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Dating the fungus-growing termites' mutualism shows a mixture between ancient codiversification and recent symbiont dispersal across divergent hosts

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Abstract

The mutualistic symbiosis between fungus-growing termites and *Termitomyces* fungi originated in Africa and shows a moderate degree of interaction specificity. Here we estimate the age of the mutualism and test the hypothesis that the major splits have occurred simultaneously in the host and in the symbiont. We present a scenario where fungus-growing termites originated in the African rainforest just before the expansion of the savanna, about 31 Ma (19–49 Ma). Whereas rough age correspondence is observed for the four main clades of host and symbiont, the analysis reveals several recent events of host switching followed by dispersal of the symbiont throughout large areas and throughout different host genera. The most spectacular of these is a group of closely related fungi (the maximum age of which is estimated to be 2.4 Ma), shared between the divergent genera *Microtermes*, *Ancistrotermes*, *Acanthotermes* and *Synacanthotermes* (which diverged at least 16.7 Ma), and found throughout the African continent and on Madagascar. The lack of geographical differentiation of fungal symbionts shows that continuous exchange has occurred between regions and across host species.

Keywords: African rainforest, age estimate, co-evolution, fungus-growing termites, mutualism origin, *Termitomyces*

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Introduction

Fungus-growing termites (subfamily Macrotermitinae) have developed a sophisticated mutualistic symbiosis with a fungus (genus *Termitomyces*), which they cultivate on combs constructed from faecal material within their nests (Wood & Thomas 1989; Darlington 1994). This mutualistic symbiosis is obligate for both partners: the termites provide a constant, highly regulated growth environment for their fungal symbionts, while the fungi provide food for the termites. Entering the symbiosis has allowed the fungi to overcome highly unfavourable ecological conditions, and the termites to exploit complex plant substrates. As in other symbioses, complex patterns of interactions among groups of multiple species are observed (e.g. leaf-cutting ants: Mikheyev *et al.* 2007; Dentinger *et al.* 2009; mycorrhiza: Brundrett 2002; Hoeksema 2010). Fungus-growing termites show moderate specificity with their symbionts: monophyletic groups of the termites (i.e. usually either a single genus or several genera) are associated with monophyletic groups of fungi (Aanen *et al.* 2002; Rouland-Lefevre *et al.* 2002).

In most species of fungus-growing termites, a new symbiont is acquired when the first foraging workers collect *Termitomyces* sexual spores from the environment, and these spores will become the inoculum for the fungus garden of the new colony. This transmission mode is referred to as horizontal symbiont transmission (reviewed in Korb & Aanen 2003). Only two cases of

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vertical transmission are known, i.e., where *Termitomyces* vegetative spores are transported in the gut of alates, from a parental colony and used to inoculate the fungus comb of the newly founded colonies. Phylogenetic reconstructions indicate that this transmission mode has originated independently twice, once in the ancestor of the genus *Microtermes* and once in the species *Macrotermes bellicosus* (Grassé & Noirot 1955; Johnson 1981; Johnson *et al.* 1981; Nobre et al. 2011). Several studies have shown that transmission mode is not a strong predictor for the levels of host–symbiont specificity observed, and other factors need to be considered to explain the observed specificity patterns between fungus-growing termites and their symbionts (e.g. Aanen *et al.* 2002; Taprab *et al.* 2002; Nobre *et al.* 2010, 2011; Osiemo *et al.* 2010).

Fungus-growing termites are restricted to the Old-World tropics (tropical Africa and parts of Arabia and Indomalaya). Phylogenetic reconstructions indicate that within the termites, the transition to fungus cultivation has occurred only once, with no reversions to free-living states (Aanen *et al.* 2002), at least 30 Ma (Brandl *et al.* 2007). This transition probably occurred in the African rainforest, and the main radiation leading to the extant genera probably took place in this habitat (Aanen & Eggleton 2005). The main African rainforest block is centred in the Congo Basin and extends to the Atlantic ocean in the west (Plana 2004), so that west-central Africa is likely to be the central ancestral area of fungus-growing termites. Furthermore, the highest generic richness of fungus-growing termites is found in the tropical forests of west-central Africa, where all the described genera are found (Eggleton *et al.* 1994; Kambhampati & Eggleton 2000; Aanen *et al.* 2002). For the termites, there is already support for this pattern of 'out-of-Africa' migrations: into Asia, with only four termite genera occurring (excluding the Asiatic genus *Hypotermes* which is derived from *Odontotermes*; Aanen *et al.* 2002) and into Madagascar, with only the genus *Microtermes* being present (Nobre *et al.* 2010), whereas all 10 genera are found in Africa. For *Termitomyces*, in contrast, the pattern is less clear as more intercontinental migrations have occurred than for their termite hosts (Aanen *et al.* 2002; Taprab *et al.* 2002; Nobre *et al.* 2010). The dynamics within Africa remain unknown for both symbiotic partners.

