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Divergence times in the termite genus *Macrotermes* (Isoptera: Termitidae)

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Abstract

The evolution of fungus-growing termites is supposed to have started in the African rain forests with multiple invasions of semi-arid habitats as well as multiple invasions of the Oriental region. We used sequences of the mitochondrial COII gene and Bayesian dating to investigate the time frame of the evolution of *Macrotermes*, an important genus of fungus-growing termites. We found that the genus *Macrotermes* consists of at least 6 distantly related clades. Furthermore, the COII sequences suggested some cryptic diversity within the analysed African *Macrotermes* species. The dates calculated with the COII data using a fossilized termite mound to calibrate the clock were in good agreement with dates calculated with COI sequences using the split between *Locusta* and *Chortippus* as calibration point which supports the consistency of the calibration points. The clades from the Oriental region dated back to the early Tertiary. These estimates of divergence times suggested that *Macrotermes* invaded Asia during periods with humid climates. For Africa, many speciation events predated the Pleistocene and fall in range of 6–23 million years ago. These estimates suggest that savannah-adapted African clades radiated with the spread of the semi-arid ecosystems during the Miocene. Apparently, events during the Pleistocene were of little importance for speciation within the genus *Macrotermes*. However, further investigations are necessary to increase the number of taxa for phylogenetic analysis.

Keywords: Cytochrome oxidase II; MULTIDIVTIME; Fungus-growing termites; Cryptic species; Molecular clock

1. Introduction

Termites (Isoptera) are an important group of tropical insects. Firstly, termites are essential for the energy flow and recycling of nutrients in tropical ecosystems (Wood and Sands, 1978; Wood, 1988; Holt and Lepage, 2000; Sugimoto et al., 2000). Thereby the role of termites depend strongly on the interaction with symbionts (Higashi and Abe, 1997; Bignell, 2000; Aanen et al., 2002). Secondly, some termite species are pests attacking crops and buildings (Spear, 1970; Su and Scheffrahn, 2000). Thirdly, termites are eusocial (Krishna, 1969; Shellman-Reeve, 1997; Higashi et al., 2000). In contrast to Hymenoptera, however, termites are diplo-diploid and comparisons between

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Hymenoptera (especially ants) and termites (''white ants'') facilitate our understanding of the general processes behind the evolution of eusociality (Husseneder et al., 1998, 1999). Despite the importance of termites in several branches of biology and a number of recent attempts (e.g. Lo et al., 2000; Aanen and Eggleton, 2005), phylogeny, diversity and radiation of termites are still poorly understood (Eggleton, 2001).

Overall, the order Isoptera consists of 7 families and 14 subfamilies (Kambhampati and Eggleton, 2000). Evidence from DNA sequences suggests that termites evolved from wood-feeding cockroaches (Lo et al., 2000), probably during the late Jurassic (Thorne et al., 2000; Grimaldi and Engel, 2005). The so-called higher termites (Termitidae) comprise about 80% of the more than 2000 living termite species (Kambhampati and Eggleton, 2000; Grimaldi and Engel, 2005). Of the five subfamilies within the Termitidae, the subfamily Macrotermitinae consists of 14 genera with about 330 species (Kambhampati and Eggleton, 2000). Recent phylogenetic analyses based on morphological and molecular characters suggested that the Macrotermitinae are monophyletic and form the basal lineage of the Termitidae (Miura et al., 1998; Donovan et al., 2000; Kambhampati and Eggleton, 2000). This subfamily is distributed across the Palaeotropics with the highest diversity of genera in Africa (Kambhampati and Eggleton, 2000; Eggleton, 2000). Therefore, the geographic origin of the Macrotermitinae is thought to be in Africa with a subsequent spread to the Oriental region (Emerson, 1955; Darlington, 1994; Thorne et al., 2000; Aanen and Eggleton, 2005). Overall, the fossil record and biogeographic patterns indicate that the forces behind termite radiation and biogeography were not continental drift, but explosive radiation and rapid dispersal in the early Tertiary (Thorne et al., 2000).

All species within the Macrotermitinae cultivate fungi in special gardens (Noirot and Darlington, 2000). Considering the monophyly of the Macrotermitinae, it is likely that the mutualistic relationship between fungi and termites evolved only once. Furthermore, the fascinating reconstruction of the ancestral habitat of fungus-growing termites by Aanen and Eggleton (2005) showed that the origin of this symbiotic relationship between termites and fungi occurred in the African rain forests. Although fungus-growing termites are very successful and important in semi-arid environments (e.g. 20% of C-mineralization is due to fungus-growing termites; Wood and Sands, 1978), the mutualistic relationship itself evolved under quite different conditions, namely, in the humid and more constant conditions of rain forests (Aanen and Eggleton, 2005). The three most species-rich genera of the Macrotermitinae are *Odontotermes* (172 species), *Microtermes* (60), and *Macrotermes* (47; Kambhampati and Eggleton, 2000). According to Aanen and Eggleton (2005), these three genera invaded Asia from Africa. The Ethiopian region is more species rich than the Oriental region in *Odontotermes* and *Microtermes* species, while the genus *Macrotermes* counters this trend (34 species in the Oriental region; 13 species in the Afrotropics; Kambhampati and Eggleton, 2000).

It has been noted that Africa is especially rich in termite genera (Eggleton, 2000). According to Eggleton et al. (1994), in Africa the severe climatic fluctuations during the Pleistocene generated in termites several pulses of speciation. Since the fossil record of termites is fragmentary (Grimaldi and Engel, 2005), molecular divergence estimates may help to test the hypothesis of Eggleton and co-workers (Eggleton et al., 1994; Eggleton, 2000). Recently, Schmitz and Moritz (1998) noted that the molecular clock might run faster for social insects than for other insects. The probability for the fixation of nearly neutral alleles by drift is expected to decrease with effective population size. Sociality reduces the effective population size and therefore mutations may become fixed quite rapidly. Recently, Luchetti et al. (2005) calibrated the molecular clock for European *Reticulitermes* taxa (Rhinotermitidae). They found a substitution rate 100-fold higher than the rate usually found for insects (2.3% My^{-1}; Brower, 1994). However, Bromham and Leys (2005) found no consistent difference between rates of molecular evolution between social and non-social lineages in a phylogenetically controlled analysis.

