

A comprehensive phylogenetic analysis of termites (Isoptera) illuminates key aspects of their evolutionary biology

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Abstract

The first comprehensive combined molecular and morphological phylogenetic analysis of the major groups of termites is presented. This was based on the analysis of three genes (cytochrome oxidase II, 12S and 28S) and worker characters for approximately 250 species of termites. Parsimony analysis of the aligned dataset showed that the monophyly of Hodotermitidae, Kalotermitidae and Termitidae were well supported, while Termopsidae and Rhinotermitidae were both paraphyletic on the estimated cladogram. Within Termitidae, the most diverse and ecologically most important family, the monophyly of Macrotermitinae, Foraminitermitinae, Apicotermitinae, Syntermitinae and Nasutitermitinae were all broadly supported, but Termitinae was paraphyletic. The pantropical genera *Termes*, *Amitermes* and *Nasutitermes* were all paraphyletic on the estimated cladogram, with at least 17 genera nested within *Nasutitermes*, given the presently accepted generic limits. Key biological features were mapped onto the cladogram. It was not possible to reconstruct the evolution of true workers unambiguously, as it was as parsimonious to assume a basal evolution of true workers and subsequent evolution of pseudergates, as to assume a basal condition of pseudergates and subsequent evolution of true workers. However, true workers were only found in species with either separate- or intermediate-type nests, so that the mapping of nest habit and worker type onto the cladogram were perfectly correlated. Feeding group evolution, however, showed a much more complex pattern, particularly within the Termitidae, where it proved impossible to estimate unambiguously the ancestral state within the family (which is associated with the loss of worker gut flagellates). However, one biologically plausible optimization implies an initial evolution from wood-feeding to fungus-growing, proposed as the ancestral condition within the Termitidae, followed by the very early evolution of soil-feeding and subsequent re-evolution of wood-feeding in numerous lineages.

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1. Introduction

1.1. Background and rationale

The order Isoptera consists of over 2600 species in around 280 genera, and seven currently recognized families (Engel and Krishna, 2004). Some 70% of these species occur in a single family, the Termitidae. Termites are recognized to be the most important decomposer animals in low-

land tropical ecosystems, where they can make up to 95% of the soil insect biomass (Eggleton et al., 1996). Termite species diversity is greatest in closed-canopy tropical rain forest, where much of the group's radiation has occurred, and where the Termitidae contribute over 90% of the species (Eggleton, 2000). The Termitidae not only constitute the majority of species, but also exhibit the widest range of ecological and behavioral diversity, including many traits fundamental to the evolution of the Isoptera, such as soil-feeding and fungus-growing. The ecological importance of termites, together with their uniquely derived social structures and cooperative behavior, makes the accurate reconstruction of termite phylogeny an important

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goal. We undertake such a phylogenetic study here based on the simultaneous analysis of molecular and morphological data.

1.2. Previous work

A number of phylogenetic studies have already been attempted (earlier studies are reviewed in Eggleton, 2001), but all have suffered from one or more fundamental problems, preventing them from being reliable and convincing. Perhaps most notably the studies have suffered from poor taxon sampling. Sometimes the studies have been heavily biased towards the less species-rich non-termitid families (Kambhampati et al., 1996), and sometimes they have dealt with just one biogeographic region (Ohkuma et al., 2004). The problem is at least partly due to the difficulty of dealing with the soldierless termites (Termitidae: Apicotermiinae), which generally lack good diagnostic characters, but which comprise large numbers of species, many of them undescribed (e.g. see Eggleton et al., 2002). Other problems include a lack of any rigorous cladistic analysis of the available data, or datasets based on relatively few characters, such as those using only mandible characters or gut anatomy in which many *a priori* assumptions of monophyly have been made without good supporting data (see Eggleton, 2001).

To date, the most comprehensive termite phylogenetic studies have tended to use morphological characters (Donovan et al., 2000; Bitsch and Noirot, 2002; Noirot, 1995a, 2001). Many of these characters suffer from a lack of independence, both from one another and from their functional roles. For example, many of the morphological characters, especially those of the soldier caste, are strongly influenced by defense or feeding strategies, so homoplasious convergence in form is frequent (Donovan et al., 2000). Molecular sequence data on the other hand, provide a wealth of characters that should be free of such non-heritable variation, and can be produced in an unambiguous manner. More recently then, a few molecular studies have been produced (e.g. Kambhampati and Eggleton, 2000; Miura et al., 1998; Ohkuma et al., 2004; Thompson et al., 2000) but these also suffer from relatively poor taxon sampling and an insufficient number of gene loci.

Our approach in this paper has been to address these methodological problems by sampling widely and comprehensively across the Isoptera and by employing multiple gene loci alongside morphological characters.

1.3. The need for a new phylogenetic analysis

A robust phylogenetic tree will allow us to address a number of taxonomic controversies, especially at the family and subfamily levels. We will test the monophyly and interrelationships of the various components of the existing classification (Table 1: the names used in this table will be the only formal family-group names used in this paper) and examine how far earlier phylogenetic hypotheses are

Table 1

Present accepted classification of termites modified from Engel and Krishna (2004) according to the scheme used in Kambhampati and Eggleton (2000)

Order Isoptera, Brullé (1832)	
Family	Mastotermitidae, Desneux (1904)
Family	Hodotermitidae, Desneux (1904)
Family	Termopsidae, Holmgren
	Subfamily Porotermitinae, Emerson (1942)
	Subfamily Stolotermitinae, Holmgren (1910)
	Subfamily Termopsinae, Holmgren (1911)
Family	Rhinotermitidae, Froggatt (1897)
	Subfamily Coptotermitinae, Holmgren (1910a)
	Subfamily Heterotermitinae, Froggatt (1897)
	Subfamily Prorhinotermitinae, Quennedey and Deligne (1975)
	Subfamily Psammotermitinae, Holmgren (1911)
	Subfamily Stylotermitinae, Holmgren and Holmgren (1917)*
	Subfamily Termitogetoninae, Holmgren (1910)
	Subfamily Rhinotermitinae, Froggatt (1897)
Family	Serritermitidae, Holmgren (1910)
Family	Termitidae, Latreille (1802)
	Subfamily Macrotermitinae, Kemner (1934)
	Subfamily Sphaerotermitinae, Engel and Krishna (2004)
	Subfamily Foraminitermitinae, Holmgren (1912)
	Subfamily Apicotermiinae, Grassé and Noirot (1954)
	Subfamily Syntermitinae, Engel and Krishna (2004)
	Subfamily Termitinae, Latreille (1802)
	Subfamily Nasutitermitinae, Hare (1937)