In Africa, the Oligocene (34–23 Ma) was characterized by widespread rainforests throughout much of the equatorial region and probably extending from coast to coast (Plana 2004). Afterwards, Africa became more arid and the savanna habitats expanded to an actual area much larger than rainforest and by the late Miocene (12–5 Ma), rainforest were limited to small patches (Lancaster 1984; Plana 2004). At present, savanna ecosystems occupy a fifth of the global land surface and cover some 65% of the land surface of sub-Saharan Africa (Scholes & Archer 1997; Sankaran *et al.* 2005). Most extant fungus-growing termite genera can be found in savanna habitats, probably due to several repeated colonizations by different genera (Aanen & Eggleton 2005). According to Eggleton *et al.* (1994), the severe climatic fluctuations during the late Miocene generated several pulses of speciation in termites.

Recently, the date of origin of taxa of fungus-growing termites has been estimated by Brandl *et al.* (2007) and Nobre *et al.* (2010). However, in both cases, the sampled taxa were biased towards the taxonomical groups being studied, the genera *Macrotermes* and *Microtermes*, respectively. No corresponding estimates for the age of the fungal symbionts have been made yet. In this study, we estimate the age of the origin of *Termitomyces*. Furthermore, we update the estimate of the age of fungus-growing termites, using new sequence data, a comprehensive sampling covering the main clades and more accurate calibration based on newly discovered fossils (Duringer *et al.* 2007). Specifically, we test the hypothesis that the main associated clades of host and symbiont have the same age. Alternatively, recent host–symbiont switches, possibly followed by dispersion of the fungal symbiont across divergent hosts, will give different age estimates of associated partner clades.

Material and methods

Taxon sampling

Samples were collected in Ivory Coast at two main localities: the reserve of Lamto located at 160 km northwestern of Abidjan (a natural reserve of 2700 ha situated in the transition zone between semi deciduous forest and Guinean savanna) and in tree forests islands of Ndouci, a city located at 125 km southeastern of Abidjan in the semi deciduous forest zone. At Ndouci, known to be a site of intense and permanent activity of termites, with mainly four sympatric fungus-growing termite genera present—*Ancistrotermes*, *Microtermes*, *Odontotermes* and *Pseudacanthotermes* (Josens 1972; Konaté 1998)—termites and nodules (modified unripe mushrooms) were sampled while excavating 12 mounds in 12 different forest islands. Several species of termites can (and were) recovered from a single mound, generally belonging to genera such as *Ancistrotermes, Microtermes* and *Pseudacanthotermes*. At Lamto reserve, three additional mounds were sampled: two colonies of *Ancistrotermes*, two colonies of *Microtermes*, one colony of *Odontotermes*, four colonies of *Pseudacanthotermes* and three colonies of *Macrotermes*. Altogether, termites and *Termitomyces* nodules were collected from 47 colonies (10 *Ancistrotermes*, 4 *Macrotermes*, 12 *Microtermes*, 11

Odontotermes, 9 *Pseudacanthotermes* and 1 *Acanthotermes*), by breaking down the mound until the comb was exposed. Termites were hand-sorted *in situ* at the genus level, and around 10 individuals per caste (workers and soldiers) were collected. Nodules on combs were directly collected using forceps. All samples were stored in 100% alcohol and kept at 4° C. *Termitomyces* basidiocarps collected in the study area were also analysed. These basidiocarps and their termite host were collected by disrupting the mounds until the comb on which the basidiocarp grew could be reached.

Data collection

Termite workers were dried on filter paper previous to DNA extraction. DNA was extracted separately from individual heads and abdomens using the Chelex method. The DNA extracted from the head capsules was used for the termites' analyses. Fungal DNA was obtained from fruiting bodies, nodules or from the termite abdomina. All extraction products were stored at −20 °C.

For the *Termitomyces* symbiont, part of the nuclear ribosomal region including the first internal transcribed spacer (ITS1), the 5.8S RNA gene and the second internal transcribed spacer (ITS2) was amplified using a standard PCR and the *Termitomyces*-specific primer ITS1FT in combination with an universal primer ITS4 as described by Aanen *et al.* (2007). The termite mitochondrial gene cytochrome oxidase subunit I (COI) was amplified using a standard PCR and the primer pair TL1862 and TH2877 (Aanen *et al.* 2002). All PCR products were then purified using the GenElute PCR clean-up kit (Sigma) and directly sequenced with the amplification primers. Sequencing was performed by MWG Biotech. Throughout this manuscript, the symbiont is identified using the name (or code) of the host with which it was associated.

From the samples collected, we obtained 40 termites' sequences and 41 *Termitomyces'* sequences. We included a selection of GenBank sequences that were chosen in such a way as to include at least one sample per clade of earlier Macrotermitinae or *Termitomyces* phylogenies and span different geographical areas (Aanen *et al.* 2002; Nobre *et al.* 2010; Osiemo *et al.* 2010) (Supporting information). In this way, a total of 80 COI termite haplotypes were straightforward aligned, as no insertions/deletions had to be inferred and 47 fungal haplotypes were aligned in MAFFT (Katoh *et al.* 2005) under the option (L-INS-I: iterative refinement method incorporating local pairwise alignments; gap opening penalty: 1.5 and gap extension penalty 0.14; 1PAM/κ = 2 scoring matrix for nucleotide sequences). Identical GenBank sequences from different geographical regions were added to the tree, so that the 80 termites' haplotypes represent 102 sequences and the 47 *Termitomyces* haplotypes correspond to 116 sequences.