Within a broader study of eusociality, speciation, and evolution of the genus *Macrotermes*, we sequenced the mitochondrial COII gene of several *Macrotermes* species from the Ethiopian and Oriental region. Furthermore, we used a fossilized termite mound to calibrate our molecular clock. This mound was found in the famous and intensively investigated Pliocene deposits of Laetoli (3°13′S; 35°13′E). The age of the deposits where the mound was found is between 3.46 and 3.76 My (Darlington, 2005). The conservation of the mound as well as the characteristic structure of the ventilation system of the mound showed that the termite species, which built this mound, was a close relative to a lineage that still occurs in Africa (Darlington, 2005). With the sequence data, recent advances in estimating divergence times (see Arbogast et al., 2002; Welch and Bromham, 2005), and the recent finding of a fossilized mound (Darlington, 2005), we are able to broaden the understanding of the genus *Macrotermes* in two directions:

1. Firstly, we are able to broaden the understanding of the phylogeny of the genus *Macrotermes*. We will show that at least in Africa several cryptic species occur.
2. Secondly, by dating the phylogeny of the genus *Macrotermes*, we broaden our understanding of the approximate time frame of speciation events within this genus. We are aware of the many problems attached to molecular time estimates. Therefore, we will use our time estimates only to outline the general time frame of the evolution of fungus-growing termites.

2. Material and methods

2.1. Specimen

The samples, sample sites and sequences accession numbers for the present analyses are listed in Table 1. The samples from Africa are from two sources: Firstly, R.B. and M.K. collected the samples from Kenya and Ivory Coast during their work on the evolution of sociality and communication in termites (voucher specimen were in collections of R.B. and M.K. as well as in the collection of the National Museums of Kenya, Nairobi). Secondly, we received material from the J.P.E.C. Darlington (voucher specimen in the collection of J.P.E.C. Darlington, Cambridge). Thirdly, several colleagues provided further material (voucher specimen in the collection of R.B. and M.K.). The samples from Asia were collected by Y.T. (voucher specimen are in the collection of Y.T.).

2.2. Samples and sequencing

For our analysis of the mitochondrial cytochrome oxidase II gene (COII), we used 35 sequences, including sequences of at least 12 supposed species of the genus *Macrotermes*. Three species (two *Cubitermes* and one *Drepanotermes*) did not belong to the Macrotermitinae and were used as outgroup (Table 1). Furthermore, we included into our analyses four other genera of Macrotermitinae (Table 1). Nevertheless, we will concentrate our discussions mainly on the genus *Macrotermes*. Some sequences were already available in GenBank (for accession numbers, see Table 1).

To scrutinize our estimates of the divergence times in termites, we used sequences of the mitochondrial cytochrome oxidase I gene (COI). Recently, Gaunt and Miles (2002) showed that this gene produced estimates of divergence times across a wide spectrum of insect lineages, which were in accord with palaeontological and biogeo-

Table 1
Name and origin of samples as well as GenBank accession numbers

Genus	Species	Continent	Location	Accession No.
Outgroup species				
Cubitermes	*spec.*	Africa	Kenya, Tsavo East	AB304486
Cubitermes	*umbratus*	Africa	Kenya, Shimba Hills	AB304487
Drepanotermes	*rubriceps*	Australia	GenBank	AB005578
Other Macrotermitinae				
Hypotermes	*xenotermitis*	Asia	GenBank	AB011409
Microtermes	*spec.*	Africa	Kenya, Kajiado	AB304488
Odontotermes	*spec.*	Asia	Thailand, Sakaerat	AB300694
Odontotermes	*javanicus*	Asia	Indonesia, Java	AB300695
Odontotermes	*tanganicus*	Africa	Kenya, Kajiado	AB304489
Pseudacanthotermes	*spiniger*	Africa	Kenya, Marigat	AB304490
Macrotermes species				
Macrotermes	*annandalei* 1	Asia	Thailand, Sakaerat	AB300696
Macrotermes	*annandalei* 2	Asia	GenBank	AB109527
Macrotermes	*bellicosus* 1	Africa	Ivory Coast, Comoé	AB304491
Macrotermes	*bellicosus* 2	Africa	Kenya, Kapenguria	AB304492
Macrotermes	*carbonarius* 1	Asia	Thailand, Sakaerat	AB300697
Macrotermes	*carbonarius* 2	Asia	GenBank	AB051878
Macrotermes	*carbonarius* 3	Asia	GenBank	AB109525
Macrotermes	*chaiglomi*	Asia	Thailand, Doi Suthep	AB300698
Macrotermes	*falciger* 1	Africa	South Africa, Krüger NP	AB304493
Macrotermes	*falciger* 2	Africa	Kenya, Shimba Hills	AB304494
Macrotermes	*falciger* 3	Africa	Malawi, Matapwata	AB304495
Macrotermes	*gilvus* 1	Asia	Thailand, Sakaerat	AB300699
Macrotermes	*gilvus* 2	Asia	GenBank	AB005582
Macrotermes	*gilvus* 3	Asia	GenBank	AB109526
Macrotermes	*herus* 1	Africa	Kenya, Marigat	AB304496
Macrotermes	*herus* 2	Africa	Uganda, Budongo	AB304497
Macrotermes	*jeanneli*	Africa	Kenya, Marigat	AB304498
Macrotermes	*malaccensis* 1	Asia	Thailand, Narathiwat	AB300700
Macrotermes	*malaccensis* 2	Asia	GenBank	AB109528
Macrotermes	*michaelseni* 1	Africa	Kenya, Tsavo East	AB304499
Macrotermes	*michaelseni* 2	Africa	Malawi, Tuchila	AB304500
Macrotermes	*cf. michaelseni*	Africa	Kenya, Gedi	AB304501
Macrotermes	*natalensis* 1	Africa	South Africa, Krüger NP	AB304502
Macrotermes	*natalensis* 2	Africa	Malawi, Matpwata	AB304503
Macrotermes	*subhyalinus* 1	Africa	Ivory Coast, Comoé	AB304504
Macrotermes	*subhyalinus* 2	Africa	Kenya, Bissl	AB304505

graphic information. We selected sequence data for 20 species of termites as well as three other species (*Blattella*, *Locusta* and *Chorthippus*) from GenBank (for accession numbers, see Fig. 4). For all termite genera, we selected arbitrarily one example from the species available in GenBank. Only for the genus *Macrotermes* we selected three species, which represent three major lineages within this genus (see below). To root this tree, we used a sequence of *Artemia* (DQ119646).