Fossil taxa not included. *Not sampled in this study.

congruent with our findings. In addition, a well-corroborated phylogenetic reconstruction will allow re-examination of some key questions concerning evolutionary transitions within the termites. These questions are:

- (1) Has the true worker caste evolved once or many times? (Noirot and Pasteels, 1988; Thompson et al., 2000).
- (2) Is dead-wood nesting and feeding an ancestral condition in termites? (Abe, 1987; Eggleton and Tayasu, 2001).
- (3) How many acquisitions and losses of wood- and soil-feeding have there been? (Donovan et al., 2001a).

2. Materials and methods

2.1. Taxon sampling

We sampled a broad range of Isoptera genera to represent the clade evenly across a wide geographic range (see [Supplementary Materials](#)). Extra attention was paid to the groups that were previously found to be morphologically intractable or highly divergent, such as the soldierless termites and some Nasutitermitinae. Termite specimens were collected by members of the Soil Biodiversity Group, with additional assistance from a number of collaborators (see Acknowledgments). We generated DNA sequences from 231 termite species in 131 genera, while an additional 11 genera are represented by GenBank sequences. Out-

groups were sequenced from the closely related cockroaches (Blattodea) and mantids (Mantodea), including the probable cockroach sister clade to the termites, *Cryptocercus* (Cleveland et al., 1934; Eggleton, 2001; Terry and Whiting, 2005).

2.2. Molecular data

Three genes were chosen for their complementary resolving power: two mitochondrial genes, ribosomal 12S and protein-coding cytochrome oxidase II (COII), and the nuclear ribosomal gene 28S. These provide a total of some 2000 base-pair characters. Total DNA was extracted from ethanol-preserved specimens using the DNeasy tissue kit by Qiagen. In some cases the presence of certain mineral or organic matter in the gut was found to inhibit PCR amplification, particularly in soil-feeding termites, so the abdomen or gut was subsequently removed from specimens before extraction. The small size of many termite workers, and their lack of either large mandibular or flight muscles, meant that there were often low DNA yields. Therefore soldiers or alates were preferentially used for DNA extraction, or, where neither soldiers nor alates were available, alternatively multiple workers from a single colony were used to increase the DNA yield.

A range of universal and termite-specific primers was used in PCR amplification (see [Supplementary Materials](#)). PCR samples were cleaned by PEG precipitation, and cycle sequenced using Applied Biosystem's BigDye Terminator v3.1 Cycle Sequencing Kit. Following purification by precipitation, automated sequencing was carried out on an Applied Biosystems 3730 DNA Analyser. GenBank accession numbers for this study are: 12S: DQ441611–DQ441841; 28S: DQ441842–DQ442052; and COII: DQ442056–DQ442267 (see [Supplementary Materials](#)).

2.3. Morphological data

We reviewed the morphological characters used in previous systematic studies of termites, primarily [Donovan et al. \(2000\)](#), [Noirot \(1995a,b, 2001\)](#) and [Sands \(1972\)](#). [Donovan et al. \(2000\)](#) reported considerable incongruence between soldier and worker characters, and this led to low overall statistical support for their cladogram. We therefore selected characters from these sources that were neither ambiguous, nor dependent upon other characters (auto-correlated), nor likely to be strongly influenced by environmental variation or their functional role. For example, we found the soldier mandible characters to contain much homoplasious signal when compared with the molecular and worker gut characters, presumably due to convergence of taxa with similar defensive requirements. Many characters originally derived for taxon identification proved to be uninformative in a phylogenetic context because they were polymorphic, invariable or impossible to code convincingly (e.g. colour characters). The majority of the 40 morphological characters selected are of the worker gut, including

those of the Malpighian tubules and enteric valve, and many of them are derived directly or indirectly from Noirot's seminal works on the termite gut ([Noirot, 1995a, 2001](#)). Character data were taken directly from [Donovan et al. \(2000\)](#) and [Sands \(1972\)](#), or coded using specimens in the collections of the Natural History Museum, London.

2.4. Phylogenetic analysis

Since length variation and mutation rate variation is considerable in the AT-rich ribosomal genes, we have chosen to explore one key parameter of uncertainty, alignment, while minimising the number of auxiliary assumptions for the phylogenetic analysis itself. The examination and modification of penalties is necessary to establish the sensitivity of the phylogenetic conclusions to alternative homology assignments at the nucleotide level. The two rRNA genes were length variable throughout while in the protein-coding COII gene locus only the presence of a length variable intergenic region before the 5' end of the gene was affected. Phylogenetic analysis of the combined molecular and morphological character data was conducted under the principle of parsimony in two contrasting ways, differing primarily in the manner of sequence alignment. In the first instance, sequence alignment was performed for each gene in turn through multiple sequence alignment using the program Clustal X ([Thompson et al., 1997](#)), followed by phylogenetic analysis on the resulting aligned data matrices, combined with the morphological data, under parsimony using PAUP* version 4.0b10 ([Swofford, 1999](#)). The second method used direct optimization ([Wheeler, 1996, 2001](#)) of length variable sequences with the program POY ([Gladstein and Wheeler, 1997](#)) conducting searches for the best trees by optimizing indels and nucleotide substitutions, plus morphological character changes, in a simultaneous analysis under parsimony.