Phylogenetic analysis

Bayesian inference was conducted using MrBayes version 3.0 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck 2003). For the ITS fungal sequences, we used the Hasegawa–Kishino–Yano model (HKY + G), and for the COI region, we used the general time-reversible (GTR) model with site-specific substitution rates (SSR, estimated separately for the three nucleotide positions) as it was previously found to be the best fit model of sequence evolution (Aanen *et al.* 2002). The default settings of MrBayes were used and with MCMC runs being repeated twice as a safeguard against spurious results. The first 10^3 trees were discarded as burn-in, and the remaining trees were used to calculate a majority rule consensus tree. Stationarity was confirmed by analysis of the log likelihoods and the consistency between runs.

Divergence dating

Based on the estimated phylogenies, a selection of representatives of the main host and symbionts' clades was made for a more in-depth analysis using additional sequence data. For the symbionts, we used the independent estimates for the age of the *Agaricus* node by Geml *et al.* (2004). Therefore, we added to the analysis one sample of *A. bisporus* and one of *A. campestris* (species belonging to two divergent clades; Geml *et al.* 2004). Data on their outgroup, *Chlorophyllum molybdites*, were obtained from the work by Johnson & Vilgalys (1998).

For 15 selected symbiont samples and the two *Agaricus* taxa, in addition to the nuclear ribosomal ITS region (ITS1, 5.8S RNA gene and ITS2), two sequences were determined: (i) 532 bp of the 25S nuclear RNA gene (nLSU-rDNA) by using the primers 25S4R and ITS4R (Aanen *et al.* 2002) and (ii) 602 bp of the 12S mitochondrial RNA gene (mtSSU-rDNA) by using the universal primers MS1 and MS2 (White *et al.* 1990), but for two samples, the specific primers ssufw105 and ssurev475 (Aanen *et al.* 2002) were used.

We reconstructed the phylogeny of the 15 selected *Termitomyces* taxa, together with the two *Agaricus* sequences and their outgroup. After alignment of data in MAFFT (as described above), the optimal model of evolution was selected, separately for each of the three regions, using MrMODELTEST v. 2.2 (Posada & Crandall 1998).

The divergence of *Agaricus* from *Chlorophyllum* was estimated using calibrations based on other molecular clock studies on fungi and fossil data, as 15.5 ± 3.8,

32.6 ± 8.1 and 73.3 ± 18.1 Ma (Geml *et al.* 2004), respectively. Although the authors refer preferably to the most ancient divergence time, and although this date has been used by Mikheyev *et al.* (2010) to date the age of the attine ant symbionts, we decided to perform three independent analyses, each using one of the three possible divergence times. Similar to the approach taken by Mikheyev *et al.* (2010), the node representing the divergence of *Agaricus* from *Chlorophyllum* was given a prior with a normal age distribution (mean = 16, SD = 2; mean = 33, SD = 4 and mean = 73, SD = 9).

For 19 selected host taxa, in addition to the 931 bp of the mitochondrial gene COI, two sequences were determined: (i) 684 bp of the mitochondrial cytochrome oxidase subunit II gene (COII) using AtLeu and B-tLys as in Brandl *et al.*(2007) and (ii) 294 bp of part of the nuclear ribosomal internal transcribe spacer (ITS2) region using the primers ITS2 and ITS2F as in Jenkins *et al.* (2001). As outgroups, three samples belonging to the *Amitermes* group—belonging to the subfamily Termitinae—were used and their sequences determined as described earlier. Separately for each gene, the optimal model of evolution was selected using MrMODELTEST v. 2.2 (Posada & Crandall 1998).

To determine divergence dates, we used the Bayesian relaxed-clock uncorrelated exponential approach implemented in BEAST, version 1.5.4 (Drummond & Rambaut 2007). For the host, the *Odontotermes* node was constrained to a minimum age of 7 Ma following a lognormal distribution (lognormal mean = 1.9, lognormal SD = 1.5, zero offset = 7) according to the dating of the fossilized fungus comb reported by Duringer *et al.* (2007). A second constrained node was used simultaneously, with a minimum age of 3.4 Ma (lognormal mean = 1.2, lognormal SD = 1, zero offset = 3.4) and corresponding to the age of the ancestor of *Macrotermes jeanneli* according to Darlington (2005).

Both for the host and for the three independent symbiont analyses, the data were constrained to the clades obtained in MrBayes, and three Markov chain Monte Carlo searches were run for 10 000 000 generations each. Convergence was assessed by using the log likelihood distributions of individual chains in Tracer v1.4 (Drummond & Rambaut 2007). Typically, the first 10 % of the trees were discarded as burn-in, prior to results being pooled in LogCombiner v1.5.4 and the trees being visualized. The topologies of the phylogenies obtained from BEAST were similar to but not identical with the MrBayes phylogenies, and the main difference was the position of the genera *Microtermes* and of the symbiont of *Pseudacanthotermes*: the first has a basal position in BEAST and not in MrBayes and the second is basal in the analysis with MrBayes and not with BEAST (see Supporting information).

Results

Phylogenetic analysis

The reconstructed phylogeny of fungus-growing termites based on COI was congruent with previous analyses (Fig. 1, left), with all genera being monophyletic, with the known exception of *Odontotermes*, which includes the exclusively Asian genus *Hypotermes* (Aanen *et al.* 2002). Apart from the distinct geographical clade formed by the Malagasy *Microtermes* samples (Nobre *et al.* 2010), and the Asiatic clades within each of the four genera *Odontotermes*, *Macrotermes*, *Ancistrotermes* and *Microtermes*, no other obvious geographical pattern was inferred.

