtermes*	101C	comb	2	chi	TA19	
MP224	*Microtermes*	101D	abdomen	1	micr	TA38	AV030
MP225	*Microtermes*	101D	abdomen	1	micr	TA38	AV007

TABLE S5-2 (page 6/8) Overview of Actinobacteria showing isolation details, assigned morphotype, and strain code used in the bioassays.

Isolate code	Termite genus	Colony	Colony part	Sample	Medium	Morphotype	Strain code
MP226	*Microtermes*	101D	abdomen	2	micr	TA8	AV080
MP227	*Microtermes*	101D	abdomen	1	chi	TA26	
MP228	*Microtermes*	101D	body	1	chi	TA19	
MP229	*Microtermes*	101D	abdomen	2	micr	TA8	AV220
MP230	*Microtermes*	101D	comb	1	micr	TA5	AV191
MP231	*Microtermes*	101D	comb	1	chi	TA29	AV075
MP232	*Microtermes*	101D	comb	2	chi	TA16	
MP233	*Microtermes*	101D	comb	3	chi	TA45	
MP234	*Microtermes*	104A	comb	1	chi	TA8/TA29/TA43	AV001
MP235	*Microtermes*	104A	comb	1	chi	TA8/TA29/TA43	AV274
MP236	*Microtermes*	104A	comb	1	chi	TA8/TA29/TA43	AV266
MP237	*Microtermes*	104A	comb	1	micr	TA6	
MP238	*Microtermes*	104B	comb	1	chi	TA4	AV197
MP239	*Microtermes*	104B	comb	2	chi	TA4	AV105
MP240	*Microtermes*	104B	comb	3	chi	TA4	AV103
MP241	*Microtermes*	104C	comb	1 & 2	chi	TA18	AV041
MP242	*Microtermes*	104C	comb	1 & 2	micr	TA16	AV168
MP243	*Microtermes*	104D	comb	1 & 2	chi	TA4	AV194
MP244	*Microtermes*	104D	comb	1	micr	TA19	AV288
MP245	*Microtermes*	104E	comb	1	chi	TA30	AV160
MP246	*Microtermes*	104E	comb	1	micr	TA47	AV275
MP247	*Microtermes*	104F	comb	1	micr	TA34	AV132
MP248	*Microtermes*	104F	comb	1	chi	TA13	AV106
MP249	*Microtermes*	104F	comb	2	chi	TA9	AV269
MP250	*Microtermes*	104G	abdomen	2	micr	TA18	AV110
MP251	*Microtermes*	104G	abdomen	1	micr	TA29	AV002
MP252	*Microtermes*	104G	abdomen	3	micr	TA38	
MP253	*Microtermes*	104G	abdomen	1	chi	TA20	AV141
MP254	*Microtermes*	104G	abdomen	3	micr	TA6	AV208
MP255	*Microtermes*	104G	abdomen	2	chi	TA38	AV284
MP256	*Microtermes*	104G	abdomen	3	chi	TA48	AV248
MP257	*Microtermes*	104G	comb	2	micr	TA18	AV176
MP258	*Microtermes*	104G	abdomen	1, 2 & 4	micr	TA13	AV268
MP259	*Microtermes*	104G	comb	2	chi	TA4	AV011
MP260	*Microtermes*	104G	comb	1	micr	TA48	AV249
MP261	*Microtermes*	104G	comb	1 & 3	chi/micr	TA8	AV086
MP262	*Microtermes*	106A	comb	2	chi	TA4	AV012
MP263	*Microtermes*	106A	comb	1	micr	TA3	AV055
MP264	*Microtermes*	106A	comb	2	micr	TA38	AV022
MP265	*Microtermes*	106B	comb	1	chi	TA19	AV231
MP266	*Microtermes*	106B	comb	2	micr	TA6	AV238
MP267	*Microtermes*	106B	comb	1	micr	TA19	AV223
MP268	*Microtermes*	106C	body	1	micr	TA3	AV053
MP269	*Microtermes*	106C	comb	1	micr	TA5	AV013
MP270	*Microtermes*	106C	comb	1	chi	TA18	

TABLE S5-2 (page 7/8) Overview of Actinobacteria showing isolation details, assigned morphotype, and strain code used in the bioassays.

Isolate code	Termite genus	Colony	Colony part	Sample	Medium	Morphotype	Strain code
MP271	*Microtermes*	106D	body	1	micr	TA12	AV107
MP272	*Microtermes*	106D	body	2	micr	TA13	AV134
MP273	*Microtermes*	106D	body	2	chi	TA28	AV056
MP274	*Microtermes*	106D	body	1	micr	TA19	AV085
MP275	*Microtermes*	106D	body	1	chi	TA37	
MP276	*Microtermes*	106D	abdomen	1	chi	TA15	
MP277	*Microtermes*	106D	body	1	chi	TA13	
MP278	*Microtermes*	106D	body	1	chi	unknown	AV127
MP279	*Microtermes*	106D	body	2	chi	TA13	AV061
MP280	*Microtermes*	106D	body	2	micr	TA19	
MP281	*Microtermes*	106D	abdomen	1	chi	TA8	AV206
MP282	*Microtermes*	106D	body	2	micr	TA11	AV113
MP283	*Microtermes*	106D	body	1	micr	TA13	
MP284	*Microtermes*	106D	body	1	chi	TA13	
MP285	*Microtermes*	106D	body	2	chi	TA13	
MP286	*Microtermes*	106D	comb	1 & 2	micr	TA10	AV282
MP287	*Microtermes*	106D	comb	1	chi	TA43	AV159
MP288	*Microtermes*	106D	comb	2	chi	TA13	
MP289	*Microtermes*	106D	comb	3	chi	TA13	
MP290	*Microtermes*	106E	abdomen	2	chi	TA13	
MP291	*Microtermes*	106E	body	1	micr	TA38	AV008
MP292	*Microtermes*	106E	body	2	chi/micr	TA11	AV260
MP293	*Microtermes*	106E	body	1	micr	TA14	AV050
MP294	*Microtermes*	106E	body	1	chi	TA14	
MP295	*Microtermes*	106E	body	1	micr	TA8	
MP296	*Microtermes*	106E	body	1	micr	TA44	
MP297	*Microtermes*	106E	comb	1	chi	TA20	AV277
MP298	*Microtermes*	106E	comb	2	chi	TA38	AV009
MP299	*Microtermes*	106E	comb	1	micr	TA8	
MP300	*Microtermes*	106E	comb	3	chi	TA14	
MP301	*Odontotermes*	111	abdomen	1	micr	TA4	AV195
MP302	*Odontotermes*	111	body	1	micr	TA3	AV255
MP303	*Odontotermes*	111	abdomen	2	micr	TA8	
MP304	*Odontotermes*	111	abdomen	1	micr	TA8	
MP305	*Odontotermes*	111	abdomen	1	chi	TA5	AV189
MP306	*Odontotermes*	111	wash	1	chi	TA1	AV054
MP307	*Odontotermes*	111	wash	1	micr	TA1	
MP308	*Odontotermes*	111	wash	1	micr	TA7	AV151
MP309	*Odontotermes*	112	body	1	micr	TA25	AV117
MP310	*Odontotermes*	112	body	1	chi	TA25	
MP311	*Odontotermes*	112	abdomen	1	chi	TA25	AV034
MP312	*Odontotermes*	112	abdomen	1	micr	TA19	AV211
MP313	*Odontotermes*	112	abdomen	1	chi	TA4	AV150
MP314	*Odontotermes*	112	body	1	chi	TA41	AV283
MP315	*Odontotermes*	112	body	2	chi	TA25	

TABLE S5-2 (page 8/8) Overview of Actinobacteria showing isolation details, assigned morphotype, and strain code used in the bioassays.

Isolate code	Termite genus	Colony	Colony part	Sample	Medium	Morphotype	Strain code
MP316	*Odontotermes*	112	abdomen	1	micr	TA38	AV006
MP317	*Odontotermes*	112	wash	1	chi	TA1	AV033
MP318	*Odontotermes*	112	abdomen	1	micr	TA19	AV236
MP319	*Odontotermes*	112	abdomen	1 & 2	chi/micr	TA28	AV081
MP320	*Odontotermes*	112	body	1	micr	TA16	
MP321	*Odontotermes*	112	wash	1	chi	TA1	AV183
MP322	*Odontotermes*	112	abdomen	1	micr	TA1	AV109
MP323	*Odontotermes*	112	abdomen	1	micr	TA1	AV031
MP324	*Odontotermes*	112	abdomen	1	chi	TA1	AV261
MP325	*Odontotermes*	112	abdomen	2	chi	TA13	AV254
MP326	*Odontotermes*	112	abdomen	1	micr	TA27	
MP327	*Odontotermes*	114	body	1	micr	TA9	AV163
MP328	*Odontotermes*	114	abdomen	2	chi	TA1	AV184
MP329	*Odontotermes*	114	abdomen	3	chi	TA2	AV203
MP330	*Odontotermes*	114	body	1	chi	TA25	AV263
MP331	*Odontotermes*	114	wash	1	chi	TA41	AV287
MP332	*Odontotermes*	114	abdomen	2	chi	TA1	AV185
MP333	*Odontotermes*	114	abdomen	1	chi	TA19	AV062
MP334	*Odontotermes*	114	wash	1	chi	TA17	AV250
MP335	*Odontotermes*	114	abdomen	2	micr	TA27	AV111
MP336	*Odontotermes*	114	abdomen	1	micr	TA41	AV130
MP337	*Odontotermes*	114	abdomen	1	chi	TA5	AV279
MP338	*Odontotermes*	114	abdomen	1	chi	TA1	AV181
MP339	*Odontotermes*	114	body	1	chi	TA9	AV162
MP340	*Odontotermes*	114	body	1	micr	TA19	AV222
MP341	*Odontotermes*	114	wash	1	chi	TA1	AV036
MP342	*Odontotermes*	114	body	1	micr	TA1	
MP343	*Odontotermes*	114	body	1	chi	TA32	AV243
MP344	*Odontotermes*	114	abdomen	1	chi	TA18	AV155
MP345	*Odontotermes*	114	body	1	chi	TA5	AV014
MP346	*Odontotermes*	114	abdomen	1	micr	TA26	AV235
MP347	*Odontotermes*	114	body	1	micr	TA32	AV246
MP348	*Odontotermes*	114	abdomen	1	chi	TA5	
MP349	*Odontotermes*	115	abdomen	2	chi	TA25	AV202
MP350	*Odontotermes*	115	abdomen	2 & 3	chi/micr	TA25	AV032
MP351	*Odontotermes*	115	abdomen	1	chi	TA19	AV226
MP352	*Odontotermes*	115	abdomen	1	micr	TA28	
MP353	*Odontotermes*	115	body	3	chi	TA16	AV201
MP354	*Odontotermes*	115	body	1	chi	TA5	AV192
MP355	*Odontotermes*	115	abdomen	1 & 2	chi/micr	TA19	AV088
MP356	*Odontotermes*	115	abdomen	1	micr	TA19	AV227
MP357	*Odontotermes*	115	abdomen	1	micr	TA25	AV264
MP358	*Odontotermes*	115	abdomen	1	micr	TA25	
MP359	*Odontotermes*	115	body	2	chi	TA7	AV152
MP360	*Odontotermes*	115	abdomen	1	chi	TA1	AV122

Table S5-3 (page 1/4) Sequenced Actinobacteria strains from fungus-growing termite nests and other sequences that were included in the estimation of the Neighbour Joining tree: First BLAST-hit of sequenced strains.