2.5. Determination of optimum Clustal alignments

For each gene we explored a range of gap opening costs in Clustal X. Gap extension penalties were held equal to the opening penalties, and all penalties were similarly held equal between the pairwise and multiple alignment steps (specific parameters are in [Table 2](#)). Choosing one Clustal alignment over another may lead to radically different tree topologies. Rather than judging the effectiveness of differing gap costs by the subsequent topologies produced to test for the quality of the homology assignments on a phylogenetic tree (e.g. [Wheeler, 1995](#)), we assessed the quality of the alignments themselves based on the recovery of the most conserved nucleotide strings (see [Simmons, 2004](#)). For each of the three genes, 10 clearly defined, length-conserved 'landmark regions' were selected. These regions, ranging from 7 to 123 base pairs and distributed throughout the length of each gene, were then used to score the effectiveness of the alignment process in and immediately adjacent to that part of the gene. The landmark regions

Table 2
Summary of Clustal parameters used and results from parsimony analyses

Analysis	Clustal alignment gap opening/extension costs & POY gap:change cost	Number of steps	Total number of characters	Number of informative characters	Consistency index	Retention index	Number of resolved nodes (strict consensus)	Rohlf's consistency index (strict consensus)	Number of nodes congruent with preferred tree	Rohlf's CI with preferred trees
<i>(A) All available data included</i>										
1	(preferred alignment) 12S = 5, 28S = 5, COII = 10	15160	1991	915	0.1414	0.5185	216	0.944	n/a	n/a
2	(suboptimal alignment 1) 12S = 2, 28S = 2, COII = 5	14671	2140	915	0.145	0.5151	221	0.868	155	0.535
3	(suboptimal alignment 2) 12S = 10, 28S = 10, COII = 10	15445	1933	909	0.1394	0.5306	221	0.847	152	0.48
<i>(B) GenBank sequences excluded</i>										
4	12S = 5, 28S = 5, COII = 10	14502	1998	911	0.1476	0.5216	213	0.95	138*	0.216*
<i>(C) POY searches</i>										
5	1:1	14744	2881	862	0.1402	0.5214	239	0.98	115	0.105
6	2:1	15551	2731	865	0.136	0.5174	242	0.999	98	0.028

* Analysis carried out with GenBank sequences deleted from preferred tree.

were selected from 'ungapped' and highly conserved sequence regions, in the same manner as one might identify suitable sites for primer binding across the same taxon sample. The landmark regions were identified in each sequence in an alignment, and all sequences scored according to the presence and degree of misalignment (see Table supp 2 in Supplementary Materials) evident within each of the landmark regions, and in the adjacent areas of sequence. The set of alignment parameters producing fewest misaligned regions was judged to be the preferred alignment. Two additional 'suboptimal' alignments, with the next best scores, were also selected for analysis, in order to examine the effect of alignment upon the tree topology.

An incongruence length difference (ILD) test was carried out using PAUP* to determine the level of congruence between the different data partitions (Mickevich and Farris, 1981; Sanderson and Shaffer, 2002; Swofford, 1991).

The preferred alignments for each gene were analysed with the morphological data in a simultaneous analysis. Phylogenetic searches were conducted under parsimony using PAUP* version 4.0b10 (Swofford, 1999). In each case, analysis was carried out as follows: heuristic search, 10,000 random addition replicates, tree bisection and reconnection (TBR) branch-swapping, all character positions weighted equally, gaps treated as missing data, saving 1 tree only at each step. The shortest tree saved was then used in a subsequent heuristic search, as a starting tree for TBR branch-swapping, saving all most parsimonious trees. Consensus trees were then produced. This methodology was adopted to maximise the number of replicates performed on the large data sets, to identify multiple 'tree islands' (Maddison, 1991), followed by a more intensive search to determine the most parsimonious trees from the initial set. The two suboptimal alignments were analysed in the same manner, as was an additional analysis in which the 13 taxa represented solely by GenBank sequences were excluded.

2.6. Tree statistics

For all Clustal and POY alignments produced, the number of parsimony-informative characters were calculated, and all trees produced were evaluated regarding their fit to the data, using the ensemble consistency index (CI) and retention index (RI). To examine the consistency of the topologies combined in the consensus trees, the number of resolved nodes and Rohlf's modified CI were calculated. The tree topologies resulting from each analysis were summarised and compared according to the key systematic relationships that were recovered in each case, enabling us to determine how robust to alignment parameters the topology is.

Decay indices (Bremer support) were calculated for each node on the preferred tree using the program AutoDecay (Eriksson, 2001) and PAUP*. AutoDecay produces constraint trees for the nodes, which are then analysed using reverse constraints in PAUP*. Phylogenetic searches were

conducted with PAUP* on a cluster of six computers, constraining each of the 243 nodes in turn, using the search parameters described above, but with 1000 replicates in the initial heuristic search to reduce computing time.

In order to examine the degree to which our analyses agree with earlier taxonomic hypotheses we used the ‘constraints’ option within PAUP* to force groups of interest to be monophyletic and then identical MP analyses as described above were undertaken on the constrained datasets so as to assess the number of extra steps needed to make the taxon of interest monophyletic. Kishino–Hasegawa tests (in PAUP*) were undertaken for statistical tests of alternative hypotheses comparing the constrained trees with the most parsimonious trees.

2.7. Mapping of biological traits—definitions and hypotheses

2.7.1. Presence of true workers

True workers are fundamental to the evolution of social systems in insects (Wilson, 1992), and are defined here as individuals which have irreversibly deviated from developing into winged reproductives (Noirot and Pasteels, 1988). More loosely, they are usually sterile, and primarily perform helper tasks. True workers are found in all termite families except all Kalotermitidae, all Termopsidae and *Prorhinotermes*, which all have pseudergates or ‘false workers’ (Noirot and Pasteels, 1988). These perform helper tasks but can continue development to become soldiers or full reproductives.

Two hypotheses compete regarding the evolution of workers in the Isoptera. The first suggests that workers developed independently on at least three separate occasions in the Mastotermitidae, the Hodotermitidae and the Rhinotermitidae + Termitidae (Noirot and Pasteels, 1987, 1988). The alternative is that workers are a primitive aspect of termite eusociality that evolved once, and were lost in the Kalotermitidae, Termopsidae and *Prorhinotermes* (Thompson et al., 2000; Watson and Sewell, 1981).