On the fungal symbiont side, the newly obtained *Termitomyces* samples were analysed together with available ITS sequences from Africa (Benin, Gabon, Kenya, Madagascar, Senegal and South Africa) and Asia. Several well-supported clades can be distinguished (Fig. 1, right), e.g., the clade comprising all symbionts of *Macrotermes* and the clade comprising all symbionts associated with *Odontotermes* and *Protermes*. The majority of fungi associated with *Microtermes* form a clade together with most symbionts associated with *Ancistrotermes*, *Synacanthotermes* and *Allodontotermes*, as well as with two samples associated with *Acanthotermes*. A further clade comprises the majority of the *Pseudacanthotermes* samples.

Divergence dating

The origin of fungus-growing termites was estimated to be at 31 Ma (credibility interval 16.7–48.8) using the two fossil calibration points available (*Odontotermes*, Duringer *et al.* 2007 and *M. jeanneli*, Darlington 2005). The independent estimates for the *Termitomyces* symbiont (Fig. 2) resulted in a large credibility interval in all calibrations used, but in particular for the calibration based on the 73-Ma divergence date of *Agaricus*. The calibration at 33 Ma dates the origin of *Termitomyces* at 49 Ma (credibility interval 23.5–79.0). This estimate overlaps with the credibility interval estimated for the origin of the fungus-growing termites. In the absence of an independent criterion to select the different estimates, we discuss this age estimate to illustrate the time dynamics of the host–symbiont relationship, but we mention also the estimates obtained with the other calibration ages.

The credibility intervals for the age of the splits into the extant termite genera are large. The support for the split between the clade comprising *Pseudacanthotermes* and *Acanthotermes* and the clade comprising all the other genera (Fig. 1) is low. Indeed, the BEAST analysis