Our own samples for sequencing the COII gene were preserved in absolute or 80% alcohol until DNA extraction. Total DNA was extracted from the head capsule of the termites using a DNasy tissue kit (Qiagen), and following the protocol recommended by the manufacturer. The COII gene was amplified by polymerase chain reaction as described by Miura et al. (1998). The reaction mix was composed of 30 μl of distilled water, 4 μl of 10× PCR buffer (Takara, Tokyo; 100 mM Tris–HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$, 0.01% (w/v) gelatin), 4 μl of dNTP mix (1 mM each dNTP), 0.2 μl of each primer (100 pM), 0.7 U of Taq polymerase (Takara, Tokyo), and 2 μl of template DNA. The tubes were placed in a thermal cycler (Gene Amp 2400, Perkin-Elmer, Norwalk, CT, USA). The conditions for amplification were as follows: 35 cycles of (i) denaturation at 94 °C for 30 s, (ii) annealing at 45 °C for 1 min and (iii) extending at 65 °C for 3 min. The primers for the amplification of a fragment ~780 bp in size were forward: 5′-ATG GCA GAT TAG TGC AAT GG-3′ (A-tLeu, 3018 ~ 3038 in *D. yakuba*, Liu and Beckenbach, 1992; Simon et al., 1994) and reverse 5′-GTT TAA GAG ACC AGT ACT TG-3′ (B-tLys, 3804–3784 in *D. yakuba*, Liu and Beckenbach, 1992; Simon et al., 1994). The amplification products were electrophoresed on a 1% agarose gel, and purified using Prep-A-Gene DNA purification kit (Bio-Rad, Hercules). The purified products were then used for the sequencing reaction.

The DNA sequencing reaction was performed using a dideoxy-nucleotide cycle sequencing procedure with a Dye-Terminater cycle sequencing kit (Perkin-Elmer, Qarrington, UK) and a GeneAmp 2400 thermal cycler. Electrophoresis was performed on 6% polyacrylamid gels (Toyobo, Tokyo, Super Reading DNA Sequence Solution), and the data were obtained using an automatic DNA sequencer (Perkin-Elmer, model 373S). Both strands of the PCR product were sequenced. The accession numbers of the COII gene sequences reported in this study are listed in Table 1.

2.3. Phylogenetic analyses and dating

We aligned sequences (COII and COI) using Clustal W (Thompson et al., 1994). In order to check for pseudogenes, we searched sequences for stop codons and unexpected deletions or insertions. The phylogenetic analyses of the COII sequences were performed under the principles of parsimony, maximum likelihood and Bayesian statistics using the most recent versions of PAUP (Swofford, 1998) and MRBAYES (Huelsenbeck and Ronquist, 2001). Gaps were treated as missing values. For the maximum likelihood approach, we first ran MODELTEST in order to select an appropriate model of sequence evolution (Posada and Crandall, 1998). The model selected by MODELTEST was then implemented with the appropriate commands in PAUP and MRBAYES. During all heuristic searches in PAUP, we used 10 rounds of random additions and 100 (maximum likelihood) or 10,000 (parsimony) rearrangements. For the Bayesian analysis, we generated 10^6 generations, which were sampled every 500 generations. We discarded the first 500 sampled trees (burn-in). We checked for convergence using the criteria suggested in the manual of MRBAYES. To evaluate the reliability of nodes, we used non-parametric bootstrapping as well as the posterior probabilities from the Bayesian analysis (1000 replicates parsimony and 500 replicates maximum likelihood; for the maximum likelihood analysis, we used the simplified search option FastStep). Nodes with bootstrap values of 70% or above were regarded as well supported (Hillis and Bull, 1993). Posterior probabilities of 0.95 and above were considered significant (for a comparison of bootstrap values and posterior probabilities, see Alfaro and Holder, 2006).

To test some alternatives of the optimal tree, we used the Shimodaira–Hasegawa-test as implemented in PAUP (Shimodaira and Hasegawa, 1999; test option FullOpt; 1000 bootstrap replicates). We generated the alternative topologies by introducing constraints during the search for the optimal tree using maximum likelihood with the model for sequence evolution suggested by MODELTEST.

To derive time estimates, we used a relaxed molecular clock (Welch and Bromham, 2005; for reviews, see Hedges and Kumar, 2003; Bromham and Penny, 2003). The selected approach implements a probabilistic model to describe the correlated change in evolutionary rate over time (program MULTIDIVTIME; Thorne et al., 1998; Thorne and Kishino, 2002). By using a Markov Chain Monte Carlo procedure, the program derives a *posterior* distribution of rates and divergence times, which can be used to construct credibility intervals for the estimates. For a step-by-step manual and other programs necessary to run MULTIDIVTIME, see Rutschmann (2004). To calibrate the clock, we used the fossilized mound described by Darlington (2005). The age of the mound is between 3.46 and 3.76 My. Further on we use the conservative values 3.4 and 3.8 My. Our COII-tree showed that *M. subhyalinus* sampled in Kenya and *M. jeanneli* are sister species (Fig. 3). Each nest of *M. jeanneli* has a single tall chimney, open at the top, through which metabolic gases are vented to the atmosphere (Fig. 1). The single chimney may reach a height of 3 m and more (own observations and Darlington, 1984, 2005; Darlington et al., 1992). *M. subhyalinus* builds a mound with many chimneys and chimneys are not tall (Fig. 1). Hence, if the mentioned mound structure is a reliable phylogenetic character, all species building open mounds must have diverged at node a in Fig. 1 from a