2.7.2. Nesting type

A wide variety of nests are found in the Isoptera, with this diversity most likely to have evolved alongside social behaviour and relating to defence strategy and the establishment of an optimal microclimate for the colony (Noirot and Darlington, 2000). We will map Abe’s (1987) three categories of nest development onto our tree. Single-piece nesting is generally thought to be the most primitive form, in which the colony nests in and is entirely restricted to a piece of dead wood. In consequence, the size of the substrate dictates the longevity of the colony (e.g. *Zootermopsis*). Intermediate-type nesting involves the production of subterranean galleries that allow colonization of new pieces of wood, but this still remains both the food source and nest site (e.g. some *Schedorhinotermes*). Conversely, separate-piece nests are completely separate from the food source (e.g. *Macrotermes*). As it can be difficult to apply

Abe’s original scheme across the whole of the Isoptera, we have added a fourth category, soil-nesting, in which the termites both feed on, and nest in the soil (e.g. *Anentotermes*, and also includes termites that build epigeal mounds from soil, such as *Cubitermes*).

The main hypothesis relating to nesting type is that the ancestral termite must have been a single-piece wood nester/feeder as no other wood-nesting Dictyoptera (from which termites presumably evolved) build nests away from their feeding substrate. A secondary hypothesis is that the loss of true workers was linked evolutionarily to the presence of the single-piece nesting type (Abe, 1987), because termites that feed and nest in the same substrate have (a) no need for a permanent foraging worker caste and (b) need to retain developmental plasticity because dead wood may easily become fragmented so that sub-colonies become isolated without fertile queens.

2.7.3. Feeding groups

Termites, uniquely among insects, feed on plant material across all parts of the humification gradient (Bignell and Eggleton, 1995), from living plants at one extreme, through to dispersed soil organic matter (SOM) at the other. A system devised by Donovan et al. (2001a) classified termite feeding groups according to their gut contents and functional morphology as follows:

Group I: taxa with relatively simple guts, and including all lower termites. Predominantly wood feeders, they feed high up the humification gradient.

Group II: these higher termites have a more complex gut, but still feed high up the humification gradient on dead wood, epiphytes or leaf litter.

Group III: having a similarly complex gut, they feed in the humus layers on organic-rich soil with a significant amount of recognisable plant material.

Group IV: these have a highly complex gut and are true soil-feeders, feeding on SOM with a high mineral content and little recognizable plant material.

Feeding behaviour is a good indicator of lifestyle and ecology (Eggleton and Tayasu, 2001), and will be mapped onto our phylogenetic tree to assess how feeding behaviour has evolved across the order. We have included an extra feeding category, group II_f, which includes just the Macrotermitinae. These share similar characteristics to the group II feeders, but have evolved the ability to cultivate the fungus *Termitomyces* inside the colony. The mutualistic fungus breaks down the ligno-cellulose in the wood, and the termites subsequently feed on the growing fungus (Rouland-Lefèvre, 2000).

Donovan et al. (2001b) presented an evolutionary scheme for the evolution of feeding groups and we test this scheme here using our new data. Specifically we test the evolutionary hypothesis that soil-feeding evolved from wood-feeding through a fungus-growing intermediate stage.

2.7.4. Coding and mapping

All species were coded according to their worker condition, their nesting strategy, and their feeding group. These data were passively mapped onto the preferred tree under the criteria of maximum parsimony, using MacClade v. 4.0 (Maddison and Maddison, 2000). Where ancestral states could not be resolved at nodes the most parsimonious states were adopted, which may be ambiguous, but also makes fewest assumptions about the evolution of these characters (i.e. when compared with using either ACC-TRAN or DELTRAN optimizations).

3. Results

3.1. Congruence and alignments

We tested which Clustal gap costs minimised the severity and number of misalignments for each gene (see Table supp 2 in the Supplementary Materials). Two 'sub-optimal' alignments were selected for further analyses (12S and 28S), while only one was chosen for COII, as all other gap costs produced particularly poor-scoring alignments. The total number of characters under the different alignment parameters varied from 1933 to 2881, with the number of parsimony informative characters ranging from 862 to 915 (Table 3). The mean overall A/T content of the loci across all taxa was 66.7% for 12S, 64.4% for COII, and 41.4% for 28S.

There was no significant incongruence between the data partitions (three gene regions and morphology, ILD test, $p = 0.93$). The MP analysis using the preferred alignment produced 187,200 equally parsimonious trees differing only in the most apical nodes. From these a strict consensus tree was constructed (Figs. 1–3).

The preferred alignment gives a tree topology (Figs. 1–3) and tree statistics (Table 3) that are very similar to both the sub-optimal alignments and the POY analyses (Table 2). Most major clades were recovered across the range of alignment parameters and between different analytical methods (Clustal and POY). However, the POY analysis gap:change cost of 2:1 produced some clearly spurious results in comparison both to the other trees and to the currently understood systematics of termites.

We can now summarise our key findings from our analyses (Figs. 1–3) in the context of the present classification (Table 1). *Mastotermes darwiniensis* is the sister group of all other extant termites. The Termopsidae are consistently recovered as a paraphyletic group, within which are nested the monophyletic family Hodotermitidae. The Kalotermitidae are also monophyletic, while the Rhinotermitidae are paraphyletic with respect to the Termitidae. The monotypic family Serritermitidae (*sensu* Emerson and Krishna, 1975) is nested within the Rhinotermitidae. Within the Termitidae, the Macrotermitinae are a monophyletic group sister to the rest of the Termitidae, and the subfamilies of Foraminitermitinae and Sphaerotermitinae form a monophyletic clade. Of the other subfamilies, we find the Termitinae to

be paraphyletic, and the soldierless termite family Apicotermiteinae, as defined by Sands (1972), to be monophyletic. One particularly interesting finding is that the Syntermitinae (the mandibulate nasutes) does not cluster with the true nasutes (Nasutitermitinae) in any analysis but is nested within the Termitinae.

The KH tests showed that most of the alternative taxonomic hypotheses (i.e. putative clades not recovered by our MP analysis) did not require significantly more steps than our MP tree. The only exception was the Termitinae (Table 3).

3.2. Mapping of ecological traits

3.2.1. True workers

True workers have evolved on three independent occasions on our tree (Fig. 4a): in the basal group of *Mastotermes*, in the Hodotermitidae, and again at the base of the Rhinotermitidae + Termitidae. There is also a single reversal event (where true workers are secondarily lost), in *Protermitermites*.