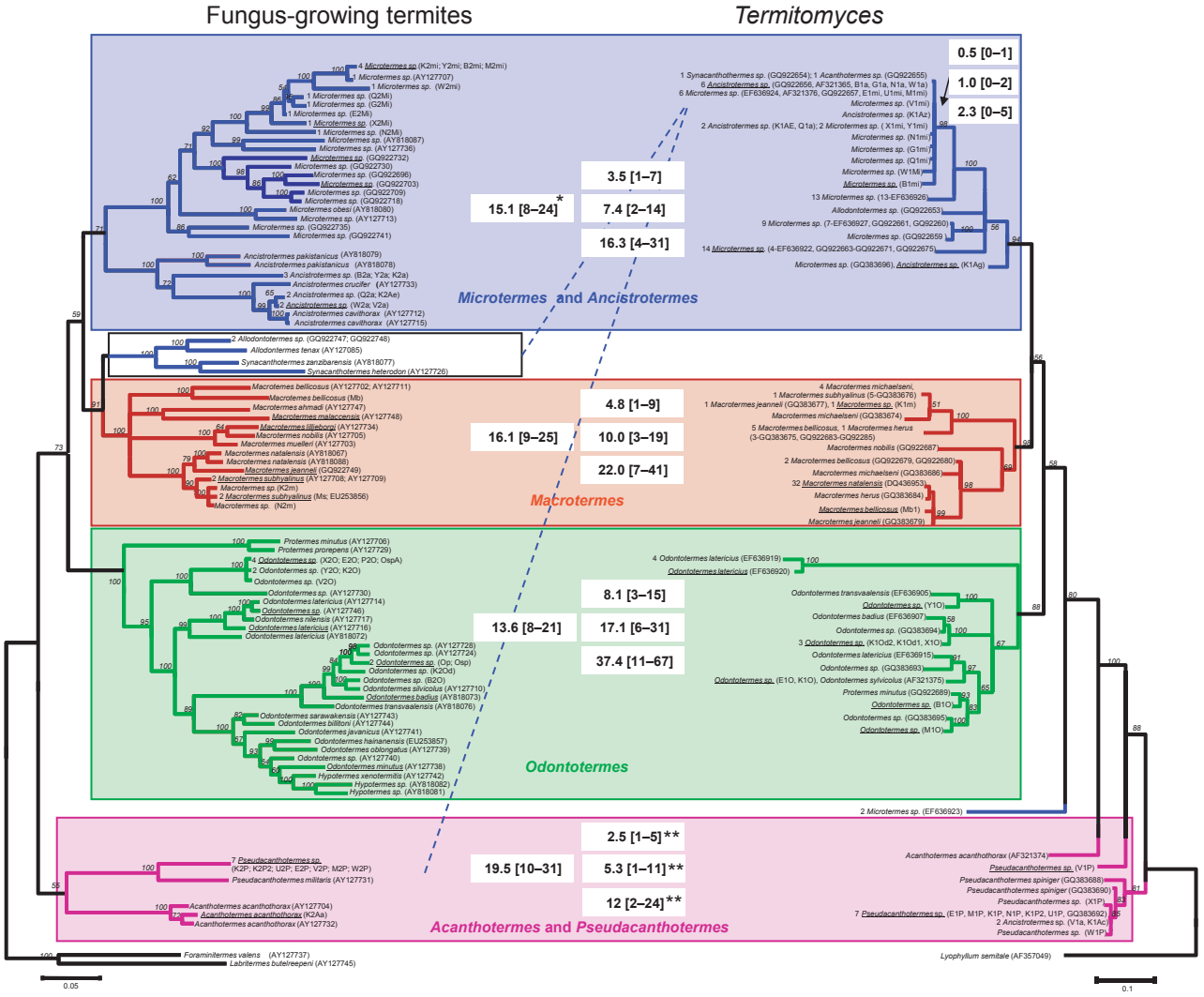

Fig. 1 Reconstructed phylogenies of fungus-growing termites (left) and their *Termitomyces* symbionts (right) included in this study (a combination of newly collected sequences and a representative selection of sequences available in GenBank). The phylogeny corresponds to the majority rule consensus tree of trees sampled in a Bayesian analysis. Two Termitidae species were used as outgroups to root the termite phylogeny and the free-living fungus *Lyophyllum semitale* as outgroup to root the *Termitomyces* phylogeny. The numbers above the branches refer to the Bayesian posterior probability of the nodes (more than 50%) derived from 19500 Markov chain Monte Carlo-sampled trees. The underlined samples were used in a separate divergence dating analysis, using two additional loci for both partners. The dates given correspond to the mean and credibility intervals obtained for the phylogenies reconstructed using BEAST (see Results and Supporting information for further details on the phylogenies). For the symbiont, the estimates resulting from the three calibration methods are presented: from top down, 16, 33 and 73 Ma as the divergence date of the genera *Agaricus* and *Chlorophyllum* (Geml *et al.* 2004). Attention is called for the widespread *Termitomyces* clade shared between the divergent host genera *Synacanthothermes*, *Acanthotermes*, *Ancistrotermes* and *Microtermes*. *Only one *Ancistrotermes* sample was used for dating the phylogeny, and in the BEAST reconstruction that sample was basal to *Odontotermes*, resulting in a somewhat older age estimate: 20.5 (13.8, 38.9). **The estimated age is based on the BEAST analysis, in which the included symbionts of *Pseudacanthotermes* formed a younger monophyletic group. For further details see Supporting information.

(Supporting information) indicates an initial split between a clade comprising *Microtermes* and a clade comprising all the other genera. However, irrespectively of the exact basal phylogenetic relationships, the initial radiation into the genera is probably to have occurred shortly after the origin of fungus-growing termites from nonfungus growers, as the age estimates for the deepest nodes largely overlap with the estimates for the origin of fungus-growing termites (Fig. 1).

The credibility intervals of the split into the main clades for the symbiotic partners are always overlapping, suggesting that these splits occurred simultaneously.

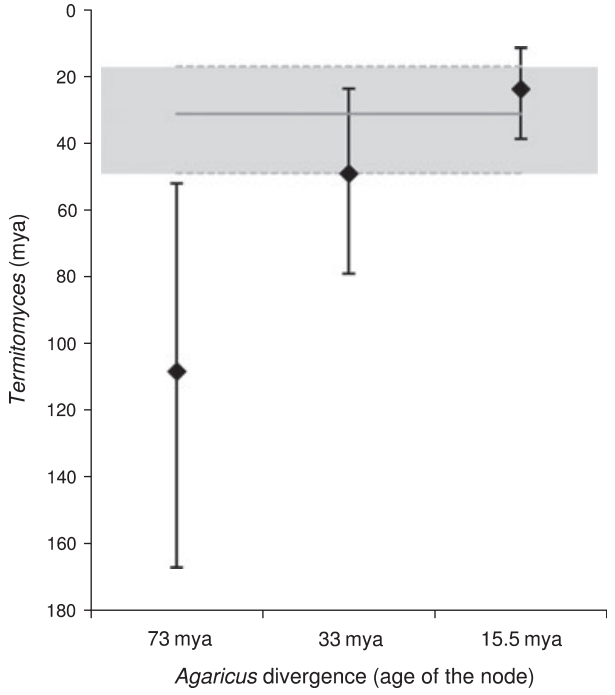

Fig. 2 Comparative dating of *Termitomyces* calibrated using three different ages for the *Agaricus* divergence according to Geml *et al.* (2004). The grey box corresponds to the credibility interval for Macrotermitinae.

However, some exceptions exist. For example, the widespread *Termitomyces* clade that is shared between the divergent host genera *Synacanthothermes*, *Acanthotermes*, *Ancistrotermes* and *Microtermes* (Fig. 1), and between regions—Ivory Coast, Senegal, Madagascar and South Africa—is of recent origin, 1.0 Ma (0.1–2.4). The most recent common ancestor of its hosts coincides with the MRCA of all fungus-growing termites, dated at 31.0 Ma (16.7–48.8).

Discussion

Phylogenetic analysis and divergence dating

Dating phylogenies still remains uncertain, with estimated divergence times varying with the DNA region(s), calibration methods and the fossil calibration point(s) used, as well as the number of taxa sampled and the accuracy of the recovered phylogeny (e.g. Magallón & Sanderson 2005; Britton *et al.* 2007; Ho & Phillips 2009, Battistuzzi *et al.* 2010; Skinner 2010). Therefore, the obtained divergence dates should be carefully interpreted as indicators of a time frame and not as absolute ages.