Strain	1st BLAST-hit	Name	Ecological origin	Geographic origin
AV013	EU741219.1	*Streptomyces spiralis* 13668B	marine sediment	Costa Rica
AV014	GU294695.1	*Streptomyces* sp. SA30	soil	China
AV025	GU722178.1	*Streptomyces* sp. JV180		
AV047	FJ222818.1	*Streptomyces* sp. HV14		
AV049	GQ495652.1[a]	*Kitasatospora* sp. MH160	soil	China
AV050	DQ663147.1	*Streptomyces* sp. 3182		China
AV056	AB331652.1	*Actinomadura bangladeshensis*	soil	Bangladesh
AV061	EU647478.1	*Streptomyces anulatus*		Malasya
AV073	EU413901.1	*Streptomyces* sp. 35-1	soil	China
AV089	HQ143632.1	*Streptomyces* sp. TZQ27		
AV101	EU908199.1	*Streptomyces* sp. MS218	sea	China
AV107	FJ972686.1	*Streptomyces fradiae* WF1	soil	China
AV109	HQ143607.1	*Streptomyces anulatus*		
AV110	FJ857946.1	*Streptomyces* sp. MV19	*Microhodotermes viator*	South Africa
AV112	HM367878.1	*Streptomyces* sp. Ank315	soil	
AV113	GU191140.1[a]	*Streptomyces* sp. SDS	soil	Egypt
AV117	GQ867034.1	*Streptomyces* sp. MP1	soil	
AV131	AB184541.1	*Streptomyces polychromogenes*		
AV135	EU714258.1	*Micromonospora* sp. R1	marine sediment	Mexico
AV137	FJ911539.1	*Micromonospora* sp. YIM 75717		India
AV186	AB448720.1	*Streptomyces* sp. TRI-11		Japan?
AV203	FJ486485.1	*Micromonospora chokoriensis*		China
AV208	AF306658.2	*Streptomyces thermosacchari*		
AV221	J486369.1	*Streptomyces ginsengisoli*		China
AV229	GU458296.2	*Streptomyces* sp. 145	soil	Thailand
AV242	HQ143606.1	*Streptomyces* sp. MTQ9		China
AV247	AB245392.1[a]	*Streptomyces ginsengisoli*	soil	Korea
AV253	AB184580.1[a]	*Streptomyces longwoodensis*		
AV258	FJ222810.1	*Streptomyces* sp. HV4		
AV259	AB184533.1	*Streptomyces misawanensis*		
AV260	HQ267533.1	*Streptomyces fradiae* RMS4	mangrove estuary	India
AV267	AY207608.1	*Streptomyces aureofaciens*		Korea
AV269	AB562508.1	*Streptomyces* sp. TA4-8	stingless bee	Thailand
AV275	EU621880.2	*Streptomyces* sp. 594	soil	Brazil
AV280	NR_026343.1	*Actinomadura madurae*		

[a]Second choice for 1st BLAST hit.

Table S5-3 (page 2/4) Sequenced Actinobacteria strains from fungus-growing termite nests and other sequences that were included in the estimation of the Neighbour Joining tree: Tenth BLAST-hit of sequenced strains.

Strain	10th BLAST-hit	Name	Ecological origin	Geographic origin
AV013	FJ797605.1	*Streptomyces* sp. DA08606	soil	China
AV014	AY999896.1	*Streptomyces* filamentosus strain AS 4.1871		
AV025	FJ830613.1	*Streptomyces* sp. 41169	endophytic	China
AV047	EU570665.1	*Streptomyces aureus* 173412		China
AV049	NR_024714.1[b]	*Kitasatospora cineracea* SK-3255		
AV050	FJ190553.1	*Streptomyces* sp. MP9C8	sea surface microlayer	Norway
AV056	NR_029090.1	*Actinomadura meyerae*	soil	
AV061	HM018106.1	*Streptomyces* sp. WALP22	potato tubers	USA
AV073	GQ924545.1	*Kitasatospora* sp. ACT-0111	endophytic	
AV089	HQ622482.1	*Streptomyces* sp. FXJ8.019	deep sea	Indian ocean
AV101	DQ167410.1	*Streptomyces* sp. WL-2		Korea?
AV107	EU368776.1	*Streptomyces* sp. A310	yellow sea	China
AV109	AM913970.1	*Streptomyces* sp. L138	brown algae	Germany
AV110	FJ857945.1	*Streptomyces* sp. MV18	*Microhodotermes viator*	South Africa
AV112	EU741225.1	*Streptomyces* sp. 13674G	beach sand	Costa Rica
AV113	FJ267616.1[b]	*Streptomyces* sp. 216701	soil	China
AV117	EU722760.1	*Streptomyces* sp. MA-G-8	endophytic	China
AV131	AB184669.1	*Streptomyces laurentii*		
AV135	EF544157.1	*Micromonospora* sp. 173314	mangrove	
AV137	AY765353.1	*Micromonospora lacunae*	estuary	South Africa
AV186	FJ481627.1	*Streptomyces pulveraceus* HBUM173130	soil	China
AV203	EU119211.1	*Micromonospora* sp. HBUM49420	soil	China
AV208	HM235467.1	*Streptomyces* sp. 31bB	Sirex noctilio	
AV221	DQ904540.1	*Streptomyces* sp. SB-B28	soil	Korea
AV229	AY237555.1	*Streptomyces* sp. YIM 30823		China
AV242	EU841575.1	*Streptomyces virginiae* HBUM174861		China
AV247	AY029696.1	*Streptomyces* sp. KN-0647		China
AV253	AY999905.1[b]	*Streptomyces bungoensis* NRRL B-24305		
AV258	EU570482.1	*Streptomyces aureus* 173414		China
AV259	GQ924490.1	*Streptomyces* sp. GSENDO-0579	endophytic	
AV260	AY944250.1	Actinomycetales bacterium N12	marine sponge	China
AV267	GU350486.1	*Streptomyces avellaneus*	soil	South Korea
AV269	EU413895.1[b]	*Streptomyces* sp. 44-A	soil	China
AV275	EU603362.1	*Streptomyces* sp. MJM6730	soil	South Korea
AV280	EU841643.1	*Actinomadura bangladeshensis*		

[b]Second choice for 10th BLAST hit.

Table S5-3 (page 3/4) Sequenced Actinobacteria strains from fungus-growing termite nests and other sequences that were included in the estimation of the Neighbour Joining tree: Morphotypes versus taxonomy of first and tenth BLAST-hit of sequenced strains.

Strain	Morphotype	Taxonomy of 1st BLAST-hit and 10th BLAST-hit
AV013	TA5[c]	Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces.
AV014	TA5[c]	Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces.
AV025	TA5/TA24[c]	Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces.
AV047	TA5/TA24[c]	Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces.
AV049	TA42[a]	Actinomycetales; Streptomycineae; Streptomycetaceae; Kitasatospora
AV050	TA14	Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces.
AV056	TA28[c]	Actinomycetales; Streptosporangineae; Thermomonosporaceae; Actinomadura.
AV061	TA13	Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces.
AV073	TA8[a]	Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces/Kitasatospora
AV089	TA28[c]	Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces.
AV101	TA21	Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces.
AV107	TA12[a]	Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces.
AV109	TA1	Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces.
AV110	TA18[c]	Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces.
AV112	TA27[a]	Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces.
AV113	TA11[a]	Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces.
AV117	TA25[a]	Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces.
AV131	TA41	Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces.
AV135	TA18[c, a]	Actinomycetales; Micromonosporineae; Micromonosporaceae; Micromonospora
AV137	TA17[a]	Actinomycetales; Micromonosporineae; Micromonosporaceae; Micromonospora
AV186	TA38	Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces.
AV203	TA2	Actinomycetales; Micromonosporineae; Micromonosporaceae; Micromonospora
AV208	TA6[c]	Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces.
AV221	TA6[c]	Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces.
AV229	TA26	Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces.
AV242	TA24/TA18[a]	Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces.
AV247	TA37	Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces.
AV253	TA20[a]	Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces.
AV258	TA3	Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces.
AV259	TA37	Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces.
AV260	TA11[a]	Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces.
AV267	TA42[a]	Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces.
AV269	TA9	Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces.
AV275	TA47	Actinomycetales; Streptomycineae; Streptomycetaceae; Streptomyces.
AV280	TA33	Actinomycetales; Streptosporangineae; Thermomonosporaceae; Actinomadura.

[c]Same morphotype in different branches of phylogenetic tree.
[d]With different morphotype in same branch of phylogenetic tree.

TABLE S5-3 (page 4/4) Sequenced Actinobacteria strains from fungus-growing termite nests and other sequences that were included in the estimation of the Neighbour Joining tree: Actinobacteria strains from other fungus-growing insects.

Sequence	Host family	Host species	Sequence taxon	Reference	
DQ360487	Curculionidae	*Dendroctonus rufipennis*	bacterium YC-4	Cardoza et al 2006	(outroup)
DQ360488	Curculionidae	*Dendroctonus rufipennis*	bacterium YC-5	Cardoza et al 2006	(outroup)
DQ232614	Curculionidae	*Dendroctonus valens*	*Leifsonia shinshuensis*	Morales-Jiménez et al 2009	
AY303668[e]	Curculionidae	*Dendroctonus valens*	*Cellulomonas xylanilytica*	Morales-Jiménez et al 2010	
EU798707	Curculionidae	*Dendroctonus frontalis*	*Streptomyces sp.*	Scott et al 2008	
EU798708	Curculionidae	*Dendroctonus frontalis*	*Streptomyces sp.*	Scott et al 2008	
AY376893	Formicidae	*Trachymyrmex zeteki*	*Pseudonocardia sp*	Cafaro & Currie 2005	
FJ490532	Formicidae	*Acromyrmex volcanus*	*Streptomyces sp.*	Haeder et al 2009	
FJ490538	Formicidae	*Acromyrmex volcanus*	*Streptomyces sp.*	Haeder et al 2009	
FJ490539	Formicidae	*Acromyrmex volcanus*	*Streptomyces sp.*	Haeder et al 2009	
FJ490540	Formicidae	*Acromyrmex volcanus*	*Streptomyces sp.*	Haeder et al 2009	
FJ490543	Formicidae	*Acromyrmex echinatior*	*Streptomyces sp.*	Haeder et al 2009	
FJ490544	Formicidae	*Acromyrmex echinatior*	*Streptomyces sp.*	Haeder et al 2009	
EF588207	Formicidae	*Atta colombica*	*Pseudonocardia sp*	Zhang et al 2007	
EF588224[e]	Formicidae	*Acromyrmex octospinosus*	*Pseudonocardia sp*	Zhang et al 2007	
EF588230[e]	Formicidae	*Trachymyrmex zeteki*	*Pseudonocardia sp*	Zhang et al 2007	

[e]Short sequence, hence replaced by its 1st BLAST-hit for calculation of the NJ tree.

Table S5-4 Complete data of screening bioassay. Effects of Actinobacteria strains AV001-AV150 on *Pseudoxylaria* (P2) and *Termitomyces* (T1), all in mm. Strains that were also tested in the detailed bioassay, selected because of their effect in this screening on either *Pseudoxylaria* (P), *Termitomyces* (T), or both (P & T), are shown in bold.