3.2.2. Nesting type

Single-piece nesting appears to be the ancestral condition for termites (Fig. 4b), and it is evident that this behaviour is only found in those groups in which true workers are absent, including *Protermitermites*. Separate piece nesting appears to have evolved at least five times.

3.2.3. Feeding groups

This is more complex (Fig. 5). The most basal group of the Termitidae, the Macrotermitinae, have the relatively simple guts of the group II feeders, although they have the highly derived fungus-gardening behaviour. Importantly, we see the acquisition of the group IV soil-feeding habit very early on in the Termitidae, and multiple evolutions of both group III and group IV soil-feeding. There seems to be considerable plasticity of feeding behaviour, which has led to the equivocal character states for much of the backbone of the topology. One striking feature is the early evolution of group IV soil-feeding, with multiple shifts from group IV to other feeding groups.

4. Discussion

4.1. Termite systematics and relation to present classification

4.1.1. Family relationships

Mastotermes has been recovered as sister to all other extant termites in nearly all previous studies, including this one, but relationships between the other families has been disputed, particularly the placement of the Termopsidae, Hodotermitidae and Serritermitidae (Table 4). Poor taxon sampling in previous studies may have led to the Termopsidae being thought monophyletic, a hypothesis which is not corroborated by our topology, which finds a monophyletic Hodotermitidae nested within this paraphyletic group,

Table 3
Results of phylogenetic analyses of the different Clustal alignments described in Table 2

Analysis	1	2	3	4	5	6	Majority consensus (%)	Step diff. if not M (KH test, <i>p</i>)
Tree length	15160	14671	15445	14503	14744	15551		
Clustal gap costs: 12S, 28S, COII/POYgap:change cost	5,5,10	2,2,5	10,10,10	5,5,10	1:1	2:1		
<i>Isoptera families</i>								
Hodotermitidae	M	M	M	M	M	M	M (100)	
Termopsidae	Pa	Pa	Pa	Pa	Pa	M	Pa (83.3)	3 ns
Kalotermitidae	M	M	M	M	M	M	M (100)	
Rhinotermitidae	Pa	Pa	Pa	Pa	Pa	Pa	Pa (100)	24 ns
Termitidae	M	M	M	M	M	M	M (100)	
Mastotermitidae sister to all other Isoptera	Y	Y	Y	Y	Y	X	Y (83.3)	
Hodotermitidae + <i>Hodotermopsis</i> (Tp)	M	M	M	M	Pa	Pa	M (66.6)	
Serritermitidae + <i>Termitogeton</i> (RT)	M	M	M	n/a	Po	Po	M (60)	
Rhinotermitidae + Termitidae	M	M	M	M	M	M	M (100)	
Coptotermitinae + Heterotermitinae	M	M	M	M	M	M	M (100)	
Rhinotermitinae	M	M	M	M	Pa	M	M (83.3)	
<i>Termitidae subfamilies</i>								
Macrotermitinae	M	M	M	M	M	M	M (100)	
Apicotermitinae	M	M	M	M	M	Pa,a	M (83.3)	
Termitinae	Pa	Pa	Pa	Pa	Pa	Pa	Pa (100)	45*
Syntermitinae	M	M	M	M	M,a	Pa	M (83.3)	
Nasutitermitinae	M,a	M,a	M,a	M,a	M,a	M,a	M (100)	
Foraminitermitinae (<i>Labritermes</i> + <i>Foraminitermes</i>)	M	M	M	M	M	Po	M (83.3)	
Foraminitermitinae + Sphaotermitinae	M	M	M	M	M	Po	M (83.3)	
Nasutitermitinae + Syntermitinae	Po	Po	Po	Po	Po	Po	Po (100)	16 ns
Macrotermitinae sister to all other Termitidae spp.	Y	Y	Y	Y	Y	Y	Y (100)	
Cubitermes group**	M	M	M	M	M	M	M (100)	
<i>Termes</i> group	Po	Pa	Po	Pa	Po	Po	Po (66.6)	19 ns
<i>Amitermes</i> group	Pa	Po	M	Po	Pa	Pa	Pa (50)	9 ns
<i>Pericapritermes</i> group	Po	Po	Po	Po	Po	Po	Po (100)	17 ns
<i>Apicotermes</i> group	Pa	M	M	Po	M	Po	M (50)	3 ns
<i>Anoplotermes</i> group (S. American Apicos)	M	M	Pa	M	M	M	M (83.3)	
<i>Astalotermes</i> group	M	M	M	M,a	M	M	M (100)	
<i>Apicotermes</i> gp + all other <i>Anoplotermes</i> gp spp.	M	M	M	Pa	M	Pa,a	M (66.6)	
<i>Neocapritermes</i> + <i>Planicapritermes</i>	Po	M	M	M	Po	M	M (66.6)	5 ns
(Protohamitermes + Orientotermes) + Nasutitermitinae	Pa	Pa	Pa	M,b	M	Po	Pa (50)	13 ns
<i>Subulitermes</i> group	M	M	Pa	M	Po	Po	M (50)	
<i>Cephalotermes</i> + <i>Cylindrotermes</i>	M	Pa	Po	M	M	M	M (66.6)	

M, monophyletic; Pa, paraphyletic; Po, polyphyletic; **, excluding *Tuberculitermes bycanistes*; a, monophyletic with inclusion/exclusion of stray group; b, *Protohamitermes* excluded in this analysis. *Apicotermes*-group, all African genera with soldiers; *Anoplotermes*-group, all South American Apicotermitinae; *Astalotermes*-group, all African soldierless genera, excluding *Adaiphrotermes*; *Subulitermes*-group: South American Nasutitermitinae genera, excluding *Nasutitermes* and *Angularitermes*; *Termes*-group: *Pericapritermes*-group plus *Spinitermes*, *Cavitermes*, *Termes*, *Dihoplotermes*, *Xylochomitermes*, *Ephelotermes*, *Macrognothotermes*, *Paracapritermes*, *Cristatitermes*; *Pericapritermes*-group: *Pericapritermes*, *Homalotermes*, *Mirocapritermes*, *Procapritermes*, *Sinocapritermes*, *Dicuspiditermes*, *Planicapritermes*, *Neocapritermes*; *Amitermes*-group: *Amitermes*, *Prohamitermes*, *Globitermes*, *Orthognathotermes*, *Promirotermes*, *Angulitermes*, *Drepanotermes*, *Cephalotermes*, *Cylindrotermes*, *Microcerotermes*, *Protohamitermes*, *Orientotermes*. KH test labels: ns, not significant.