Based on a phylogenetic analysis of a comprehensive sample of species of fungus-growing termites, using two calibration points, we estimated the origin of the fungus-growing termites at 31.0 Ma (16.7–48.8). This is younger than the previous estimation at 62 Ma (30–108) (Brandl *et al.* 2007), although credibility intervals overlap. The difference in both estimates results from the use of a second calibration point in the present study and from a better coverage of Macrotermitinae species and genera, as Brandl and coworkers focused their work on the genus *Macrotermes*. Another difference is the methods used: whereas Brandl *et al.* (2007) used MultiDivTime, which assumes that the rate variation is autocorrelated in ancestral and descendant lineages, we have used BEAST, which allows rates to vary independently.

For the first time, we attempted to date the most recent common ancestor of *Termitomyces*. However, in the absence of *Termitomyces* fossils, we used an indirect calibration—the divergence of *Agaricus* from *Chlorophyllum* (Geml *et al.* 2004). Uncertainty on the estimated age of this node contributed to the uncertainty of the estimation of the age of *Termitomyces*.

The present age estimates for the origin of the fungus-growing termite symbiosis (both for the host and for the symbiont) coincides with the beginning of the Oligocene, when the rainforests (the reconstructed ancestral habitat of the group; Aanen & Eggleton 2005) were at the maximum of covered area in Africa (Plana 2004). Termite fungiculture is thus likely to have originated just before the expansion of the savanna began (at the cost of rainforest area). The ambiguity in the phylogenetic identification of the first radiation event —reflected in basal relationships with low branch support (e.g. this work, Aanen *et al.* 2002; Aanen & Eggleton 2005)—is consistent with a fast radiation into the main genera shortly after the origin of the fungus-growing termites and therefore still in the original habitat.

Whereas there is congruence between host and symbiont estimates at the level of the main clades (Fig. 1), a comparison of the estimated age of particular host and symbiont clades also revealed several inconsistencies, probably reflecting recent events of host switching followed by dispersal through specific groups of the termite population. For example, most of the *Microtermes* species have symbionts with a relatively recent origin (about twice as young as the genus *Microtermes*). However, even more striking is the *Termitomyces* clade of identical sequences shared between regions and between divergent genera (*Synacanthothermes*, *Acanthotermes*, *Ancistrotermes* and *Microtermes*), of which the maximum age is estimated to be 2.4 Ma. The minimum age of the most recent common ancestor of all these termite genera would only correspond to the lower boundary for the origin of the symbiosis, i.e., 16.7 Ma.

Differences in interaction specificity and recent host switches and dispersion events

Transmission mode is not a strong predictor for the observed degree of host–symbiont specificity and geographical patterns are not either. Paradoxically, our reconstructions show that on the one hand, the main radiations into the extant genera of fungus-growing termites have occurred simultaneously with their symbiont, but on the other hand, several recent host switches of symbionts seem to have occurred across divergent genera and throughout geographical areas. How can we explain these differences in interaction specificity and which factors determine whether specificity will be maintained or lost? One factor may be the role of the fungal symbiont.

The fungal symbiont is thought to be the main decomposer of lignocellulose, although the exact function of the symbiont is still unclear. Several functions have been proposed (recently reviewed in Bignell 2011)—such as being an additional source of protein-rich and higher nitrogen value food (Collins 1983; Tayasu *et al.* 1997) and/or an extra digestive chamber (Wood & Thomas 1989), helping in cellulose degradation through providing cellulases and xylanases to work synergistically and/or complementarily with endogenous termites enzymes (Martin & Martin 1978). In any case, the role of the symbiont seems to differ between genera and species (Rouland-Lefevre *et al.* 1991; Bignell 2000; Hyodo *et al.* 2003; Ohkuma 2003). Differences in nutritional requirements of termites between genera and even species and hence, differences in demands of the termites on their fungal symbiont to fulfil these requirements, may be the main factor maintaining host specificity. For example, *Macrotermes natalensis* is associated with a single lineage of *Termitomyces*, a specialist symbiont as defined by Rouland-Lefèvre *et al.* (2006), and this symbiont is transmitted horizontally. This means that in each generation, a new association is established, so that there must be strong selection mechanisms, either by the termites or by their fungal symbionts to maintain this specific association through generations. Alternatively, the specificity of this interaction may be because of different selection regimes occurring near the southern distribution limit of *Macrotermes* termites in South Africa and not an inherent property of this particular species (*M. natalensis*) having a more specific symbiont association. On the other side of the specificity spectrum, species within the genera *Microtermes* (with vertical symbiont transmission), *Synacanthotermes*, *Ancistrotermes* and *Acanthotermes* (with horizontal symbiont transmission) share the same—more generalist—symbiont. Apparently, a highly successful symbiont has recently dispersed across diverse host genera, becoming associated with several different hosts across a large geographical area. To test how associations arise and how specificity is maintained, future studies should focus on the functional patterns of the interaction between fungus-growing termites and their symbiont, on differences in behaviour, nest architecture and microclimate and on the details of symbiont transmission and the frequency of horizontal vs. vertical transmission across genera, as the existing evidence is based on only few studies (Grassé & Noirot 1955; Johnson 1981; Johnson *et al.* 1981; Nobre *et al.* 2011).