Strain	P2 ZOI	P2 ZOE	T1 ZOI	T1 ZOE	Selected for effect on:
AV001	2	**15**	10	**18**	P & T
AV002	0	0	6	7	
AV003	0	0	1	4	
AV004	5	**20**	5	**15**	P & T
AV005	0	6	0	7	
AV006	0	5	0	7	
AV007	0	**20**	6	**16**	P & T
AV008	0	0	0	2	
AV009	0	0	0	1	
AV010	0	0	0	5	
AV011	0	0	0	9	
AV012	0	**7**	1	**3**	P
AV013	0	0	0	4	
AV014	0	0	0	5	
AV015	0	7	8	22	
AV016	0	3	2	10	
AV017	0	2	5	7	
AV018	0	6	5	5	
AV019	0	3	0	15	
AV020	0	0	2	3	
AV021	0	5	0	20	
AV022	0	5	0	0	
AV023	0	0	0	3	
AV024	0	10	0	13	
AV025	0	7	0	15	
AV026	0	5	5	17	
AV027	0	**3**	7	**25**	T
AV028	0	0	2	11	
AV029	0	0	0	5	
AV030	14	**14**	7	**21**	P & T
AV031	0	0	2	3	
AV032	0	5	6	9	
AV033	0	0	2	2	
AV034	5	5	7	15	
AV035	0	**12**	1	**16**	P & T
AV036	0	6	0	3	
AV037	0	**7**	1	**6**	P
AV038	0	1	2	5	
AV039	0	**8**	0	**12**	P
AV040	4	**10**	2	**3**	P
AV041	0	2	3	5	
AV042	1	6	0	4	
AV043	0	3	0	3	
AV044	0	**8**	0	**6**	P
AV045	0	6	4	8	
AV046	0	2	3	7	
AV047	1	2	0	2	
AV048	1	3	0	6	
AV049	0	0	0	4	
AV050	0	0	0	2	
AV051	0	5	7	16	
AV052	0	**12**	0	**12**	P
AV053	6	**14**	7	**18**	P & T
AV054	3	**8**	2	**9**	P
AV055	5	**14**	8	**18**	P & T
AV056	0	0	0	2	
AV057	6	**9**	5	**15**	P & T
AV058	0	0	3	14	
AV059	0	2	1	5	
AV060	3	3	0	6	
AV061	0	2	0	0	
AV062	0	**2**	0	**20**	T
AV063	11	**11**	6	**18**	P & T
AV064	0	0	0	3	
AV065	0	2	4	4	
AV066	0	1	1	5	
AV067	1	**11**	0	**20**	P & T
AV068	0	3	0	8	
AV069	0	**0**	0	**18**	T
AV070	0	0	1	5	
AV071	0	0	0	2	
AV072	5	**9**	1	**18**	P & T
AV073	0	**8**	0	**4**	P
AV074	0	3	2	6	
AV075	0	2	2	3	
AV076	0	2	0	6	
AV077	0	2	2	5	
AV078	0	6	0	11	
AV079	0	1	1	4	
AV080	8	**20**	8	**18**	P & T
AV081	0	**1**	0	**20**	T
AV082	5	**10**	0	**11**	P
AV083	0	0	2	2	
AV084	0	3	0	3	
AV085	0	0	0	3	
AV086	0	**15**	8	**18**	P & T
AV087	0	3	0	3	
AV088	0	**12**	0	**5**	P
AV089[a]	3	7	5	10	P
AV090	15	**25**	8	**18**	P & T
AV091	0	5	0	2	
AV092	0	**13**	0	**15**	P & T
AV093	1	1	0	10	
AV094	0	0	0	3	
AV095	0	**0**	0	**15**	T
AV096	0	4	0	21	
AV097	0	3	3	10	
AV098	0	3	2	4	
AV099	0	0	0	3	
AV100	0	0	0	4	
AV101	0	0	0	1	
AV102	0	0	0	7	
AV103	0	6	5	10	
AV104	0	3	1	3	
AV105	1	4	0	6	
AV106	0	1	2	5	
AV107	0	2	1	4	
AV108	0	0	0	7	
AV109	0	0	1	6	
AV110	0	0	0	5	
AV111	0	8	0	17	
AV112	0	0	2	13	
AV113	0	0	0	2	
AV114	7	7	3	16	
AV115	0	3	0	20	
AV116	0	0	0	2	
AV117	2	6	4	17	
AV118	0	**9**	0	**17**	P & T
AV119	0	**1**	0	15	
AV120	0	2	0	5	
AV121	0	0	0	3	
AV122	0	6	1	8	
AV123	2	**7**	5	**9**	P
AV124	0	3	5	11	
AV125	0	0	0	1	
AV126	0	0	0	1	
AV127	0	0	0	2	
AV128	0	0	3	5	
AV129	0	0	2	3	
AV130	0	4	0	9	
AV131	0	0	3	14	
AV132	5	4	0	10	
AV133	0	0	0	1	
AV134	0	0	0	1	
AV135	0	**14**	0	**2**	P
AV136	0	0	0	0	
AV137	0	0	0	2	
AV138	0	**0**	1	**17**	T
AV139	0	4	0	18	
AV140	0	0	0	1	
AV141	0	0	0	3	
AV142	0	0	0	1	
AV143	0	0	0	0	
AV144	0	0	0	4	
AV145	0	**10**	1	**8**	P
AV146	0	0	0	2	
AV147	0	2	0	6	
AV148	0	0	0	7	
AV149	0	**0**	0	**18**	T
AV150	0	0	0	0	

[a]Strain AV089 was selected with 'P', but not used due to lack of growth after culture transfer.

TABLE S5-4 (page 2/2) Complete data of screening bioassay. Effects of Actinobacteria AV151-AV288, on *Pseudoxylaria* (P2) and *Termitomyces* (T1), all in mm. Strains that were also tested in the detailed bioassay, selected because of their effect in this screening on either *Pseudoxylaria* (P), *Termitomyces* (T), or both (P & T), are shown in bold. At the lower right total ZOE per fungal strain.

Strain	P2 ZOI	P2 ZOE	T1 ZOI	T1 ZOE	Selected for effect on:
AV151	0	1	0	6	
AV152	0	7	0	17	
AV153	0	1	3	6	
AV154	0	6	4	20	
AV155	0	1	0	1	
AV156	0	**10**	0	**0**	P
AV157	0	6	10	11	
AV158	0	1	3	10	
AV159	0	0	0	1	
AV160	0	3	3	7	
AV161	0	6	6	14	
AV162	0	0	0	3	
AV163	6	6	0	10	
AV164	5	5	4	5	
AV165	0	6	2	4	
AV166	0	**0**	0	**20**	T
AV167	0	5	0	10	
AV168	0	0	0	0	
AV169	0	0	0	1	
AV170	3	3	3	10	
AV171	0	1	1	17	
AV172	0	1	2	11	
AV173	0	2	0	10	
AV174	0	0	0	3	
AV175	1	3	1	10	
AV176	0	1	0	4	
AV177	0	0	0	0	
AV178	0	2	3	11	
AV179	0	1	0	5	
AV180	0	2	0	4	
AV181	0	0	0	1	
AV182	0	0	0	0	
AV183	0	0	0	9	
AV184	0	0	0	5	
AV185	0	0	0	10	
AV186	0	1	0	4	
AV187	0	3	0	16	
AV188	0	2	2	8	
AV189	0	4	5	12	
AV190	0	3	4	7	
AV191	0	0	0	0	
AV192	0	0	0	0	
AV193	0	0	0	12	
AV194	0	0	0	0	
AV195	0	0	0	0	
AV196	0	1	0	1	
AV197	0	0	0	3	
AV198	0	3	3	11	
AV199	0	3	1	8	
AV200	0	0	0	0	
AV201	0	2	0	12	
AV202	0	0	0	1	
AV203	0	1	0	2	
AV204	0	**0**	0	**16**	T
AV205	0	2	3	25	
AV206	0	**8**	0	**12**	P
AV207	0	2	0	5	
AV208	0	1	4	7	
AV209	0	7	3	15	
AV210	3	**14**	4	**15**	P & T
AV211	0	2	0	2	
AV212	0	**20**	4	**19**	P & T
AV213	0	**15**	6	**17**	P & T
AV214	0	1	3	5	
AV215	0	**10**	4	**15**	P & T
AV216	4	**10**	0	**2**	P
AV217	0	0	0	0	
AV218	1	1	0	14	
AV219	0	3	0	13	
AV220	0	2	1	6	
AV221	0	5	2	6	
AV222	0	**10**	0	**7**	P
AV223	0	0	0	6	
AV224	0	0	0	3	
AV225	0	8	4	22	
AV226	0	2	0	10	
AV227	0	3	0	5	
AV228	0	0	0	2	
AV229	0	**0**	0	**20**	T
AV230	0	0	0	2	
AV231	0	3	1	5	
AV232	0	1	0	2	
AV233	0	0	2	5	
AV234	0	2	2	4	
AV235	0	5	0	3	
AV236	0	2	1	2	
AV237	0	6	7	10	
AV238	0	6	8	12	
AV239	8	8	7	18	
AV240	0	**0**	3	**18**	T
AV241	0	4	0	3	
AV242	0	0	0	2	
AV243	0	0	0	1	
AV244	0	0	0	0	
AV245	0	0	0	0	
AV246	0	0	0	1	
AV247	0	2	0	5	
AV248	0	0	0	0	
AV249	0	0	0	1	
AV250	0	0	0	5	
AV251	0	0	0	2	
AV252	0	0	0	0	
AV253	0	7	5	13	
AV254	0	0	0	0	
AV255	0	**17**	6	**15**	P & T
AV256	0	1	0	7	
AV257	0	7	5	15	
AV258	0	0	0	0	
AV259	0	2	1	14	
AV260	0	0	0	0	
AV261	0	0	3	8	
AV262	0	0	2	11	
AV263	0	5	5	11	
AV264	0	**3**	5	**15**	T
AV265	0	3	5	8	
AV266	0	**0**	14	**18**	T
AV267	0	3	4	8	
AV268	0	2	2	6	
AV269	0	3	0	6	
AV270	0	**7**	2	**8**	P
AV271	0	0	0	10	
AV272	0	**10**	8	**11**	P
AV273	0	0	0	4	
AV274	0	8	10	20	
AV275	0	1	0	6	
AV276	0	0	0	2	
AV277	0	0	0	0	
AV278	0	0	0	4	
AV279	0	2	0	3	
AV280	0	0	0	2	
AV281	0	2	0	0	
AV282	0	0	0	0	
AV283	0	1	0	8	
AV284	0	0	0	5	
AV285	0	0	0	0	
AV286	0	0	0	3	
AV287	0	0	2	5	
AV288	0	0	2	4	

Strain	Sum ZOE P2	Sum ZOE T1
AV001-AV050	229	416
AV051-AV100	257	473
AV101-AV150	106	345
AV151-AV200	100	338
AV201-AV250	168	383
AV251-AV288	84	252
Total ZOE	944	2207

Table S5-5 (page 1/2) Complete data of detailed bioassay. Average effects of Actinobacteria on *Pseudoxylaria* and *Termitomyces*. Second page show effects per individual fungal strain, and total ZOE per strain, all in mm.

Strain	Screening effect on:	*Pseudoxylaria* ZOI	ZOE	*Termitomyces* ZOI	ZOE	P - T ZOI	ZOE
AV138	T	0	4.5	2.2	17.8	-2	-13
AV270	P	2.63	4	13.9	16.3	-11	-12
AV166	T	0.25	1.75	0.5	11.2	0	-9
AV035	P & T	1.5	4	1.17	13	0	-9
AV063	P & T	7.5	8	13	15	-6	-7
AV007	P & T	10	12.5	16.3	18.3	-6	-6
AV027	T	3.5	10	11	15.7	-8	-6
AV039	P	3.5	4	4.33	9.5	-1	-6
AV001	P & T	10	16	18	20.8	-8	-5
AV072	P & T	6.25	6.25	8	10.8	-2	-5
AV037	P	2.25	6	2.33	10.3	0	-4
AV272	P	2.75	6.75	7.67	11	-5	-4
AV030	P & T	9.5	14.3	16.7	18.5	-7	-4
AV206	P	3.75	6.5	6.83	10.5	-3	-4
AV264	T	3.25	3.25	5	7	-2	-4
AV086	P & T	10.8	11	12.3	14.7	-2	-4
AV082	P	3.5	8.75	9.83	12.4	-6	-4
AV255	P & T	10.8	13	13.5	16.5	-3	-4
AV053	P & T	7	12.5	10.7	15.8	-4	-3
AV149	T	0	0.25	0	3.2	0	-3
AV052	P	0.5	4	1.67	6.67	-1	-3
AV215	P & T	4.25	10	10.3	12.5	-6	-3
AV062	T	0.5	3.5	3.33	6	-3	-3
AV054	P	0.5	7.25	2.67	9.67	-2	-2
AV057	P & T	11.3	16.7	12.3	19	-1	-2
AV266	T	8.5	14.8	13.7	17.1	-5	-2
AV145	P	2	4.33	3.2	6.5	-1	-2
AV118	P & T	1	7	1.6	8.8	-1	-2
AV123	P	0.5	3	1.67	4.67	-1	-2
AV004	P & T	1	2	1.17	3.5	0	-2
AV080	P & T	5.75	16.3	12.7	17.7	-7	-1
AV090	P & T	10.3	13.7	10.3	15	0	-1
AV088	P	0	4.25	1.33	5.33	-1	-1
AV040	P	2.25	8.33	5.17	9	-3	-1
AV055	P & T	3.75	10.5	6.5	11	-3	-1
AV213	P & T	12	19	16.5	19.3	-5	0
AV135	P	0	0	0.17	0.2	0	0
AV073	P	0	0.25	0	0.17	0	0
AV012	P	0.25	1	0.17	0.83	0	0
AV240	T	12.5	17	12.3	16.8	0	0
AV044	P	0	5	0.5	4.2	-1	1
AV222	P	0.75	6.33	0.83	5.5	0	1
AV081	T	2.25	7	1.17	6	1	1
AV095	T	2	4	0	3	2	1
AV229	T	0.25	1.75	0	0.67	0	1
AV067	P & T	1.25	4	0.83	2.8	0	1
AV069	T	0.75	2.67	0.17	1.33	1	1
AV210	P & T	14.8	19.7	14.8	18.3	0	1
AV204	T	0.25	1.5	0	0	0	2
AV212	P & T	11.3	15.7	12	14	-1	2
AV216	P	0.75	3.5	1.67	1.67	-1	2
AV092	P & T	7	11	1	7	6	4
AV156	P	8	10.5	0.4	1.25	8	9

Table S5-5 (page 2/2) Complete data of detailed bioassay. Effects per individual fungal strain, and total ZOE per strain, all in mm. First page shows average effects of Actinobacteria on *Pseudoxylaria* and *Termitomyces*. *Part of the combinations could not be measured due to contamination.