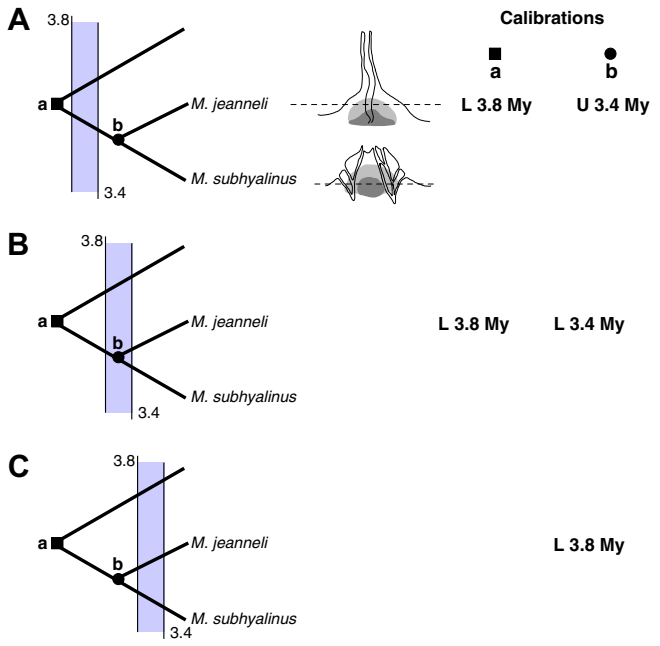

Fig. 1. Three possibilities to calibrate the molecular clock using a fossil termite mound found in Laetoli. The age of this mound is between 3.4 and 3.8 My. This time interval is shown by the shaded rectangle. From our phylogenetic analyses, we assume that the two termite species which build open mounds (*M. subhyalinus* and *M. jeanneli*) are sister species (node b). The monophyletic clade of termites with open mounds diverged at node a. The relative position of the fossil mound in relation to the phylogeny determines the lower (L) and/or upper (U) time estimates for nodes a and b. For example, possibility A assumes that mounds with one ventilation chimney are the primitive state, whereas possibility C assumes that one chimney is the derived state. For further details, see Material and methods. The simplified line-drawings visualize the basic mound structure of *M. jeanneli* and *M. subhyalinus*. The dark shaded areas symbolize the central nest including the queen cell, whereas the light shading indicates the position of the fungus gardens.

stock of ancestors which build closed mounds. We do not know which of the two open mound types present the primitive state. Assuming that a tall chimney is the primitive state (Fig. 1A), the fossilized mound is a lower estimate of the age of node a and an upper estimate of the node b. If a tall chimney is the derived state (Fig. 1C), then 3.8 My is a lower estimate of node b. In the case of Fig. 1B, 3.8 My is a lower estimate of a and 3.4 My a lower estimate of b. We experimented with the various possibilities to calibrate nodes (see Fig. 1), but results were not very much different. In Table 4, we present the time estimates for all nodes using the possibility of Fig. 1B (including standard deviation and credibility intervals) as well as Fig. 1C. During all runs, other input values used by the program MULTIDIVTIME were default values. To set the time steps between root and tip, we used the value 50 My (arbitrary standard deviation 20). We checked carefully for convergence by running the program several times changing certain priors (e.g. time unites between tip and root and corresponding rates).

For further comparison, we used a tree of the COI-sequences to estimate the divergence times of major lineages of Termitidae and Macrotermitinae. The COI-tree was calibrated with the split between *Locusta* and *Chorthippus*. This split is well supported by fossil remains (Grimaldi and Engel, 2005). Gaunt and Miles (2002) list in their Table 1 an age from fossil remains between 144 and 150 My for this split. Using a molecular clock these authors generated an estimate between 88 and 98 My, which gives at least the correct order of magnitude (see Table 1 in Gaunt and Miles, 2002). We used both possibilities to calibrate our clock and the comparison of results between the two calibrations gave a hint about the robustness of the results from the COI analyses. To set the time steps between root and tip, we used the value 323 My (Gaunt and Miles, 2002; arbitrary standard deviation 50). Note that the COI and the COII gene are together part of the mitochondrial genome and therefore share a common evolutionary history. Hence the comparison of the COI and COII dates test the consistency of the calibration points.

3. Results

3.1. Phylogeny derived from the COII gene

Base frequencies were skewed (Table 2) and differed between as well as within genera. All species of the *Macrotermitinae* had a deletion of 6 base pairs (Miura et al., 1998). From the 681 positions submitted to the analyses, 221 were parsimony informative. Position three of the reading frame showed some signs of saturation (Fig. 2). Analyses using only the first two positions of a reading frame or runs with various weighting schemes showed that the topologies of trees were very similar to the topologies of trees retrieved using the full data set with equal weights for all three codon positions. Thus, we decided to submit all three positions to the final analyses.

Using the parsimony criterion, PAUP retrieved two trees (CI = 0.43, RI = 0.63). MODELTEST selected via the hierarchical likelihood ratio tests the general time-reversible model with among site rate heterogeneity (GTR + Γ; shape parameter = 0.2092; rate matrix: [A–C] = 1.0490; [A–G] = 12.7488; [A–T] = 1.3576; [C–G] = 0.6232; [C–T] = 19.2577; [G–T] = 1.0000; Yang et al., 1994; for estimated base composition, see Table 2). The topology of the tree found using the maximum likelihood criterion (Fig. 3; heuristic search with default options; −ln likelihood = 4876.103) differed from the tree generated by the Bayesian approach only in the position of *Pseudacanthotermes* and *Microtermes*, which form sister lineages in the latter approach (the Bayesian topology was also one of the best trees found with the parsimony criterion). The topology of the tree originating from the log-likelihood criterion was not significantly different from the two trees generated under the parsimony criterion (Table 3). Therefore, we used the tree from the maximum likelihood analysis as our master tree (Fig. 3). For a closer comparison of the three methods, we list in Table 4 information about the bootstrap values using par-

Table 2
Base composition of the sequences (see Table 1; for species)

Species	A	C	G	T
Outgroup species	39.2 ± 0.6%	20.9 ± 1.1%	14.0 ± 0.2%	26.0 ± 1.0%
Other Macrotermitinae	39.6 ± 0.8%	24.8 ± 1.1%	13.9 ± 0.6%	21.7 ± 1.0%
All *Macrotermes*	41.0 ± 0.6%	22.9 ± 0.6%	13.8 ± 0.4%	22.2 ± 0.8%
Frequencies estimated by MODELTEST	41.05%	28.55%	9.22%	21.18%

We give the mean ± standard deviation across all species of each group as well as the estimated base frequencies reported by MODELTEST for the most appropriate model of sequence evolution (GTR + Γ).