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References

Aanen DK, Eggleton P (2005) Fungus-growing termites originated in African rain forest. *Current Biology*, **15**, 851–855.

Aanen DK, Eggleton P, Rouland-Lefevre C, Guldberg-Froslev T, Rosendahl S, Boomsma JJ (2002) The evolution of fungus-growing termites and their mutualistic fungal symbionts. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 14887–14892.

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

Battistuzzi FU, Filipski A, Blair Hedges S, Kumar S (2010) Performance of relaxed-clock methods in estimating evolutionary divergence times and their credibility intervals. *Molecular Biology and Evolution*, **27**, 1289–1300.

Bignell DE (2000) Introduction to symbiosis. In: *Termites: Evolution, Sociality, Symbioses, Ecology* (eds Abe T, Bignell DE, Higashi M), pp. 189–208. Kluwer Academic Publishers, Dordrecht.

Bignell DE (2011) Morphology, physiology, biochemistry and functional design of the termite gut. In: *Biology of Termites: A Modern Synthesis* (eds Bignell DE, Roisin Y, Lo N), pp. 375–412. Springer, Dordrecht.

Brandl R, Hyodo F, Korff-Schmising M *et al.* (2007) Divergence times in the termite genus *Macrotermes* (Isoptera: Termitidae). *Molecular Phylogenetics and Evolution*, **45**, 239–250.

Britton T, Anderson CL, Jacquet D, Lundqvist S, Bremer K (2007) Estimating divergence times in large phylogenetic trees. *Systematic Biology*, **56**, 741–752.

Brundrett MC (2002) Coevolution of roots and mycorrhizas of land plants. *New Phytologist*, **154**, 275–304.

Collins NM (1983) The utilization of nitrogen resources by termites (Isoptera). In: *Nitrogen as an Ecological Factor* (eds

Lee JA, McNeill S, Robison IH), pp. 381–412. Blackwell Scientific Publications, Oxford.

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

Darlington J (2005) Distinctive fossilised termite nests at Laetoli, Tanzania. *Insectes Sociaux*, **52**, 408–409.

Dentinger BTM, Lodge DJ, Munkacsi AB, Desjardin DE, McLaughlin DJ (2009) Phylogenetic placement of an unusual coral mushroom challenges the classic hypothesis of strict coevolution in the *Apterostigma pilosum* group ant–fungus mutualism. *Evolution*, **63**, 2172–2178.

Drummond A, Rambaut A (2007) BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology*, **7**, 214.

Duringer P, Schuster M, Genise JF, Mackaye HT, Vignaud P, Brunet M (2007) New termite trace fossils: galleries, nests and fungus combs from the Chad basin of Africa (Upper Miocene-Lower Pliocene). *Palaeogeography, Palaeoclimatology, Palaeoecology*, **251**, 323–353.

Eggleton P, Williams PH, Gaston KJ (1994) Explaining global termite diversity: productivity or history? *Biodiversity and Conservation*, **3**, 318–330.

Geml J, Geiser D, Royse D (2004) Molecular evolution of *Agaricus* species based on ITS and LSU rDNA sequences. *Mycological Progress*, **3**, 157–176.

Grassé P-P, Noirot C (1955) La fondation de nouvelles sociétés par *Bellicositermes natalensis* Hav. *Insectes Sociaux*, **2**, 213–220.

Ho SYW, Phillips MJ (2009) Accounting for calibration uncertainty in phylogenetic estimation of evolutionary divergence times. *Systematic Biology*, **58**, 367–380.

Hoeksema JD (2010) Ongoing coevolution in mycorrhizal interactions. *New Phytologist*, **187**, 286–300.

Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics*, **17**, 754–755.

Hyodo F, Tayasu I, Inoue T, Azuma JI, Kudo T, Abe T (2003) Differential role of symbiotic fungi in lignin degradation and food provision for fungus-growing termites (Macrotermitinae: Isoptera). *Functional Ecology*, **17**, 186–193.

Jenkins TM, Dean RE, Verkerk R, Forschler BT (2001) Phylogenetic analyses of two mitochondrial genes and one nuclear intron region illuminate European subterranean termite (Isoptera: Rhinotermitidae) gene flow, taxonomy, and introduction dynamics. *Molecular Phylogenetics and Evolution*, **20**, 286–293.

Johnson R (1981) Colony development and establishment of the fungus comb in *Microtermes* sp. nr. *usambaricus* (Sjöstedt) (Isoptera: Macrotermitinae) from Nigeria. *Insectes Sociaux*, **28**, 3–12.

Johnson J, Vilgalys R (1998) Phylogenetic systematics of *Lepiota* sensu lato based on nuclear large subunit rDNA evidence. *Mycologia*, **90**, 971–997.

Johnson RA, Thomas RJ, Wood TG, Swift MJ (1981) The inoculation of the fungus comb in newly founded colonies of some species of the Macrotermitinae (Isoptera) from Nigeria. *Journal of Natural History*, **15**, 751–756.

Josens G (1972) *Etudes biologique et écologique des termites (Isoptera) de la savane de Lamto-Pakobo (Cote d'Ivoire). Thèse d'Etat*, Université de Bruxelles, 262 pp.

Kambhampati S, Eggleton P (2000) Phylogenetics and Taxonomy. In: *Termites: Evolution, Sociality, Symbioses, Ecology* (eds Abe T, Bignell DE, Higashi M), pp. 1–24. Kluwer Academic Publishers, Dordrecht.