Strain	P1 ZOI	P1 ZOE	P3 ZOI	P3 ZOE	P4 ZOI	P4 ZOE	P5 ZOI	P5 ZOE	T1 ZOI	T1 ZOE	T2 ZOI	T2 ZOE	T3 ZOI	T3 ZOE	T4 ZOI	T4 ZOE	T5 ZOI	T5 ZOE	T6 ZOI	T6 ZOE
AV001	7	7	13	13	10	14	10	18	16	20	17	22	16	19	19	22	20	20	20	20
AV004	1	2	0	2	1	2	2	2	0	6	2	3	1	4	1	2	2	4	1	2
AV007	13	13	9	12	11	11	7	13	16	20	16	20	12	15	20	20	17	17	17	17
AV012	1	1	0	2	0	1	0	0	0	0	1	2	0	0	0	0	0	2	0	1
AV027	6	6	5	13	3	10	0	7	12	19	14	17	10	17	7	11	13	16	10	14
AV030	15	15	10	14	10	12	3	17	16	16	18	18	16	16	20	20	15	20	15	17
AV035	1	5	0	3	3	3	2	2	2	11	0	17	0	10	1	1	4	12	0	15
AV037	2	5	2	6	3	3	2	7	2	2	3	12	2	8	2	2	3	3	2	11
AV039	5	5	3	4	3	3	3	3	5	5	5	10	4	5	3	3	5	12	4	11
AV040	4	4	1	7	2	10	2	8	4	9	7	7	4	4	5	5	6	6	5	5
AV044	0	5	0	6	0	2	0	7	0	11	0	7	0	0	0	2	3	3	0	1
AV052	0	3	1	7	1	6	0	0	0	8	0	8	6	9	1	8	3	5	0	2
AV053	9	13	8	12	6	6	5	5	10	20	14	20	9	10	8	8	11	15	12	14
AV054	2	3	0	12	0	8	0	6	5	15	3	13	2	11	4	9	1	4	1	6
AV055	9	14	2	7	4	14	0	7	5	12	7	13	7	10	8	14	8	8	4	6
AV057	13	17	12	17	10	16	10	10	10	18	12	20	15	20	17	23	12	17	8	16
AV062	1	4	0	5	1	3	0	2	0	2	1	2	8	10	3	10	3	5	5	7
AV063	9	9	7	7	8	8	6	8	11	11	15	15	12	13	13	15	15	17	12	12
AV067	0	0	2	2	3	4	0	8	0	2	0	1	1	1	1	5	2	3	1	3
AV069	1	1	1	2	0	2	1	4	0	0	0	1	0	1	0	2	1	2	0	2
AV072	10	10	5	5	8	8	2	2	6	23	7	8	8	10	13	13	14	14	0	2
AV073	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
AV080[a]	8	23	0	*	7	11	8	15	12	17	15	19	13	15	15	19	16	19	5	17
AV081[a]	3	8	0	*	4	6	2	2	1	4	2	7	2	15	2	3	0	3	0	4
AV082	2	3	7	22	3	6	2	4	9	12	11	13	8	10	11	11	12	16	8	11
AV086[a]	13	13	15	15	10	12	5	10	*	*	14	17	10	12	*	*	*	*	13	15
AV088[a]	0	8	0	4	0	2	0	3	*	*	2	8	0	4	*	*	*	*	2	4
AV090[a]	11	15	10	14	9	12	11	11	*	*	10	19	11	13	*	*	*	*	10	13
AV092[a]	5	5	8	12	8	10	7	11	*	*	3	12	0	5	*	*	*	*	0	4
AV095[a]	3	5	4	4	1	1	0	3	*	*	0	6	0	0	*	*	*	*	0	3
AV118[a]	3	8	0	6	1	4	0	10	*	*	2	18	0	2	2	7	3	10	1	7
AV123	2	9	0	2	0	0	0	1	0	0	0	4	1	4	3	10	1	3	5	7
AV135	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	1	0	0	0	0
AV138[a]	0	2	0	7	0	2	0	7	*	*	0	20	0	14	9	23	0	15	2	17
AV145[a]	1	1	3	5	2	3	2	5	*	*	3	6	2	3	6	12	2	2	3	5
AV149[a]	0	0	0	0	0	1	0	0	0	0	*	*	0	8	0	8	0	0	0	0
AV156[a]	8	11	8	10	9	9	7	7	2	2	*	*	0	1	0	0	0	2	0	2
AV166	1	4	0	3	0	0	0	0	0	12	0	12	3	15	0	14	0	10	0	4
AV204	1	3	0	2	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AV206	10	11	3	6	2	5	0	4	7	7	12	12	5	10	6	10	6	12	5	10
AV210	24	27	15	20	15	15	5	12	19	19	16	21	11	15	14	19	15	18	14	14
AV212	14	14	14	20	12	15	5	12	14	14	10	15	8	12	13	13	16	16	11	15
AV213	15	19	16	16	9	9	8	8	17	20	15	20	15	15	17	17	19	19	16	18
AV215	4	4	4	8	6	12	3	3	10	13	11	14	9	11	10	12	12	14	10	11
AV216	1	1	1	7	1	1	0	0	1	1	2	2	2	2	2	2	2	2	1	1
AV222	3	3	0	11	0	5	0	3	0	7	0	5	2	5	0	6	2	5	1	5
AV229	0	0	1	5	0	2	0	0	0	0	0	3	0	0	0	0	0	1	0	0
AV240	18	21	13	19	13	17	6	11	10	15	13	25	10	13	14	17	14	16	13	15
AV255	17	20	9	12	11	12	6	8	18	18	15	20	9	12	12	20	15	15	12	14
AV264	13	13	0	4	0	2	0	1	6	6	8	8	2	7	3	6	8	10	3	5
AV266	6	6	11	14	11	17	7	14	13	13	15	21	11	14	15	15	15	17	14	17
AV270	6	6	3	5	2	5	0	3	14	14	15	19	12	14	15	15	15	15	15	16
AV272	5	9	3	7	3	6	0	5	6	6	10	15	8	11	10	10	7	8	5	10
Total ZOE		415		417		349		309		430		598		456		464		453		448

Chapter 6 - General Discussion:

Termite Fungiculture Revisited

Mutualism, cooperation between species where they benefit reciprocally from exchanging goods or services, can immensely increase productivity and diversity in an ecosystem (Leigh 2010). The list of studied examples of mutualism is ever-increasing, which suggests that the more we look, the more we will find mutualisms playing an essential role in all the worlds ecosystems (Leigh 2010). It seems unlikely that any organism is left unaffected by mutualistic interactions. Even species that display most antagonistic types of behaviour often thrive by the grace of goods produced in mutualistic alliances between other species. The fungus *Escovopsis weberi*, for example, has specialised on parasitizing the cultivar fungus of attine ants in the fungus-growing ant mutualistic symbiosis (Currie *et al.* 1999). Indeed, Herre *et al.* (1999) state that, either directly or indirectly, most organisms are involved in mutualistic interactions.

In this thesis, I have studied fungus-growing termites to learn what factors play a role in stability of cooperation between species. A colony of fungus-growing termites (Blattodea: Macrotermitinae) lives in a reciprocally obligate mutualistic symbiosis with a monoculture of the basidiomycete genus *Termitomyces* (Sands 1960; Katoh *et al.* 2002). The termite-fungus agricultural (or fungicultural) practice has persisted for about 30 million years already (Aanen *et al.* 2002; Mueller *et al.* 2005). What makes the fungus-growing termite mutualism successful to such extent that it dominates semi-arid ecosystems in sub-Saharan Africa and South Asia? How does this cooperation between species remain stable over evolutionary time scales? Monocultures in human agriculture are very susceptible to weeds and pathogens. Hence, what about the weed and pathogen pressure on the *Termitomyces* monoculture; are there other organisms besides macrotermitine termites and *Termitomyces* that play a role in the symbiosis? How are conflicts of interest between symbiotic partners resolved? These questions form the foundation of this

thesis on the ecology and evolution of micro-organisms associated with fungus-growing termites.

Pseudoxylaria: an additional mutualist, a parasite, or a weed?

Species of *Pseudoxylaria* (Ascomycota, Xylariales, genus *Xylaria*) associated with fungus-growing termites received particular attention, because after termites and their *Termitomyces* cultivar, *Pseudoxylaria* species are the most prominent inhabitants of termite nests. Though inconspicuous in an active termite nest, dead nests and their fungus gardens are typically covered with a dense mycelium and fructifications of *Pseudoxylaria* (Chapter 2, 4; Rogers *et al.* 2005; Ju & Hsieh 2007). Moreover, often, *Pseudoxylaria* also covers healthy fragments of fungus-garden incubated in the lab in a matter of days (Thomas 1987a). Because of this prominence of *Pseudoxylaria* in termite nests, some authors have considered it a mutualistic symbiont of fungus-growing termites (Sannasi 1969; Batra & Batra 1979), while others considered it a 'very minor inhabitant' (Thomas 1987a).

What little has been reported on the role of this prominent associate of fungus-growing termite nests was in conflict with each other. Therefore, I focussed on old questions that were still unanswered. What is the status of *Pseudoxylaria* species in the fungus-growing termite nest – are they present by chance or as symbiont? If *Pseudoxylaria* is a symbiont, is it an additional mutualist, a parasite, or a weed? How do termites manage the *Termitomyces* fungus-garden; what prevents *Pseudoxylaria* growth or take-over in active termite nests? Are there other microorganisms that play a role in the fungus-growing termite nest?

Field and experimental observations revealed fascinating interactions among symbionts and associates of fungus-growing termite nests. Over hundred and fifty termite nests were sampled for fungi, termites, and bacteria. As many students before me, during the work I became increasingly intrigued by the social organization of the termites and the apparent nest hygiene. When opening the termite mound, soldiers instantly came to defend the openings against intruders, while workers appeared shortly after with mouthfuls of clay to repair the damage. In all those nests –

except one – the sole microorganism visible was *Termitomyces*. The exception was a dead nest, which contained a fungus garden that was overgrown with *Pseudoxylaria* fruiting bodies indeed.

The results of Chapter 2 (Visser *et al.* 2009) show that *Pseudoxylaria* constitutes a species-rich (16 OTUs) monophyletic group, the large majority of which is associated with fungus-growing termites (only three of the twenty-three closest BLAST-hits of isolates that occur in this clade were not associated with fungus-growing termites). Within the *Pseudoxylaria* clade, specificity for different macrotermitine genera is low. This result has been confirmed twice since (Guedegbe *et al.* 2009a; Hsieh *et al.* 2010). The data of Chapter 3 suggest that *Pseudoxylaria* is a low-profile (inconspicuously or latently present) weed, rather than a mutualist or mycoparasite. An analogy is drawn between *Pseudoxylaria* and foliar endophytic fungi, which have adopted a 'sit-and-wait strategy' (Herre *et al.* 1999). The experiment described in Chapter 4 shows that *Macrotermes natalensis* workers play a crucial role in maintaining the *Termitomyces* monoculture, as in their absence *Pseudoxylaria* and other fungi soon cover the fungus comb, similar to what Shinzato *et al.* (2005) observed in *Odontotermes formosanus*. Comparisons were made with other fungus-growing insects. In Chapter 5, it was tested whether antibiotic-producing Actinobacteria play a role in the defence against unwanted fungi, as has been found in several other insect mutualisms (Kaltenpoth 2009). The data suggest that Actinobacteria do not play a significant defensive role in fungus-growing termites. Actinobacteria isolated from the termite nest are not specific for fungus-growing termites. Additionally, the isolated Actinobacteria inhibit the cultivar fungus more strongly than *Pseudoxylaria*, which argues against a specific role in the fungus-growing termite nest.

As the previous chapters developed, it became clear that the role of microorganisms in the fungus-growing termite mutualism cannot be deduced in a forthright way from the roles observed in similar insect symbioses, which was also noted by Aanen (2006). At the same time, some remarkable analogies can be drawn between termite fungiculture and human agriculture, and between *Pseudoxylaria* in the fungus-growing termite nest and foliar endophytic fungi in plants. In the following discussion I reflect more

deeply on some of these and other general findings from previous chapters. Directions for future research are given at the end of sections. Finally, this chapter closes with the main conclusions of this thesis.