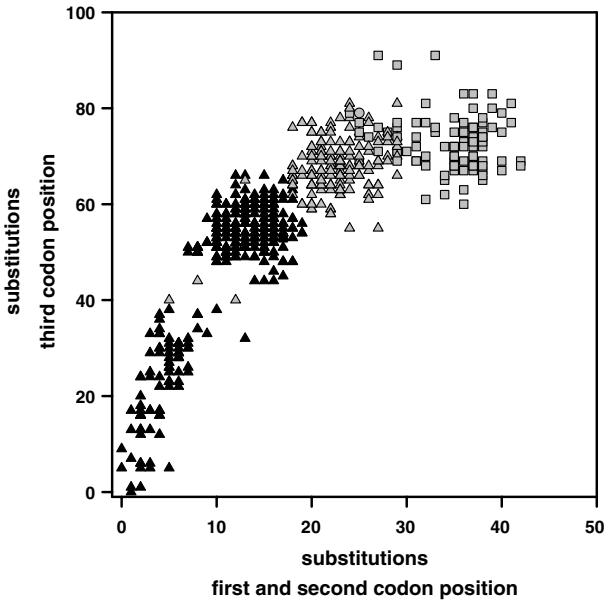

Fig. 2. Plot of number of substitutions on the first and second codon position *versus* substitution on the third position for the mitochondrial COII gene. Grey squares: comparisons to the outgroup species; grey triangles: comparisons between *Macrotermes* and the other Macrotermitinae as well as within the other species; black triangles: comparisons between *Macrotermes* species.

simony, maximum likelihood as well as the *posteriori* probabilities for the nodes.

Not all methods supported unambiguously the monophyly of the genus *Macrotermes*. At least the Bayesian approach supported node 25 (see Table 4 and Fig. 3). Furthermore, heuristic searches with a constrained phylogenetic position for the genus *Pseudacanthotermes spiniger* (either as a sister group to *M. malaccensis* or a sister group to all *Macrotermes* except *M. malaccensis*) produced trees with a log-likelihood not significantly different from the topology shown in Fig. 3 (Table 3).

The genus *Macrotermes* consisted of at least six distantly related clades: clade I – *M. malaccensis*; clade II – *M. bellicosus*; clade III – *M. falciger, M. herus, M. jeanneli, M. michaelseni, M.natalensis*, *M. subhyalinus*; clade IV – *M. carbonarius;* clade V – *M. gilvus*; clade VI – *M. ananndalei* and *M. chaiglomi*. The low support for the nodes leading to these clades (nodes 17, 18, 24, 25) suggested a star-like evolution. Only node 23 which leads to clades V and VI had some support from the maximum likelihood and Bayesian approaches (Table 4). An analysis forcing all Oriental species and all Ethiopian species into two monophyletic groups produced a tree, which was not significantly different from the master tree (Table 3).

Clade III included all African *Macrotermes* species (except *M. bellicosus*). The phylogeny of this clade points to some interesting details: Firstly, the population from West Africa (Ivory Coast) named *M. subhyalinus* was not related to the population from East Africa (Kenya) supposed to be the same species (see Ruelle, 1970). The population from the Ivory Coast was close to *M. herus*. A constrained search which forced the putative *M. subhyalinus* sequences to be sister groups produced a tree with a log-likelihood significantly less than the log-likelihood of the master tree (Table 3). Secondly, the two species *M. subhyalinus* (from Kenya) and *M. jeanneli* were closely related (Fig. 3). These two only species build mounds with open ventilation chimneys. Furthermore, these two species occur across East Africa with little overlap of the distributional ranges. Overall this suggests that open mounds developed only once during the evolution of *Macrotermes*. Thirdly, the two samples named *M. natalensis* comprised two distinct species. However, forcing the two sequences to form one group produced a tree not significantly different from the master tree (Table 3).

3.2. Estimates of divergence times

Although most of the credibility intervals for the estimates of the divergence times were very broad (Table 4), the dates suggested that the basal splits within the genus *Macrotermes* fall into the early Tertiary. Many of the splits within African clade III predated the Pleistocene and are older than 6 My.

To search for the best tree using the COI sequences, we used the GTR + I + Γ model of sequence evolution (MODELTEST using the hierarchical likelihood ratio tests). Note that the intention of this analysis was not to generate a phylogenetic hypothesis but to generate a tree for the dating of certain splits (for the tree, see Fig. 4). Hence we refrain from discussing the details of the tree. We only note that the branching pattern within the genus *Macrotermes* is not consistent with the pattern derived from our COII sequences. Overall we found that the time estimates differed not very much between the two possibilities to calibrate the clock (Table 5) and we use in our sub-