* $p < 0.1$ in a one-tailed test.

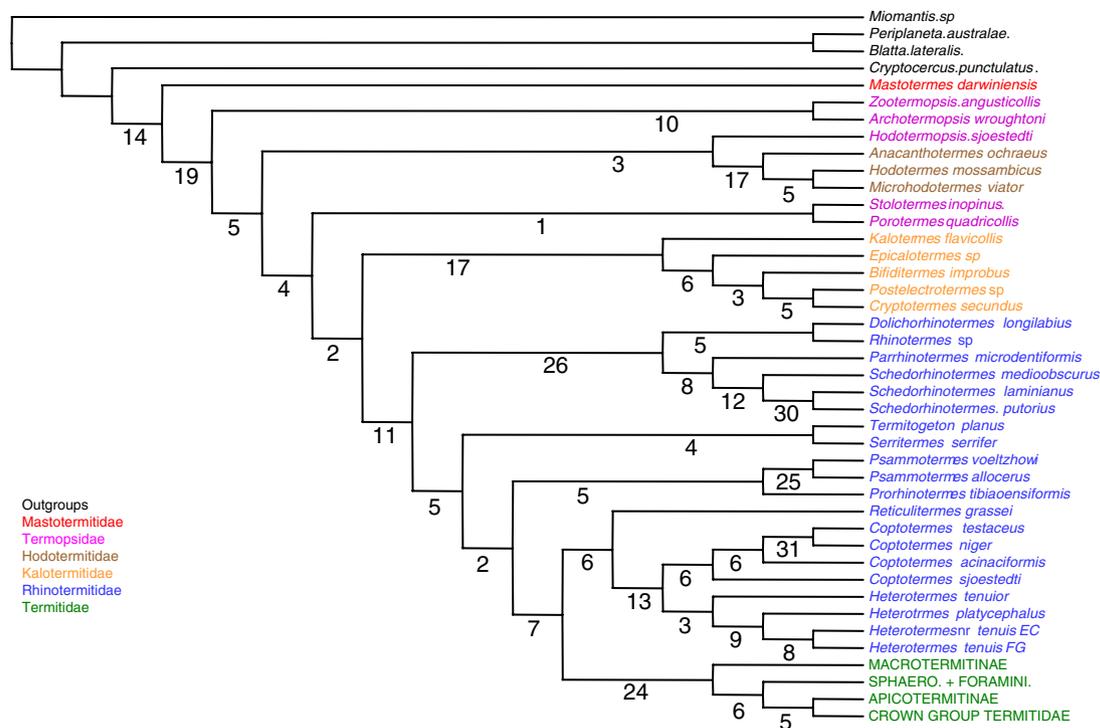


Fig. 1. The phylogeny of Isoptera inferred from simultaneous analysis of molecular and morphological data. The tree shown is based on the preferred Clustal alignment, as a strict consensus of the 187,300 equally parsimonious trees. Numbers on the branches represent Bremer support values. The existing classification is indicated by coloured species names. With few exceptions, the preferred tree represents very well the consensus of key relationships recovered by the different analyses (Table 4). The trees are cladograms and so the branch lengths shown are arbitrary. Leaves without species names are generally undescribed species, which have been given a code according to where they were collected. Termitidae tree continued in Fig. 2. (For interpretation of the references to colors in this figure legend, the reader is referred to the web version of this paper.)

rather than basal to it (Donovan et al., 2000; Kambhampati and Eggleton, 2000), or sister to it (Thompson et al., 2000). However, only three additional steps are required to make the Termopsidae monophyletic (Table 3). Another family which may also warrant reclassification is the enigmatic Brazilian Serritermitidae, which has been consistently recovered within the part of the (paraphyletic) Rhinotermitidae in all of our analyses, rather than sister to the Rhinotermitidae + Termitidae as suggested by several of the previous studies. The Rhinotermitidae is paraphyletic on our tree, just as has been found in most recent studies (Ohkuma et al., 2004). The sister group of the Termitidae on our tree is one subset of the Rhinotermitidae: the Coptotermitinae plus Heterotermitinae. The Kalotermitidae is, as expected, found to be monophyletic, although our intra-familial taxon sampling of the group is not as complete as for other clades. Their position on the tree now seems to be well corroborated: the kalotermitids are the sister group of the rhinotermitids (including the serritermitids) plus termitids.

4.1.2. Relationships within the Rhinotermitidae

Our taxon sampling within the Rhinotermitidae is not as good as in the Termitidae, and we have failed to sequence one subfamily, the Stylotermitinae (Table 1). Our findings, which are in line with other research (e.g. Ohkuma et al., 2004), show a paraphyletic Rhinotermitidae, with strong

support for only two major clades: the early branching Rhinotermitinae and the late branching [Coptotermitinae plus Heterotermitinae]. The other rhinotermitid clades are an apparent ragbag of poorly supported groups consisting of monotypic subfamilies.

4.1.3. Relationships within the Termitidae

The monophyly of the Termitidae is well established, and is supported by our topology. Within the higher termites, we find the fungus-growing termites (Macrotermitinae) to be a clade, sister to the remaining termitids. *Sphaerotermes*, which shares many plesiomorphies with the Macrotermitinae but does not cultivate *Termitomyces* fungus (Sands, 1972), has recently been placed in its own subfamily, Sphaerotermitinae (Engel and Krishna, 2004). We recover this monotypic group as sister to the Foraminitermitinae, a group first proposed by Holmgren (1912), and re-established by Engel and Krishna (2004). It comprises two enigmatic genera (*Foraminitermes* and *Labritermes*) that have some symplesiomorphic gut characters (Krishna and Adams, 1982), but are functionally group III soil-feeders.