Fungus monoculture revisited

Agriculture revolves around optimal crop production; all farmers seek to benefit maximally from what they cultivate. Humans now seek the solution in immense monocultures that produce maize, rice, soybean, wheat; beef, eggs, fish, pork; cotton, silk and eucalypt wood – to name just a few vegetable, animal, and fibre products. Humans have specialised intensely in managing their mass-production units, and have developed an endless array of equipment and logistics to accommodate, feed, harvest, multiply, and process their crops. Additionally, taking the ever-increasing wheat yield (Tilman *et al.* 2002) as an example, selective breeding has resulted in cultivars that meet the demands of humans increasingly well.

Similar to many human examples of agriculture, fungus-growing termites grow their crop, *Termitomyces*, in monoculture (Aanen *et al.* 2002; Katoh *et al.* 2002; Moriya *et al.* 2005; Aanen *et al.* 2009). Additionally, like agriculture allowed the human population to increase from 4 million to more than 6,000 million individuals (Tilman *et al.* 2002), monopolizing and exploiting a crop has made fungus-growing termites ecologically extremely successful (Wood & Thomas 1989; Korb & Aanen 2003). Among mound-building termite species, some termite species create an optimal climate of 28(±2)°C and 94(±2)% humidity for their fungiculture (Agarwal 1980; Thomas 1987c). *Termitomyces* uses (and respires!) a major part of the carbon when breaking down lignocellulose and other wood components (Rouland *et al.* 1991; Kuyper 2004), achieving a net increase in the nitrogen mass fraction of the substrate, and resulting in a high quality resource (with low C:N ratio) for the termites derived from the low quality wood (high C:N ratio). This gave the ancestors of Macrotermitinae and *Termitomyces* an advantage over other detritivores, an advantage that increased as termite and

fungus evolved more and more adaptations to meet each other's demands, to such extent that presently they cannot survive without each other – like in human agriculture the fate of non-shattering cereal cultivars and the fate of human populations have become increasingly intertwined.

Individual termite nests contain a single strain monoculture of *Termitomyces* (Aanen *et al.* 2002; Katoh *et al.* 2002; Aanen *et al.* 2009). Single-nucleate *Termitomyces* spores that the first workers collect from the environment during foraging germinate, mate and eventually form a heterokaryotic monoculture on the termite faecal pellets in the nest (Darlington 1994; de Fine Licht *et al.* 2005). See Chapter 1 and Korb & Aanen (2003) for more details in the life history see of *Termitomyces*. To avoid confusion in this discussion, 'monoculture' needs to be defined. Monoculture means the presence of one cultivar strain (or genet) within a unit of production, or like in fungus-growing termites one strain of *Termitomyces* in a single nest. Therefore, a monoculture does not necessarily mean absence of other organisms.

Besides farmers and their cultivar, other organisms tend to be present. We are only too familiar with weeds and pathogens that can attack the crop such as bird flu virus, *Phytophthora infestans*, *Salmonella*, *Striga* and swinepox virus. Especially cultivars grown in monocultures are susceptible to these parasitic organisms, as they have little or no genetic variation in resistance (Odorfer *et al.* 1994; Piper *et al.* 1996; de Bellaire *et al.* 2010). Humans breed new cultivar varieties to replace varieties that suffer from weeds and pathogens. New varieties are genetically different from the used cultivars such that they are less susceptible to weeds and pathogens. The latter, however, rapidly evolve to re-infest or re-infect contemporary monocultures. Hence, humans are in a constant race to stay ahead of adaptations by the weeds and pathogens that threaten their monocultures, like in Lewis Carroll's *Through the Looking-Glass* (1871) where the Red Queen said, "It takes all the running you can do, to keep in the same place".

Fungus-growing termites are expected to be in a similar evolutionary race against antagonists. Being in close contact with the soil and dead vegetation teeming with microorganisms, termites continuously pick up contaminants (Thomas 1987b). In the absence

of genetic diversity in *Termitomyces*, and additionally a genetically uniform population of farmers, fungus-growing termite nests could form an even easier target for weeds and pathogens (Chapter 4). While humans may opt for monoculture to save on labour input, to use machines efficiently, and to optimise retail of their produce, what advantage do termites gain from growing *Termitomyces* in monoculture to compensate for this magnitude of contaminant susceptibility that makes the evolutionary race against parasites extra hard? Though nowadays the *Termitomyces* monoculture appears to arise by default from the way it is propagated in the fungus-garden in fungus-growing termites (Chapter 1), there must have been strong forces in the past that selected this practice.

Advantages of Termitomyces *monoculture*

On the short term, monoculture cropping of fungi has a large advantage in terms of yield. Growing a monoculture avoids costly antagonism among different cultivar genets (Kennedy *et al.* 2007), or reduced growth in border areas (Bleiker & Six 2009). Besides the energy and cultivar tissue that gets lost in the war for territory in the fungus-garden (Kennedy *et al.* 2007), warfare among fungi often involves production of hazardous substances (Boddy 2000), which could result in low quality and possibly toxic fungus comb for the termites. Also for fungus-growing ants (Poulsen & Boomsma 2005) and woodboring beetles (Bleiker & Six 2009), avoiding competition among cultivars has been identified as an advantage of monoculture. An additional advantage for the termites, and typical for basidiomycete fungi, is that hyphae of the same *Termitomyces* genotype – but separate mycelia – fuse to form one interactive mycelium, which amplifies the productivity of fungus (Aanen *et al.* 2009). On the long term, high symbiont relatedness selects for prudent reproductive strategies to the benefit of the host (Aanen *et al.* 2009).

Conflict of interest between termites and Termitomyces

When organisms are associated in time and space, and dependent on each other for nutrition and accommodation, their

interests are not automatically aligned. For example, the fig wasp has interest in maximizing the number of fig tree seeds in which an egg is laid and minimizing the pollination task, while the fig tree has interest in minimizing the number of seeds that is sacrificed to wasps while maximizing pollination by the wasps (Jandér & Herre 2010). Ultimately both symbionts should maximise their own reproductive success (Darwin 1859). Having their ultimate fates connected does not explain how meanwhile any conflict of interest is resolved. In fungus-growing termites, producing alates (winged reproductive termites) that leave the nest, or producing a stipe that pierces the mound to form basidiocarps (sexual fruiting bodies), means consuming resources against the other partners short-term, within-generation, fitness (Aanen 2006).

Law and Lewis (1983) showed that in mutualistic symbioses the inhabitants tend to have less sex than their free-living relatives. The genus *Termitomyces* fits this hypothesis only partly, as vertical transmission of the fungal symbiont, without formation of fruitbodies and sexual reproduction, has evolved twice. In *Macrotermes bellicosus* and in species of *Microtermes* vertical transmission occurs (Johnson 1981; Johnson *et al.* 1981). The fungus-growing ant cultivar fits the hypothesis better, as fructification is almost completely suppressed in *Leucoagaricus gongylophorus* by the higher attine ants (Korb & Aanen 2003). So what keeps the termite-*Termitomyces* mutualism in balance?

Several mechanisms can stabilise mutualism within and among species. Mutualistic interactions are stabilised by high symbiont relatedness (Hamilton 1964; Brock *et al.* 2011), sanctioning of cheaters (Foster & Kokko 2006; Kiers *et al.* 2006), partner choice to opt out of mutualistic interaction (Mathew & Boyd 2009), and high probability of continued interaction (Axelrod & Hamilton 1981). Now, let us find out how they apply to fungus-growing termites. On the one hand, the ultra-high kinship within the termite and *Termitomyces* of a single nest unites the interests within these partner groups via kin selection. For example, if a fungus evolves a trait that benefits the host and receives a benefit from this host in return, this benefit is shared with clonal relatives so that such a trait can be kin selected. On the other hand, because of the complete absence of kinship between termites and *Termitomyces* they do not

compete for the same resources, which favours cooperation between the partners (Leigh 2010). However, there is a difference between the short term (within nest lifetime) and long term (beyond nest lifetime) effects, which will be discussed subsequently.

On the short term, cheating, non-performing of either termite or *Termitomyces*, seems unlikely. Presuming that within a nest there is only one genotype of *Termitomyces*, cheating would be en-masse, leading to reduced return of benefits from the termites. The sanction is direct, so there seems to be no incentive for cheating. Opting out is an option for neither termites, nor *Termitomyces*. Macrotermitine termites have lost the typical gut-microbiota that allows the non-fungus growers to digest wood. Switching to a new nest or eradicating the comb to build a new fungus-garden would leave the termites without proper nutrition for too long and is thus very unlikely. *Termitomyces* apparently has such a low efficiency during the degradation of lignocellulose (it respires a very large part of its substrate, resulting in a remaining substrate with a low C:N ratio) that it is an extremely weak competitor and has a 'pitiful' performance in competition with other fungi (Darlington 1994). Host switching by *Termitomyces* through contact between the fungus gardens of different termite nests is highly unlikely. Even if there was possibility of transfer, frequency-dependent selection would probably prevent the incoming strain from becoming established in the visited fungus garden. No free-living macrotermitine termites or *Termitomyces* have been described, hence the mutualism is considered obligate (Aanen *et al.* 2002). As a result, the probability of continued interaction in the fungus-growing termite nest approaches hundred per cent. The strong connection between the fate of *Termitomyces* monoculture and that of termites ensures fidelity towards each other (Axelrod & Hamilton 1981).

On the long term, cheating by directing resources to dispersal (either alates or basidiocarps) while the other partner invests in maintenance of the colony, seems tempting for both partners. However, if the probability of continued mutualistic interaction diminishes, the other partner is likely to do the same, which would soon exhaust and finish the termite nest. Contrastingly, fungus-growing termite mounds are very long-lived, which encourages mutualistic behaviour (Axelrod & Hamilton 1981). Queens of

Macrotermes species live 15-20 years (Keller 1998) and, if dead queens are succeeded (Uys 2002), colonies may exist for several decades, but we remain with the question how this conflict of long-term interest is resolved.

What forms in mutualisms the point of no return to a free-living state, enslaving the partners? Determining which enzymes are produced by termites and which by *Termitomyces*, and quantifying their respective importance for substrate decomposition, is necessary to understand more about what benefits and other evolutionary forces shape this mutualistic symbiosis, as was done by Martin & Martin (1978) and Sen *et al.* (2009) for example. Combining data of all fungus-growing termite species and *Termitomyces* cultivar and aligning those with their respective phylogenetic trees as in de Fine Licht *et al.* (2010), is necessary to understand how the benefits evolved and what stabilises the fungus-growing termite symbiosis.

Associated microorganisms and commensalists

When reading the former section or opening active termite nests, one easily forgets that besides termites and *Termitomyces* there are other organisms that are of significance for the symbiosis. In all but one of the over hundred and fifty termite nests that were sampled for fungi, termites and bacteria, the sole microorganism visible was *Termitomyces*. The single inactive nest that we mistakenly sampled though, contained a fungus garden that was covered with *Pseudoxylaria* stromata and fruiting bodies (Chapter 2). When incubating healthy fungus comb in the lab, *Pseudoxylaria* species were quick to appear in the majority of cases during all sampling seasons (Chapter 2) but *Pseudoxylaria* could not be isolated from all nests, and within nests it did not appear on all combs (Chapter 2). These observations confirmed earlier reports that *Pseudoxylaria* species are present but suppressed in favour of *Termitomyces* in active termite nests, and that *Pseudoxylaria* species can rapidly overgrow the fungus comb thereby smothering *Termitomyces* (Sands 1969, Batra & Batra 1979; Thomas 1987a, b, c).

Though less prominent, I observed also other arthopods and microorganisms besides *Pseudoxylaria* (Chapter 2, 4, 5).

So, combining observations (Kistner 1969; Thomas 1987a, b, c ; Gumming 1996; Shinzato *et al.* 2005; Hongoh *et al.* 2006), there is a myriad of organisms that await their chance to profit from goods and services of the fungus-growing termite nest. These put a large pressure on the intrinsically susceptible symbiosis. Therefore, highly efficient mechanisms to prevent weeds and pathogens must be present in the fungus-growing termite nest, which are discussed extensively in Chapter 4 & 5. Most surprising perhaps is that the class of Actinobacteria – considered an essential symbiont for a number of insects as protective mutualist – do not seem to aid the termites in suppressing *Pseudoxylaria* (Chapter 5).