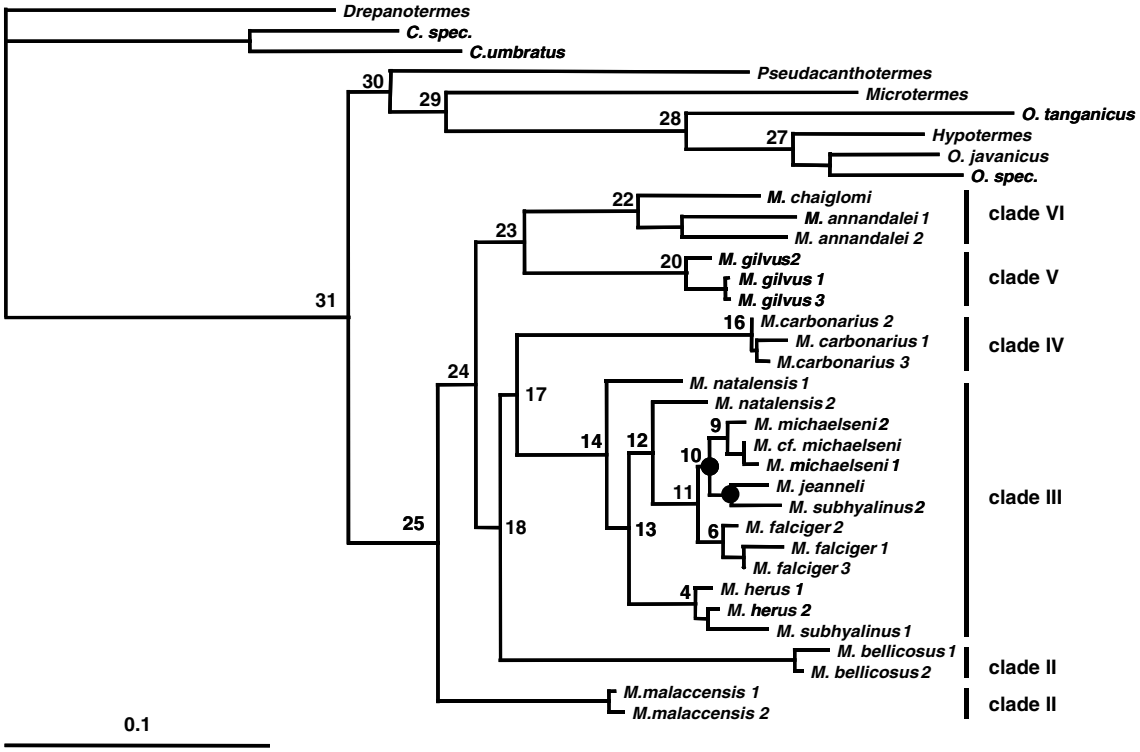

Fig. 3. Tree generated under the criterion of maximum likelihood (using PAUP). For details of species, see Table 1. The tree was rooted with *Drepanotermes*, *Cubitermes spec* and *C. umbratus*. The numbers of the nodes correspond to Table 4, where we give bootstrap values as well as posterior probabilities and age estimates of the nodes. We also pinpoint the six distantly related clades within the genus *Macrotermes*. The nodes used for dating are marked with a dot. For full species names, see Table 1.

Table 3
Tests of the optimal topology *versus* alternative topologies derived from other optimization methods (parsimony trees) or approaches (Bayesian analysis), as well as of maximum likelihood searches with constraints

Topology	−ln *L*	Difference	KH-test	SH-test
Maximum likelihood tree	4876.10384			
Parsimony and MRBAYES trees versus maximum likelihood tree				
Parsimony tree #1	4879.48835	3.38451	0.55	0.74
Parsimony tree #2 and MRBAYES tree	4877.81370	1.70986	0.58	0.89
Constrained trees versus maximum likelihood tree				
All Asian taxa of the genus *Macrotermes* and all African taxa forced into monophyletic lineages	4881.73220	5.62835	0.34	0.55
Samples supposed to be*M. subhyalinus* forced to be sister taxa	4918.35962	42.25577	<0.001	<0.001
Samples of *M. natalensis* forced to be sister taxa	4879.55734	3.45350	0.35	0.74
Pseudacanthotermes within *Macrotermes* (C1)	4886.11688	10.01304	0.14	0.37
Pseudacanthotermes within *Macrotermes* (C2)	4886.63901	10.53517	0.09	0.35

The presented analyses test *a posteriori* hypotheses, a situation in which the Kishino–Hasegawa-test (KH-test; Kishino and Hasegawa, 1989) is not appropriate. Therefore, we also used the Shimodaira–Hasegawa-test (SH-test; one-tailed error probability; Shimodaira and Hasegawa, 1999). Nevertheless, for comparative reasons, we give the two-tailed error probabilities for the KH-test. For the last two constraints (C), we broke up the monophylogeny of the genus *Macrotermes* by forcing *Pseudacanthotermes spiniger* to be a sister taxon of *M. malaccensis* (C1) or to be a sister taxon to the other investigated *Macrotermes* species (C2).

sequent discussion the time estimates derived from the age of the fossils. The COI tree dates the origin of the termites to 229 Mya (credibility interval (143–342 Mya; Fig. 4). The origin of the fungus-growing termites was estimated to have occurred around 62 Mya (30–108 Mya) and the genus *Macrotermes* around 47 Mya (22–84 Mya). Note that the latter estimate is very close to the estimate derived from the COII sequences using different calibration dates (node 31 in Fig. 3; estimate 43 Mya; credibility interval 20–79 Mya; Table 4). The COI sequences date the splits between the major clades of *Macrotermes* between 33 and 41 Mya, again these estimates are consistent with the estimates presented in Table 4).

4. Discussion

The taxonomy and phylogeny of the genus *Macrotermes* have puzzled biologists for quite a while (Ruelle, 1970;

Table 4
Information on the reliability of the nodes of the phylogenetic tree presented in Fig. 2

# Node	Parsimony	Maximum likelihood	*A posteriori* probability	Divergence time[1]	Divergence time[2]	Standard deviation	Lower boundary	Upper boundary
M. malaccensis 1 – M. malaccensis 2	**100**	**98.6**	**1.00**	5.2	4.9	4.9	0.4	18.9
M. bellicosus 1 – M. bellicosus 2	**100**	**100**	**1.00**	3.3	3.3	2.9	0.3	11.3
M. herus 2 – M. subhyalinus 1	33.8	25.8	0.43	4.3	4.3	3.3	0.5	12.8
4	**84.1**	**73**	**1.00**	6.4	6.5	4.1	1.6	17.0
M. falciger 1 – M. falciger 3	**97.7**	**88.4**	**1.00**	2.3	2.2	2.0	0.1	7.7
6	**93.5**	**78**	**1.00**	5.4	5.5	3.2	1.6	14.0
M. subhyalinus 2 – M. jeanneli	**74.7**	**76**	**1.00**	6.4	6.6	2.9	3.5	14.0
M. michaelseni 1 – M. cf. michaelseni	**83.4**	**70.4**	**1.00**	2.4	2.4	1.9	0.2	7.4
9	67.9	45.8	0.98	5.2	5.4	3.0	1.7	13.0
10	60.4	31.2	0.74	8.7	9.0	3.9	4.2	19.0
11	80.7	50.4	1.00	10.4	10.7	4.6	4.8	22.4
12	51.1	25.2	0.70	14.7	15.1	6.3	6.6	30.7
13	46.1	24.8	0.76	17.9	18.3	7.3	8.1	36.5
14	97.6	58.4	1.00	20.6	21.1	8.2	9.4	41.8
M. carbonaria 1 – M. carbonaria 3	10.2	49.8	0.30	3.3	3.3	2.9	0.3	11.2
16	**100**	**97.2**	**1.00**	4.8	4.8	3.8	0.8	15.1
17	25.4	12	0.42	29.8	31.0	11.0	14.0	56.3
18	16.5	15.8	0.88	32.4	33.8	11.8	15.3	60.6
M. gilvus 1 – M. gilvus 3	**100**	**92.6**	**1.00**	1.9	1.8	2.2	0.1	7.6
20	**100**	**92.6**	**1.00**	8.3	8.1	5.5	2.0	23.0
M. annandalei 1 – M. annandalei 2	66.9	77.6	0.93	13.1	13.6	6.4	4.4	28.9
22	**94**	**96.4**	**1.00**	18.3	19.0	8.0	7.0	37.6
23	26.9	67.2	1.00	29.5	30.6	11.2	13.3	56.3
24	13.5	16.4	0.91	34.8	36.3	12.5	16.6	64.6
25	50.3	28.6	1.00	37.6	39.2	13.4	17.8	69.4
O. javanicus – O. spec.	69.9	51.6	0.96	11.5	11.8	5.9	3.6	25.9
27	**96.1**	**88.2**	**1.00**	14.6	15.0	7.0	4.9	31.7
28	**98.3**	**99.2**	**1.00**	22.8	23.7	9.8	8.6	46.5
29	15	29.6	0.33	34.7	36.0	13.0	15.0	65.7
30	50	30.8	0.76	39.4	41.0	14.2	17.9	73.2
31	**98.9**	**98.8**	**1.00**	43.2	45.1	15.2	19.9	79.2