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

Konaté S (1998). *Structure, dynamique et rôle des buttes termitiques dans le fonctionnement d'une savane pré forestière (Lamto, Côte d'Ivoire): le termite champignonniste* Odontotermes *comme ingénieur de l'écosystème*. Thèse de doctorat de l'Université Paris VI, 252 pp.

Korb J, Aanen DK (2003) The evolution of uniparental transmission of fungal symbionts in fungus-growing termites (Macrotermitinae). *Behavioral Ecology and Sociobiology*, **53**, 65–71.

Lancaster N. (1984) Aridity in southern Africa: age, origins and expression in landforms and sediments. In: *Late Cainozoic Palaeoclimates of the Southern Hemisphere. Proceedings of an International Symposium by the South African Society for Quaternary Research* (ed. Vogel JC), pp. 433–444. A. A. Balkema, Rotterdam.

Magallón SA, Sanderson MJ (2005) Angiosperm divergence times: the effect of genes, codon positions, and time constraints. *Evolution*, **59**, 1653–1670.

Martin MM, Martin JS (1978) Cellulose digestion in the midgut of the fungus-growing termite *Macrotermes natalensis*: the role of acquired digestive enzymes. *Science*, **199**, 1453–1455.

Mikheyev AS, Mueller UG, Boomsma JJ (2007) Population genetic signatures of diffuse co-evolution between leaf-cutting ants and their cultivar fungi. *Molecular Ecology*, **16**, 209–216.

Mikheyev AS, Mueller UG, Abbot P (2010) Comparative dating of Attine ant and lepiotaceous cultivar phylogenies reveals coevolutionary synchrony and discord. *The American Naturalist*, **175**, 126–133.

Nobre T, Eggleton P, Aanen DK (2010) Vertical transmission as the key to the colonization of Madagascar by fungus-growing termites? *Proceedings of the Royal Society B: Biological Sciences*, **277**, 359–365.

Nobre T, Fernandes C, Boomsma JJ, Korb J, Aanen DK (2011) Farming termites determine the genetic population structure of *Termitomyces* fungal symbionts. *Molecular Ecology*, doi: 10.1111/j.1365-294X.2011.05064.x.

Ohkuma M (2003) Termite symbiotic systems: efficient bio-recycling of lignocellulose. *Applied Microbiology and Biotechnology*, **61**, 1–9.

Osiemo Z, Marten A, Kaib M, Gitonga L, Boga H, Brandl R (2010) Open relationships in the castles of clay: high diversity and low host specificity of Termitomyces fungi associated with fungus-growing termites in Africa. *Insectes Sociaux*, **57**, 351–363.

Plana V (2004) Mechanisms and tempo of evolution in the African Guineo-Congolian rainforest. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, **359**, 1585–1594.

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

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

Rouland-Lefevre C, Lenoir F, Lepage M (1991) The role of the symbiotic fungus in the digestive metabolism of several species of fungus-growing termites. *Comparative Biochemistry and Physiology*, **99A**, 657–663.

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

Rouland-Lefèvre C, Inoue T, Johjima T (2006) *Termitomyces*/termite interactions. In: *Intestinal Microorganisms of Termites and Other Invertebrates* (eds König H, Varma A), pp. 335–350. Springer, Berlin, Heidelberg.

Sankaran M, Hanan NP, Scholes RJ *et al.* (2005) Determinants of woody cover in African Savannas. *Nature*, **438**, 846–849.

Scholes RJ, Archer SR (1997) Tree–grass interactions in savannas. *Annual Review of Ecology and Systematics*, **28**, 517–544.

Skinner A (2010) Rate heterogeneity, ancestral character state reconstruction, and the evolution of limb morphology in *Lerista* (Scincidae, Squamata). *Systematic Biology*, **59**, 723–740.

Taprab Y, Ohkuma M, Johjima T *et al.* (2002) Molecular phylogeny of symbiotic basidiomycetes of fungus-growing termites in Thailand and their relationship with the host. *Bioscience Biotechnology and Biochemistry*, **66**, 1159–1163.

Tayasu I, Abe T, Eggleton P, Bignell DE (1997) Nitrogen and carbon isotope ratios in termites: an indicator of trophic habit along the gradient from wood-feeding to soil-feeding. *Ecological Entomology*, **22**, 343–351.

White T, Bruns T, Lee S, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: A Guide to Methods and Applications* (eds Innis M, Gelfand D, Shinsky J, White T), pp. 315–322. Academic Press, San Diego.

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

T.N.'s main interest is in evolution and stabilization of symbioses, and has been focusing on the fungus-growing termites' mutualism. N.A.K. is interested in the ecology and conservation of wild useful fungi and their habitats, and has been focusing on *Termitomyces* spp. S.K. works on the diversity and functional role of termites and ants in West Africa, with a focus on the fungus-growing termite as ecosystem engineer. K.E.L. is mainly interested in biodiversity questions, behavioural, physiological and community ecology with special focus on tropical systems. D.K.A. is interested in the evolution of conflict and cooperation using fungi as model organisms.

Supporting information

Additional supporting information may be found in the online version of this article.

Data S1 Details on methodology and samples used.

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