During the fieldwork and primary isolations, three types of commensals (inquilines) of the fungus-growing termite nests were repeatedly found: inquiline flies (S. Dupont, University of Copenhagen, unpublished), 'small arthropods' (ersonal observation), and beetles (Kistner 1969). Particular was the abundance of small arthropods on termite workers from a certain nest, of which the combs at a later stage in the laboratory were covered with *Ophiostoma* species (Chapter 4). Ambrosia beetles (Coleoptera, Curculionidae), which depend on fungi for digesting the wood for their larvae, are typically hitchhiked by certain mites. Those mites are the vector by which *Ophiostoma* species, detrimental to the beetle larvae, are transmitted from tree to tree (Klepzig *et al.* 2001). Hence, an interesting line of research could be to investigate if inquiline insects play a role as vectors for microorganisms to enter the fungus-growing termite nest.

The role of *Pseudoxylaria* in fungus-growing termite nests

In Chapter 2 it became clear that a group of *Xylaria* species specifically occur in fungus-growing termite nests, subgenus *Pseudoxylaria* after Hsieh *et al.* (2010). The interactions observed between *Pseudoxylaria* and *Termitomyces* suggest that *Pseudoxylaria* is a weed, competing with *Termitomyces* for the comb-substrate, rather than a mutualistic symbiont (Chapter 3). Observations of Chapter 4 demonstrate that termites play a role in suppressing *Pseudoxylaria*, which in their absence rapidly covers the whole

fungus comb, leaving no space for *Termitomyces*. Along the way, the idea that *Pseudoxylaria* acts like a weed in the fungus-growing termite garden grew stronger. The previous, with an increasing amount of publications that prove the dominance of *Termitomyces* mycelium in fungus combs from active fungus-growing termite nests, and the observation that *Pseudoxylaria* does not emerge from all fungus-growing termite nests or all parts of the fungus comb (Chapter 2), imply strongly that *Pseudoxylaria* is a weed instead of a mutualist in the fungus-growing termite nest.

But could *Pseudoxylaria* perhaps be the lesser antagonist in the nest, compared to other weeds and pathogens? Colonizing a plant as the lesser antagonist, thereby preventing more pathogenic species to colonise the plant, seems to apply for a number of endophytes (Arnold *et al.* 2003; Herre *et al.* 2007). However, it remains highly unlikely that *Pseudoxylaria* has evolved towards the role of mutualist in termite nests, since its presence is not guaranteed. Besides, its presence is extremely limited as long as the nest is healthy. Over the past decade quantitative molecular methods have shown that *Pseudoxylaria* and other alien fungi only form a marginal part of the fungal biomass, as analyses of healthy comb material often only detect *Termitomyces* (Shinzato *et al.* 2005; Guedegbe *et al.* 2009a; Guedegbe *et al.* 2009b).

Life history of Pseudoxylaria

Why does *Pseudoxylaria* only appear after termites have abandoned the nest? Xylariaceous fungi grow better at low water potential than basidiomycetes (Boddy 2000). Batra & Batra (1979) suggested high levels of CO_2 and other gas concentrations inside the termite mound could suppress certain fungi. When the nest is devoid of termites, damage causing holes in the mound is no longer repaired, giving rise to a drier microclimate and lower CO_2 levels. Is this how *Pseudoxylaria* gains advantage over *Termitomyces* and covers the fungus comb? I consider this unlikely, because during our incubations the comb was kept moist and *Pseudoxylaria* still appeared in a matter of days. Also changing concentrations of carbon dioxide or other gasses or volatile compounds is unlikely the cause of the emergence of *Pseudoxylaria*, as in the experiment described

in Chapter 4 cups were opened regularly and *Pseudoxylaria* did still not develop unless termite workers were absent. Currently, the most plausible explanation is that termite themselves suppress *Pseudoxylaria* (Chapter 4, Chapter 5).

How do *Pseudoxylaria* species survive in the fungus garden, how do they transmit from one colony to the next? For survival within the nest, *Pseudoxylaria* should stay low-profile, like a stowaway, but at the same time either continuously grow enough to stay ahead of fungus-comb replacement, or survive termite gut passage (Chapter 2, 3; Rogers *et al.* 2005; Batra & Batra 1979; Hsieh *et al.* 2010). For transmission between colonies, *Pseudoxylaria* spores of fruiting bodies that are produced soon after termites have ceased to control the fungus-garden need to escape the termitarium and gain access to active termite nests. The dead termite mound with *Pseudoxylaria* fructifications encountered during fieldwork was occupied by an array of insects such as larvae of beetles that presumably will leave the nest again at a certain stage of their life cycle. Also vertebrates like mongoose, fox and aardvark are known to use dead termite nests for accommodation, so getting aboveground seems not the biggest challenge. How *Pseudoxylaria* subsequently enters an active termite mound might be sought in the direction of inquilline insects of fungus-growing termite nests. Rogers *et al.* (2005) and Hsieh *et al.* (2010) hypothesised that the atypical small spores of *Pseudoxylaria* as compared to other *Xylaria* species, could be the result of adaptation towards ingestion by invertebrates for the purpose of transmission. Batra & Batra (1979) suggested that *Escovopsis* – a parasite of the ant fungus cultivar – is perhaps not transmitted vertically, but that inquilines (beetles) associated with fungus-growing ants carry the relatively small spores outside the nest and thus transmit *Escovopsis* horizontally.

Future research needs to establish if *Pseudoxylaria* indeed depends on inquiline insects for between-colony transmission. Concerning within-colony transmission, the problem whether *Pseudoxylaria* survives termite gut passage as mycelium, as asexual spores, or that it depends on vegetative growth in the fungus comb, needs to be addressed.

What determines parasitic and mutualistic behaviour?

An important question that has remained untouched so far: what makes *Termitomyces* a mutualist and *Pseudoxylaria* a weed? Both *Termitomyces* and *Xylaria* can degrade lignin. Perhaps *Pseudoxylaria* did not arrive in the termite nest after *Termitomyces*, but was there too from the beginning, only somehow termites evolved into selectively growing *Termitomyces* (see Sands 1960), whilst suppressing *Xylaria* species that entered the nest. Termites had many opportunities of collecting *Xylaria* species, as they are common in the decaying wood that termites prefer to forage on. This is reflected in that *Xylaria* species have also been found associated with non-fungus-growing termites on Martinique (Hsieh *et al.* 2010), and subterranean ant nests (Dennis 1958; 1961).

So which factors make an organism to evolve mutualistic behaviour, under what conditions does an organism become antagonistic? In some ecological niches the road of mutualism seems one without return. *Termitomyces* seems trapped in the termite-fungus mutualism, as its species never reversed to a free-living life-style (neither the termites left their symbiont, see Aanen *et al*. 2002). Ectomycorrhizal symbioses evolved independently and persisted at least sixty-six times in fungi, but also here that road is without return; the vast majority of genera has no known reversals to the saprotrophic life-style of their ancestors (Tedersoo *et al.* 2010). Contrastingly, Sachs & Simms (2006) presented ample examples where members of mutualistic clades have rebounded to a free-living or parasitic life-style. Moreover, a single organism can behave beneficially at certain times and detrimentally at other times in its life. Promphutta *et al*. (2007) provided evidence for the possibility that the same fungus first exhibits an endophytic and later, at host senescence, a saprotrophic life style. *Pseudoxylaria* is quite like the endophytes in that it applies the sit-and-wait strategy; it is latently present and invisible in active nests, but seizes the opportunity to smother the comb showing its negative side as a weed as soon as the termites are gone or no longer in control of their fungus-garden. Hughes *et al.* (2010) state that parasites should grow fast and use a hit-and-run strategy (Wulff 2008) if the host is likely to die early from another cause, but exploit slowly and sustainably if you can

control its demise yourself. The fact that the termite nest is long-lived may thus explain why *Pseudoxylaria* stays low-profile in active nests.

To come back to the question why *Termitomyces* has become a symbiont and *Pseudoxylaria* not, we may look at their intrinsic traits. Two traits could cause termites to prefer one over the other. First, Xylariaceae are renowned for their secondary metabolites (Whalley & Edwards 1995; Schneider *et al.* 1996; Stadler *et al.* 2004), some of which might repel or even be toxic for the termites. Second, Xylariaceae tend to make sclerotia and stromata (Rogers 2000), tough structures which are probably not nutritious for the termites. Lyophyllaceae to which *Termitomyces* belongs, on the other hand, are often not toxic, tend to make white and soft tissue, and several species are even highly valued for human consumption. Perhaps, as in human agriculture, the most docile and versatile/mendable organisms are most likely to be seized by another species. At present, with only soft unsclerotised mycelium *Termitomyces* does fit better in the picture of a cultivar than *Pseudoxylaria* that typically grows into tough sclerotised structures. Though, the latter trait can also have evolved as defence against the termites' aim to eradicate *Pseudoxylaria* (Chapter 4).

Many questions mentioned by Sachs & Simms (2006) on shifting between mutualism and parasitism are still up to date, and addressing those will shed more light on the traits and circumstances that determine the role an organism plays in a symbiosis.

Symbiont role versus host specificity

What means the role of symbiont for the level of host-specificity (and the other way around)? What is the relative importance of coevolution for the productivity of the mutualism? Why do fungus-growing termite species often harbour only one, or a few very closely related types of *Termitomyces*, while the latter may occur in completely different host genera (Aanen *et al.* 2002)? Why do certain *Pseudoxylaria* OTU's occur almost exclusively with *Microtermes* species, while others occur in *Odontotermes* and *Macrotermes* alike

(Figure 2-1), noting that the latter two termite genera do no share their fungal cultivar (Figure 3-1; Aanen *et al.* 2002)?

There are many examples of gene-for-gene evolution in parasitic (pathogenic) relationships between species (Bird *et al.* 2009; Champouret *et al.* 2009; Dodds & Thrall 2009), while mutualistic symbionts often exhibit more moderate host specificity (Aanen *et al.* 2002; den Bakker *et al.* 2004; Mikheyev *et al.* 2007). However, the mycoparasite *Escovopsis* has turned out to be less strictly coevolved with fungus-growing ants than previously thought (Taerum *et al.* 2007; Mueller *et al.* 2008). Also *Pseudoxylaria*, though not parasitizing on *Termitomyces* but benefitting from the comb-substrate, seems without high levels of host specificity (Chapter 2). Hence, the level of antagonism is not an ultimate proxy for the level of specificity between species, rendering this issue a fertile ground for future research.

Conclusions

– Fungus-growing termites are so successful in maintaining a *Termitomyces* monoculture, that for human agricultural interests we may further study how exactly termites manage their fungiculture.

– *Pseudoxylaria* species occur specifically in fungus-growing termite nests, where they are suppressed by termites while awaiting an opportunity to overgrow the fungus garden.

– While working with termites may provoke tunnel vision, previous sections show that a broad variety of evolutionary ecological questions need to be addressed in order to explain the evolution and ecology in fungus-growing termite nests.

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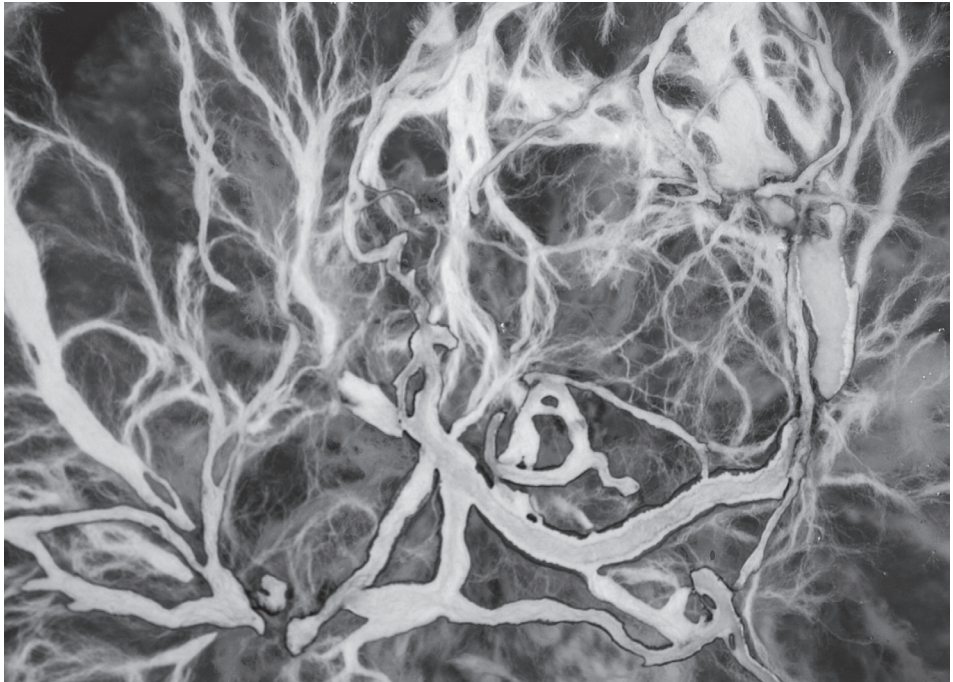
Summary

Organisms living in symbiosis fascinate us with their adaptations to live in extreme proximity to, or even inside, a partner that may be from a completely different Class, Phylum or Kingdom. Combinations of organisms that live in mutualistic symbiosis seem very exceptional, but when studying any organism more closely one may find involvement in mutualistic symbiosis to be the rule rather than an exception. For example, most of the animals have microorganisms in their guts that help digestion, and many plants have fungi around their roots that aid in uptake of nutrients from the soil. Having complementary traits and reciprocally benefitting each other, cooperating organisms may evolve into extremely successful species.