We give the bootstrap values for the nodes from the parsimony and maximum likelihood analyses. Furthermore, we give the posterior probabilities of the nodes. For nodes leading to tips, we note the names of the tips. Nodes supported by all three methods are in bold (70% bootstrap support and 0.95 posterior probabilities). For full species names, see Table 1. The last five columns give the estimated divergence times (in Mya) with associated standard deviation using the COII sequences. We also give the lower and upper boundaries of the credibility intervals (in Mya). We give two estimates of divergence times. The first estimate is the result of a single run of the program MULTIDIVTIME using the possibility B in Fig. 1. The second estimate shows the results when using possibility C in Fig. 1. Note the small difference between the two estimates, especially when judged against the often very large credibility intervals. The standard deviation as well as the lower and upper bound of the credibility interval are from possibility B (Fig. 1).

Fig. 4. Tree used to estimate divergence times for events in the evolution of termites. The tree is based on published COI sequences (accession number follows genus name). For estimates of the divergence times of the nodes marked with a filled circle, see Table 5.

Table 5
Divergence times, standard deviations as well as credibility intervals (in Mya) for the nodes marked in Fig. 4

# Node	Mean	SD	Boundary	
			Lower	Upper
M. natalensis – M. malaccensis	33.0	12.8	14.0	62.8
	31.0	12.2	13.8	61.0
2	40.6	14.7	18.3	75.7
	39.3	14.1	18.0	72.6
3	46.7	16.1	21.7	84.0
	45.3	15.5	21.1	81.0
4	62.1	20.0	30.2	108.8
	60.6	19.7	29.3	105.0
5	229.0	51.0	143.0	341.6
	228.7	51.1	139.1	336.8

For each node we give two estimates. The upper estimate was generated by using an age of the split between *Chorthippus* and *Locusta* between 144 and 150 My as suggested by fossils, whereas the second estimate was generated using an age between 88 and 98 My. Note that the two calibrations generated very similar time estimates of the ages for the splits.

Bagine et al., 1989). However, mitochondrial DNA sequences generated recently considerable progress (e.g. Aanen and Eggleton, 2005). The tree of Aanen and Eggleton (2005) is in general agreement with our tree (e.g. *Hypotermes* within *Odontotermes*; position of *M. bellicosus*). However, the two trees have only few species in common.

One major problem in the taxonomy of the genus *Macrotermes* is that only a few taxonomic characters are available for a reliable identification of species. Taxonomists had problems in assigning collected material to described species (van der Werff, 1981). So populations with very different biologies (e.g. species building fundamentally different mound types) were often combined in one species (Darlington, 1984). This problem is clearly shown by our analysis. Populations of termite species sampled in East Africa and West Africa, which were assigned to the species *M. subhyalinus*, are definitely two different species. This is consistent with biological information: *M. subhyalinus* mounds in Kenya have many open ventilation chimneys, which give these mounds a typical appearance (Darlington, 1984). In contrast, mounds found in the Ivory Coast lack such chimneys (personal observation, RB and MK) and the appearance of these West African mounds is very similar to the mounds of *M. herus*. This fits our phylogenetic tree. But mounds which are similar from outside may have quite different internal structures. Furthermore, mound size and shape may change within certain limits with climatic conditions (Korb and Linsenmair, 1998).

There are other examples of cryptic species in our data set. The two samples both described to be *M. natalensis* may be two different species, although this is far from clear (see Table 3). Another example is the species pair *M. subhyalinus* (from Kenya) and *M. jeanneli*. Previously, Ruelle (1970) synonymized the two species, although they build very different mounds. *M. jeanneli* occurs in arid regions of Eastern Africa and builds impressive mounds with only one tall chimney (Darlington, 1984, 2005; Darlington et al., 1992). In contrast, the mounds of *M. subhyalinus* have numerous openings (Darlington, 1984). Our analyses suggest that the two species with open mound types are distinct sister taxa with a genetic distance larger than the distance between populations within species (Fig. 5). Furthermore, our tree suggests that the open ventilation system evolved only once. The two species are distributed in the more arid savannah regions of Eastern Africa and the open ventilation system may be an adaptation to hot climates.

Our analysis using the COII gene allowed no convincing resolution of deep splits in the genus *Macrotermes*. We cannot resolve the origin of the Oriental species (note that the COI sequences suggested a different branching pattern). Nevertheless, our data show that all the analysed Oriental species belong to clades, which date back to the early Tertiary. Furthermore, the deep genetic difference and the age of the clades (Table 4) may justify treating these clades as separate genera. In fact, the species *M. bellicosus* was assigned formerly to a separate genus, *Bellicositermes* (Ruelle, 1970). The deep genetic differences between *M. bellicosus* and the other species of African *Macrotermes* sequenced during our study are correlated with differences in biology: in *M. bellicosus* the small workers are the main mound builders, whereas in all other investigated Ethiopian species this is performed by the major workers.