The Apicotermitinae, as defined by Noirot & Grassé (1954) and Sands (1972), form a well-supported monophyletic group on our tree, consisting of several African species groups, the Oriental *Speculitermes*-group, and the Neotropical *Anoplotermes*-group. Together they form a clade



Fig. 2. Tree covering Termitidae from Macrotermitinae to the *Termes*-group. Features as in Fig. 1. Black arrow, acquisition of asymmetrical snapping mandibles in the soldiers. Sub-group 1 continued in Fig. 3. (For interpretation of the references to colors in this figure legend, the reader is referred to the web version of this paper.)

with a widespread lack of soldiers, and with group III and group IV soil-feeding habits. Of the genera sampled here, only the African genera *Jugositermes*, *Eburnitermes*, *Coxotermes* and *Allognathotermes* retain the soldier caste, and these are found as a paraphyletic group basal to the soldierless termite clade. This suggests a single loss of soldiers in this group. This is the first study to have confirmed unequivocally the monophyly of the Apicotermiinae, vindicating at least this part of the present classification.

The subfamily Termitinae is consistently found to be a paraphyletic group with respect to the Nasutitermitinae and the *Syntermes*-group. The Termitinae consists of a number of distinctive species groups of which several form well supported clades. The most basal of these is the *Cubitormes*-group, unique in that it consists entirely of group IV soil feeders, with highly derived and distinctive guts. The Termitinae is the only group which fails the KH test of congruence with our preferred tree (Table 3). However, this

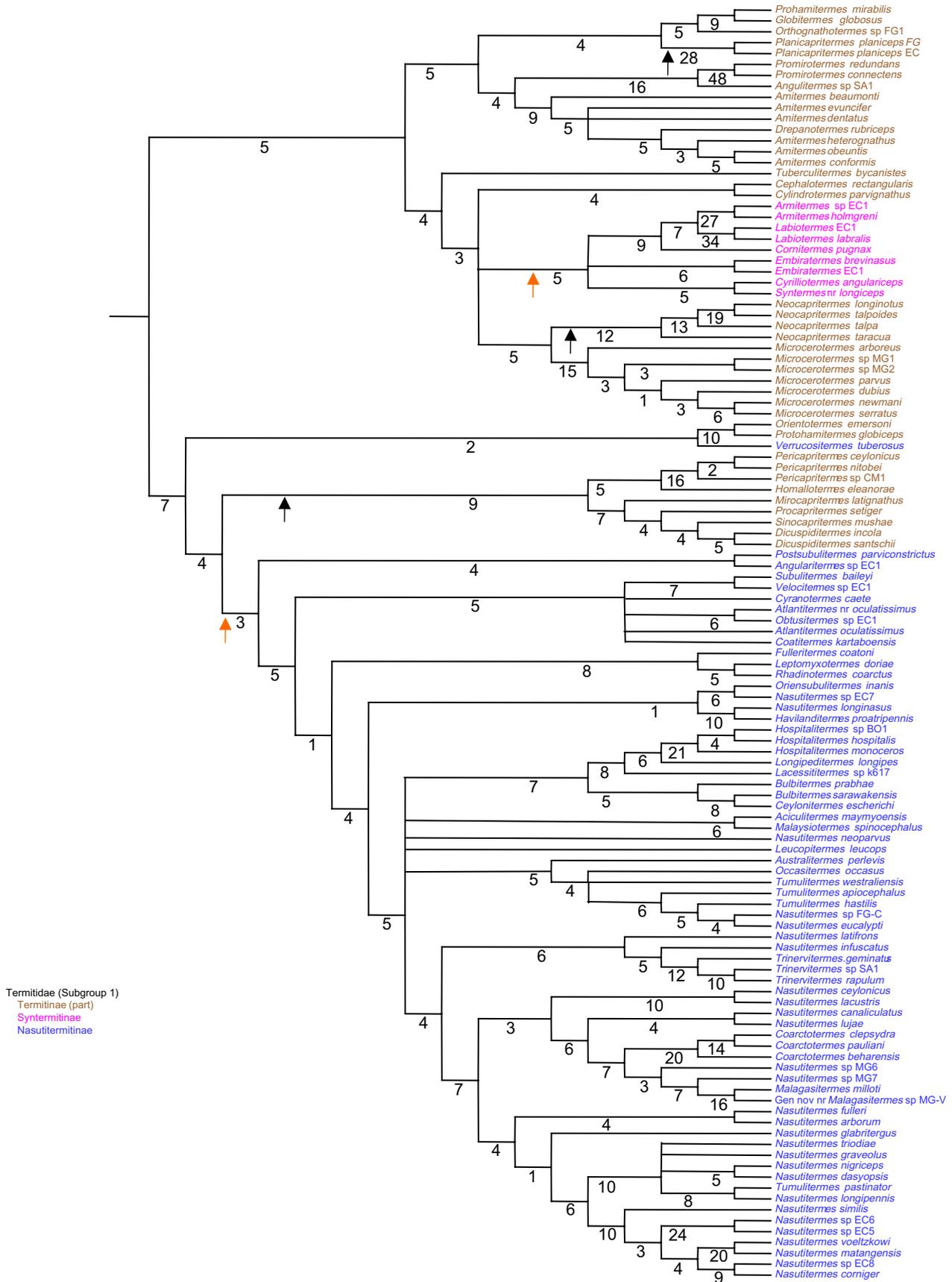


Fig. 3. Tree covering the rest of Termitidae. Features as in Fig. 1. Black arrow, acquisition of asymmetrical snapping mandibles in the soldiers. Orange arrows, acquisition of a nasus. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