Chapter 1 introduces the topic of this thesis: fungus-growing termites. Fungus-growing termites play a dominant role as ecosystem engineers in sub-Saharan Africa and South Asia. They change soil properties by their building and foraging activities, and are major players in decomposition of wood and dead vegetation. Though they are often regarded as a pest, termites can be very useful for people. Besides eating the termites and mushrooms that emerge from the termite mound, people use termite soil-engineering to improve the fertility of agricultural fields.

The termite and fungus live in obligate mutualistic symbiosis. Termites (Blattodea: Termitidae, subfamily Macrotermitinae) provide the fungus *Termitomyces* (Basidiomycota: Agaricales: Lyophyllaceae) with fragmented dead plant material and create a controlled environment perfect for the fungus, whereas *Termitomyces* decomposes the low-quality matter into a nutritious food source and produces mushroom primordia both of which are eaten by the termites.

The symbiosis exists in a world where other organisms are awaiting their chance to exploit the richness of the termite nests. Hence, one could expect to find other organisms in the nest, next to termites and *Termitomyces*. There is at least one fungus associated with fungus-growing termites that emerges very prominently after termites are no longer active: species of *Xylaria* (Ascomycota: Xylariales: Xylariaceae, subgenus *Pseudoxylaria*) are frequently

overgrowing the fungus gardens of dead termite nests. What is the status of *Pseudoxylaria* in the fungus-growing termite symbiosis, does it play a role? How are the fungus-growing termite gardens kept free of weeds, parasites and pathogens? These questions form the foundation of this thesis on the ecology and evolution of microorganisms associated with fungus-growing termites, with particular focus on the role and interactions with associated *Pseudoxylaria*.

Chapter 2 investigates the specificity of *Pseudoxylaria* for fungus-growing termites. I hypothesise that specificity or selectivity for fungus-growing termites would mean that *Pseudoxylaria* is not present coincidentally as opportunist, but truly associated with fungus-growing termite symbiosis. Hundred and eight South-African fungus-growing termite nests were sampled for *Pseudoxylaria*, and it was found in most of the nests. Partial rDNA sequences of the obtained isolates were compared with those of *Xylaria* from the environment and isolates from other parts of the world. I found 16 different molecular types ('species') of *Pseudoxylaria*. They formed a separate group, showing that *Pseudoxylaria* specifically occurs in fungus-growing termite nests indeed. No specificity for the termite genus or species was found, implying that *Pseudoxylaria* may have specialised on the fungus garden substrate, rather than on the termite host or the mutualistic fungus *Termitomyces*.

Chapter 3 focuses on the role of *Pseudoxylaria* in the fungus-growing termite nest. *Pseudoxylaria* is inconspicuous in healthy termite nests and usually only occurs when termites are no longer present in the nest, or when pieces of fungus garden are incubated without termites in the lab. Therefore, it seems to be suppressed and an unwelcome nest inhabitant. I postulate that *Pseudoxylaria* is a benign stowaway that practices a sit-and-wait strategy to survive in the termite nest. First, *Pseudoxylaria* and *Termitomyces* were grown independently on different carbon sources, to test if they have a complementary diet preference, degrading complementary substrate components as had been suggested previously. The carbon source use of both fungi overlapped, implying that *Pseudoxylaria* is not a beneficial or benign symbiont. Second, the role of *Pseudoxylaria* in termite nests was inferred from interactions between mycelia of *Pseudoxylaria*, *Termitomyces*, and their free-living relatives.

Both fungi were grown on the same plate, and also combinations with each other's free-living relatives were tested. This revealed that *Pseudoxylaria* is not parasitizing *Termitomyces*. Furthermore, *Pseudoxylaria* grew relatively less than its free-living relatives when combined with *Termitomyces*. This result suggests that the symbiotic lifestyle adopted by *Pseudoxylaria* went together with adaptations that changed the interaction between both fungi, consistent with *Pseudoxylaria* being a stowaway.

Chapter 4 tests the hypothesis that termite workers play a crucial role in maintaining the fungus garden hygiene. The occurrence of microorganisms other than *Termitomyces* was monitored for pieces of fungus garden that were incubated with, without, or temporarily without termite workers. The effect that workers had on the fungus-comb hygiene, as well as observations on worker cleaning behaviour and their response to mycelium tissue of *Pseudoxylaria* and *Termitomyces*, show that termites play an important role in maintaining the fungus-garden hygiene indeed.

Chapter 5 explores the potential of Actinobacteria for a mutualistic role as defensive symbiont against *Pseudoxylaria* in the fungus-growing termite nest. Actinobacteria play a mutualistic role as defensive symbionts in many biological systems. It was unclear by which mechanism the termites suppress *Pseudoxylaria*. Thirty fungus-growing termite colonies from two geographically distant sites were sampled for Actinobacteria. Resulting isolates were characterised based on morphology and 16S rRNA sequences. Next, the obtained Actinobacteria were tested for their antibiotic effect on both *Pseudoxylaria* and *Termitomyces*.

This chapter describes the first discovery of an assembly of Actinobacteria occurring in fungus-growing termite nests. Actinobacteria were found throughout all sampled nests and materials, and in the phylogenetic tree their 16S rRNA sequences were interspersed with those of Actinobacteria from origins other than fungus-growing termites. The bioassays showed that many Actinobacteria inhibited both the substrate competitor *Pseudoxylaria* and the termite cultivar *Termitomyces*. The lack of specificity of the Actinobacteria for fungus-growing termites, and lack of specific defence against *Pseudoxylaria*, make it unlikely that Actinobacteria play a role as defensive symbionts in fungus-growing termites.

Final CHAPTER 6 reflects on the previous chapters, focussing on underlying mechanisms. What caused fungus-growing termites to survive for thirty million years already, and what makes them so successful that they dominate semi-arid ecosystems in sub-Saharan Africa and South Asia? How are conflicts of interest between symbiotic partners resolved? How does cooperation between termites and *Termitomyces* remain stable over evolutionary time scales? The roles of termites, *Termitomyces*, *Pseudoxylaria*, and other organisms in the fungus-growing termite nest are discussed more elaborately. Also, the question to what extent certain aspects determine whether an organism behaves parasitically or mutualistically, and the question whether symbiont role affects the level of specificity between symbiotic partners, are examined. An analogy is drawn with human agriculture and directions for future research are given.

The chapter ends with main conclusions of this thesis. Fungus-growing termites are so successful in maintaining a *Termitomyces* monoculture that the means by which they accomplish this may be further studied for human agricultural interests. *Pseudoxylaria* species occur specifically in fungus-growing termite nests, where they are suppressed by termites while awaiting an opportunity to overgrow the fungus garden.

Samenvatting

Organismen die in symbiose leven fascineren ons met hun aanpassingen om te leven in extreme nabijheid van, of zelfs binnen in, hun partner die tot een heel andere Klasse, Fylum of Rijk van organismen kan behoren. Combinaties van organismen die in mutualistische symbiose leven lijken vaak heel bijzonder, maar wanneer men willekeurige organismen beter bestudeert blijkt dat betrokkenheid bij een mutualistische symbiose eerder regel is dan uitzondering. De meeste dieren hebben bijvoorbeeld micro-organismen in hun darmstelsel die helpen bij de vertering, en bij veel planten groeien schimmels in en rond de wortels die helpen bij de opname van voedingsstoffen uit de bodem. Met eigenschappen die elkaar aanvullen en door elkaar wederzijds te begunstigen, kunnen samenwerkende organismen tot zeer succesvolle soorten evolueren.

Hoofdstuk 1 introduceert het onderwerp van dit proefschrift: schimmelkwekende termieten. Schimmelkwekende termieten spelen een dominante rol als ecosysteemvormgevers in Afrika bezuiden de Sahara en zuidelijk Azië. Ze veranderen bodemeigenschappen door hun bouw- en forageerwerkzaamheden, en ze spelen een dominante rol bij de afbraak van hout en afgestorven vegetatie. Hoewel ze vaak als een plaag worden beschouwd, kunnen termieten ook erg bruikbaar zijn voor mensen. Naast het eten van termieten en paddestoelen die uit termietenheuvels groeien, gebruiken mensen termieten ook als bodembewerkers om de vruchtbaarheid van agrarische percelen te verbeteren.

De termiet en de schimmel leven in obligate wederzijds voordelige (mutualistische) symbiose. Termieten (Blattodea: Termitidae, onderfamilie Macrotermitinae) voorzien de schimmel *Termitomyces* (Basidiomycota: Agaricales: Lyophyllaceae) van gefragmenteerd dood plantenmateriaal en creëren een stabiele omgeving die perfect is voor de schimmel. *Termitomyces* verteert het materiaal van lage voedingskwaliteit tot voedzaam eten en vormt kiemen van paddestoelen. Zowel het halfverteerde plantenmateriaal als de schimmel worden door de termieten gegeten.

De symbiose bevindt zich in een wereld waar andere organismen op de loer liggen om de rijkdom van termietennesten te

exploiteren. Het is daarom te verwachten dat er zich naast termieten en *Termitomyces* ook andere organismen in het nest bevinden. Er is tenminste één schimmel gelieerd aan schimmelkwekende termieten die prominent tevoorschijn komt nadat termieten niet langer actief zijn: soorten behorend tot *Xylaria* (Ascomycota: Xylareales: Xylariaceae, subgenus *Pseudoxylaria*) overwoekeren vaak de schimmeltuinen van dode termietennesten. Wat is de positie van *Pseudoxylaria* in de schimmelkwekende termieten symbiose en speelt deze een rol? Hoe worden de schimmeltuinen gevrijwaard van onkruiden, parasieten en ziekten? Deze vragen vormen de basis van dit proefschrift over de ecologie en evolutie van micro-organismen gelieerd aan schimmelkwekende termieten, met speciale aandacht voor de rol van en interacties met geassocieerde *Pseudoxylaria*.

Hoofdstuk 2 onderzoekt de specificiteit van *Pseudoxylaria* voor schimmelkwekende termieten. Ik beweer dat specificiteit of selectiviteit voor schimmelkwekende termieten zou betekenen dat *Pseudoxylaria* niet toevallig als gelukszoeker in termietennesten aanwezig is, maar werkelijk gelieerd aan de schimmelkwekende termieten symbiose. Honderd-en-acht Zuid-Afrikaanse termietennesten werden bemonsterd voor *Pseudoxylaria*, en in de meeste werd *Pseudoxylaria* aangetroffen. De basenpaarvolgorden van een deel van het rDNA van de verkregen isolaten werden vergeleken met die van *Xylaria* uit de omgeving en isolaten uit andere delen van de wereld. Ik vond 16 verschillende rDNA types ('soorten') van *Pseudoxylaria*. Ze vormden een aparte groep, wat aangeeft dat *Pseudoxylaria* inderdaad specifiek voorkomt in termietennesten. Er werd geen specificiteit voor termietengeslacht of -soort gevonden, wat suggereert dat *Pseudoxylaria* zich eerder heeft gespecialiseerd op het schimmeltuinsubstraat dan op de termietengastheer of de mutualistische schimmel *Termitomyces*.