Darlington (2005) described a fossilized mound found in Pliocene deposits with a single and tall chimney (1.5 m) as well as an internal structure very similar to the typical mounds built by *M. jeanneli*. The striking similarity between the recent and fossilized mounds shows that species building open mounds existed for a long period of

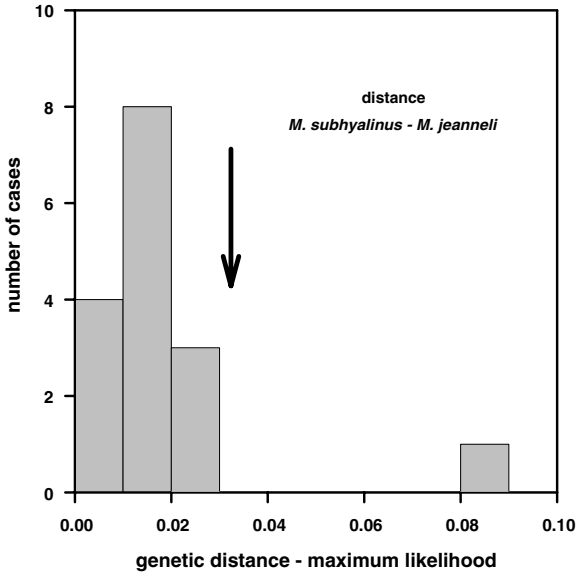

Fig. 5. Distribution of genetic distances within species (8 species; 16 cases; see Table 1). The arrow indicates the genetic distance between the sibling species *M. subhyalinus* and *M. jeanneli*, two species lumped by previous authors (see also Darlington, 2005). Genetic distances are maximum likelihood distances calculated with the settings suggested by MODELTEST.

time. Furthermore, the observations that the species building open mounds are sister species allowed to use the age of the fossil mound to date our tree, although there are several possibilities to calibrate nodes (Fig. 1). Clearly, one fossil remain provides no convincing basis to calibrate a molecular clock. Hence, we attempted to derive further estimates using the COI sequences, at least to check the calibration procedure. The COI and COII share the same evolutionary history (the two genes are linked on the mitochondrial genome) and therefore really independent time estimates would need genes with an independent evolutionary history. Regretfully, at present such data are not at hand. There are two results that suggest that the divergence times of the COI gene provide the correct order of magnitude for important events in the evolution of termites. Firstly, our analyses suggested a divergence time between cockroaches and termites of 229 Mya (credibility interval 143–342 Mya). Although the credibility interval is large, this estimate is consistent with an origin of the termites in the upper Jurassic (Thorne et al., 2000; Grimaldi and Engel, 2005). Second, the origin of the fungus-growing Macrotermitinae is estimated to have occurred around 62 Mya (credibility interval 30–108 Mya). Again this is consistent with the assumption that most of the subfamilies of the Termitidae appeared at the turn from the Cretaceous to the Tertiary (Thorne et al., 2000). Accepting that the estimates from the COI sequences are reliable, we compare the age of the genus *Macrotermes* as derived from the COI as well as COII sequences using different information for the calibration: the origin of the genus *Macrotermes* (node 31 in Fig. 3 and node 3 in Fig. 4) differs at maximum by only 4 My between the two analyses! Overall we are fairly convinced that our time estimates provides the correct order of magnitude.

Beside the reliability of the calibration dates (see also Fig. 1), Bayesian methods call for a number of priors which need to be specified at the beginning of an analysis (Alfaro and Holder, 2006). This is often an arbitrary step. Wahlberg (2006) showed that priors for the rate of molecular evolution at the ingroup may have substantial effects on the posterior estimates of dates for the nodes. Therefore, we run the program using extreme priors for the parameter of the rate of molecular evolution (parameter rrate between 0.2 and 0.002) as well as the time steps between root and tips (time steps between 20 and 100). As expected we found a decrease of the time estimates with increasing rrate as well as with decreasing the prior for the time steps between root and tips (see also Wahlberg, 2006). For example, for node 12 in Fig. 3, the time estimates varied from 12.8 (rrate = 0.2; time steps = 20) to 16.9 (rrate = 0.002, time steps = 100) and for node 31 between 36.9 and 52.2. Although these variations are substantial, they fall with the credibility intervals of the time estimates given in Table 3. Therefore, the main conclusions of our paper are robust against changes of the numerical values of the priors specified for the run of the program.

The phylogeny and the estimated dates of *Macrotermes* lineages allow to comment on two important issues in the biogeography and diversity of *Macrotermes*. First, according to our dating attempt, the basal splits within the genus *Macrotermes* occurred in the early Tertiary, a period with humid tropical climates. The habitats of the Asian *Macrotermes* examined in our study are forests. This is consistent with the suggestion that the original habitats of the fungus-growing termites were forests and that the Asian clades split off during humid climates. Second, the cycles of humid and arid periods during the Pleistocene were hypothesized to have triggered speciation in African termites (Eggleton et al., 1994; Brandl and Kaib, 1995; Eggleton, 2000). This idea is in line with a long tradition that interprets climatic fluctuations of the Pleistocene as an important driving force of genetic divergence and speciation (Rand, 1945; recent examples: Sullivan et al., 2000; Austin et al., 2002; Zamudio and Savage, 2003; Johnson and Cicero, 2004; Weir and Schluter, 2004). However, the study by Weir and Schluter (2004) showed that there is a latitudinal trend in the age of speciation events in birds: the ages of many tropical lineages predate the Pleistocene (see also Gaston and Blackburn, 1996). Our time estimates for the splits between *Macrotermes* species of clade III (Africa) show that at least several events predated the Pleistocene. Despite the large confidence intervals of our dates, many of the intervals exclude the Pleistocene. However, our taxon sampling is limited. For Africa we analysed 7 species. Kambhampati and Eggleton (2000) note that at least 13 species occur in Africa and therefore we covered only little more than 50% of the known species. The inclusion of more taxa as well as the possibility of the discovery of new species may change our conclusions. Furthermore,

our analysis is based on only one short stretch of mtDNA. Although several nodes are well supported, there is still much uncertainty. Nevertheless, the dated phylogeny of the genus *Macrotermes* supports the hypothesis that many splits in the African clade III occurred somewhere between 6 and 23 Mya (see Table 4). During that time savannahs spread over Africa (Coetzee, 1993).

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