Hoofdstuk 3 richt zich op de rol van *Pseudoxylaria* in het nest van schimmelkwekende termieten. *Pseudoxylaria* is onzichtbaar in gezonde termietennesten en komt gewoonlijk alleen tevoorschijn als de termieten niet langer in het nest aanwezig zijn, of wanneer stukken schimmeltuin zonder termieten wordt gehouden in het lab. *Pseudoxylaria* lijkt dus een ongewenste nestbewoner die onderdrukt wordt. Ik stel dat *Pseudoxylaria* een onschuldige verstekeling is

die een kijk-de-kat-uit-de-boom strategie heeft om te overleven in het termietennest. Eerst zijn *Pseudoxylaria* en *Termitomyces* apart op verschillende koolstofbronnen gekweekt om te kijken of ze misschien een tegenovergestelde voedingvoorkeur hebben, dus complementaire onderdelen van het substraat verteren, zoals eerder is voorgesteld. Het koolstofgebruik van beide schimmels overlapte, wat suggereert dat *Pseudoxylaria* niet een gunstige of onschuldige symbiont is. Daarna is de rol van *Pseudoxylaria* in termieten nesten afgeleid uit interacties tussen mycelia van *Pseudoxylaria*, *Termitomyces*, en hun vrij-levende verwanten. Beide schimmels werden op dezelfde plaat gekweekt, en ook combinaties met elkaars vrij-levende verwanten werden getest. Dit wees uit dat *Pseudoxylaria* niet op *Termitomyces* parasiteert. Bovendien groeide *Pseudoxylaria* relatief gezien minder goed dan zijn vrij-levende verwanten in combinatie met *Termitomyces*. Dit resultaat suggereert dat het leven als symbiont van *Pseudoxylaria* is samengegaan met aanpassingen die de interactie tussen beide schimmels veranderden, wat strookt met de hypothese dat *Pseudoxylaria* een 'verstekeling' is.

Hoofdstuk 4 test de hypothese dat werkers van termieten een essentiële rol spelen bij de handhaving van de hygiëne in de schimmeltuin. Het vóórkomen van andere micro-organismen dan *Termitomyces* is gepeild in stukken schimmeltuin met, zonder, of tijdelijk zonder werkertermieten. Het effect van de werkers op de schimmeltuinhygiëne, evenals het waargenomen schoonmaakgedrag en de reactie van werkers op *Pseudoxylaria* en *Termitomyces* weefsel, laten zien dat termieten inderdaad een belangrijke rol spelen in het handhaven van de schimmeltuinhygiëne.

Hoofdstuk 5 onderzoekt de mogelijkheid dat actinobacteriën mutualistische symbionten zijn met afwerende werking tegen *Pseudoxylaria* in het nest van schimmelkwekende termieten. Dertig kolonies van schimmelkwekende termieten van twee geografisch ver van elkaar verwijderde locaties werden bemonsterd voor actinobacteriën. De resulterende isolaten werden getypeerd op basis van morfologie en 16S rRNA sequenties. Vervolgens werden de verkregen actinobacteriën getest op antibiotisch effect op *Pseudoxylaria* en *Termitomyces*.

Het hoofdstuk beschrijft de eerste ontdekking van een verzameling actinobacteriën die voorkomen in schimmelkwekende

termietennesten. Actinobacteriën werden gevonden in alle nesten en bemonsterde onderdelen van de nesten, en in de fylogenetische boom worden hun 16S rRNA sequenties afgewisseld met die van actinobacteriën van andere origine dan termietennesten. De test liet zien dat vele bacteriën zowel de substraat-concurrent *Pseudoxylaria* als de termieten-cultivar *Termitomyces* remden. Het ontbreken van specificiteit van de actinobacteriën voor schimmelkwekende termieten en het ontbreken van specifieke afweer tegen *Pseudoxylaria*, maken het onwaarschijnlijk dat actinobacteriën een rol spelen als afweer-symbiont in schimmelkwekende termieten nesten.

Afsluitend HOOFSTUK 6 beschouwt de voorgaande hoofdstukken, en richt zich op onderliggende mechanismen. Wat zorgde ervoor dat schimmelkwekende termieten al 30 miljoen jaar overleven, en wat maakt hen zo succesvol in gebieden met weinig regenval? Hoe zijn belangenconflicten tussen de partner-symbionten opgelost? Hoe heeft de samenwerking tussen termieten en *Termitomyces* gedurende evolutionaire tijdschalen stand gehouden? De rollen van termieten, *Termitomyces*, *Pseudoxylaria*, en andere organismen in het nest van schimmelkwekende termieten worden uitgebreider besproken. Verder wordt van een aantal aspecten onderzocht in hoeverre ze bepalen of een organisme zich mutualistisch of parasitair gedraagt, en wordt onderzocht of de rol in de symbiose ook invloed heeft op de mate van specificiteit tussen de symbionten. En vergelijking met landbouw door mensen wordt gemaakt en suggesties voor toekomstig onderzoek worden gegeven.

Het hoofdstuk sluit met de hoofdconclusies van het onderzoek. Schimmelkwekende termieten zijn zo succesvol in het kweken van een *Termitomyces* monocultuur, dat het voor menselijke doeleinden interessant kan zijn ze verder te bestuderen. *Pseudoxylaria* soorten komen specifiek in termietennesten voor, waar ze door de termieten onderdrukt worden terwijl ze wachten op een kans om de schimmeltuin te overwoekeren.

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Thanks to Mike Wingfield, Wilhelm de Beer, Bernard Slippers, and all other 'FABIans', I felt more than welcome and experienced very successful fieldwork periods in South Africa. Thank you for sharing facilities, time, energy, enthusiasm, and knowledge about fungi. All other colleagues, in Wageningen and abroad, thank you for sharing good times during meetings, coffee, and lunch breaks; discussing work, life, and everything in between.

Also family, friends, and housemates have been a great support. Thank you for all the nice times and making me put work in proper perspective. When starting the PhD I expected to learn about science. Looking back, though, it seems I learned more about myself.

Finally, I would like to thank my parents, sisters and brother for being who they are and for supporting me in whatever I do. Heit en mem, tank foar jimme stipe. Of it paad dat ik berin no rjocht & sljocht of ûnbegeanber is, jimme hawwe my altiid bystien.

Anna   Wageningen, May 2011

Curriculum Vitae

On May 19th 1982, a girl was born in Sneek and named Anna Alida Visser. De Gaastmar, a Frisian village where roads end and lakes begin, is where she grew up. She obtained an athenaeum-diploma at Bogerman in Sneek in summer 2000, after which she studied Biology at Wageningen University.

During MSc studies, unknowingly, a basis was laid for the PhD project that followed. To have a look into the kitchen of microbiology, she did a minor thesis at the laboratory of Biological Farming Systems, Wageningen University (WU), under supervision of Anne van Diepeningen and Eelco Franz. The survival of the human pathogens *E. coli* and *S. enterica* in manure-soil mixtures and their potential to infect lettuce were tested. To learn more about animal ecology, she did a major thesis at the Resource Ecology Group (WU), under supervision of Sip van Wieren and Fred de Boer. In cooperation with Maaike Renard, the use of the 'Woeste Hoeve' wildlife overpass by mammals was investigated by means of track counts. To finish, an internship was done in Kenya, under supervision of Karlé Sykora from Nature Conservation & Plant Ecology (WU) and Rosemary Groom who was a PhD-candidate at that time at the Mamal Research Unit, Bristol University. The vegetation survey on Maasai rangeland taught her several fieldwork skills. The MSc degree was obtained in November 2005.

In summer 2006, Anna started working on a PhD project at the Laboratory of Genetics, the results of which can be read in this thesis. Further, in the years 2009-2010 she was member of the PE&RC PhD student council, where she helped organising the PE&RC-day of 2009.

Publications

Franz E, **Visser AA**, van Diepeningen AD, Klerks MM, Termorshuizen AJ, van Bruggen AHC, 2007. Quantification of contamination of lettuce by GFP-expressing *Escherichia coli* O157 : H7 and *Salmonella enterica* serovar Typhimurium. *Food Microbiology* **24**: 106-112.

Renard M, **Visser AA**, de Boer WF, Wieren SE, 2008. The use of the 'Woeste Hoeve' wildlife overpass by mammals. *Lutra* **51**: 5-16.

Visser AA, Ros VID, de Beer ZW, Debets AJM, Hartog E, Kuyper TW, Læssøe T, Slippers B, Aanen DK, 2009. Levels of specificity of *Xylaria* species associated with fungus-growing termites: a phylogenetic approach. *Molecular Ecology* **18**: 553-567.

PE&RC PhD Education Certificate
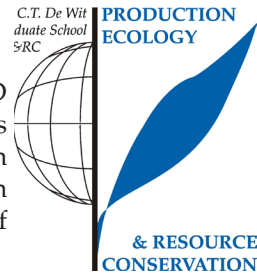
With the educational activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (6 ECTS)
– Weeds, diseases and the evolution of sustainable agriculture in fungus-growing termites

Post-graduate courses (10.5 ECTS)
– Molecular phylogenies: reconstruction and interpretation; EPS (2006)
– Soil ecology: crossing the frontier between below- and above-ground; FE/PE&RC/SENSE (2007)
– The biology of social insects; Centre for Social Evolution (CSE), University of Copenhagen (2008)
– Advanced statistics; PE&RC (2008)
– Land dynamics: getting to the bottom of Mount Kenya; CERES/MG3S/PE&RC/SENSE (2009)

Laboratory training and working visits (1.2 ECTS)
– Fungal taxonomy, identification; Forestry and Agricultural Biotechnology Institute (FABI), Microscopy, specimen preparation; FABI, University of Pretoria (2008)

Invited review of (unpublished) journal manuscripts (2 ECTS)
– *Molecular Phylogenetics and Evolution*: phylogenetic status of *Xylaria* subgenus *Pseudoxylaria* among taxa of the subfamily Xylarioideae (2009)
– *European Journal of Plant Pathology*: detection of Botryosphaeriaceae within grapevine woody tissues by nested PCR (2010)

Competence strengthening / skills courses (4.8 ECTS)
– Information literacy; Wageningen UR Library (2006)
– PhD Competence assessment; WGS (2006)
– Scientific writing; WGS/The Language Centre (2007)
– Project- and time management; WGS/Valley Consult (2009)
– Advanced course guide to scientific artwork; Wageningen UR Library (2009)
– Career assessment; WGS (2009)

PE&RC Annual meetings, seminars and the PE&RC weekend (3.3 ECTS)
– Current themes in ecology (2006 and 2007)
– PE&RC Day (2007, 2008 and 2009)
– PE&RC Weekend (2008 and 2009)

Discussion groups / local seminars / other scientific meetings (9.3 ECTS)
– Experimental Evolution Discussion Group; Wageningen (2006-2011)
– Symposium Bioscience Showtime; Netherlands Institute for Biology (NIBI); Lunteren, the Netherlands (2007)
– Autumn meeting of Dutch Association of Microbiology section Mycology (NVvM), CBS-KNAW Fungal Biodiversity Centre; Utrecht, the Netherlands (2007)
– Ecological Theory and Application (previously Forest and Conservation Ecology) Discussion Group; Wageningen (2007-2010)
– Seminar Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria; South Africa (2008); oral presentation

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- Autumn meeting of Dutch Association of Microbiology section Mycology (NVvM), CBS-KNAW Fungal Biodiversity Centre; Utrecht, the Netherlands (2008); oral presentation
- Future for nature day; Burgers Zoo; Arnhem, the Netherlands (2009)
- Symposium 'Floradag, thema Ascomyceten en Myxomcyeten'; Netherlands Mycological Society (NMV); Leiden, the Netherlands (2009); oral presentation
- Wageningen Evolution and Ecology Seminars (WEES) (2009-2011)
- Seminar series Interdisciplinary Research; Technology and Agrarian Development (TAD) Group, Wageningen University (2010)

International symposia, workshops and conferences (10.5 ECTS)
- 11th Congress European Society for Evolutionary (ESEB); Uppsala, Sweden (2007); poster presentation
- International Congress of Entomology (ICE); Durban, South Africa (2008); oral presentation
- Annual meeting of Netherlands Ecological Research Network (NERN); Lunteren, the Netherlands (2008); oral presentation
- 15th Annual European Meeting of PhD Students in Evolutionary Biology (EMPSEB); Schoorl, the Netherlands (2009); oral presentation

Lecturing / supervision of practical's / tutorials (9.3 ECTS)
- Capita Selecta Genetics, teaching BSc student molecular techniques and phylogenetic analysis (2007)
- Molecular and Evolutionary Ecology, supervision student group (2008 and 2009)
- Molecular and Evolutionary Ecology, seminar (2010)