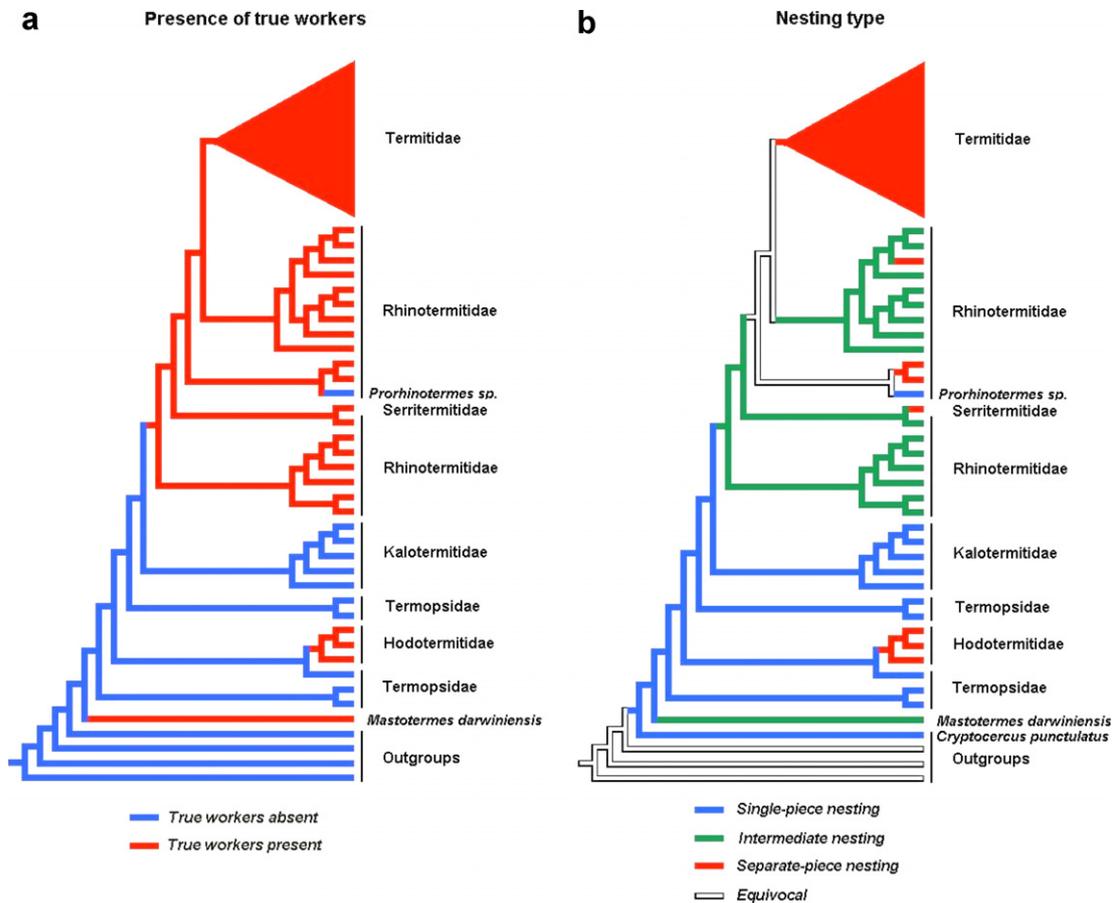
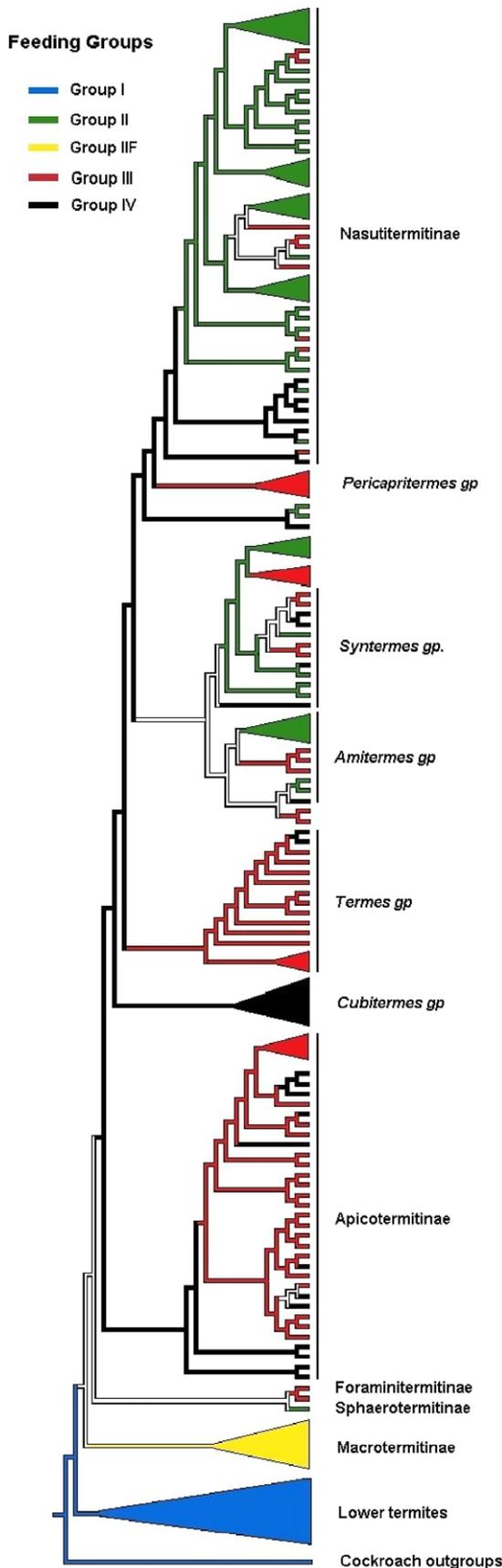


Fig. 4. Tree with characters mapped on using maximum parsimony, comparing presence of (a) true workers and (b) nesting type. (For interpretation of the references to colors in this figure legend, the reader is referred to the web version of this paper.)

may not be particularly meaningful as the KH test is known to be very conservative.

Soldier mandible characteristics have been used in the past to help define species groups within the Termitinae, such as the symmetrical snapping mandibles of the *Termes*-group soldiers, and the distinctive asymmetrical snapping mandibles of the *Capritermes*-group soldiers. These mandibular structures have been thought to form a character gradient of increasingly asymmetrical mandibles within a single clade (e.g. Krishna, 1968). The most extensive previous study using such mandibular characters recovered the *Termes* and *Capritermes* groups as sister (Donovan et al., 2000). However, our new topology suggests a clade of *Termes*-group species, consisting of predominantly group III feeders of wide biogeographic origins, but a polyphyletic *Capritermes*-group. On our trees, then, the asymmetrical snapping mandibles have evolved four times (indicated by black arrows on Figs. 2 and 3), and this supports our assertion that soldier mandible characters are rather plastic and may be influenced more strongly by environmental pressures (in this case adaptive constraints on defensive strategy) than by shared ancestry. This is perhaps seen most strikingly in the South American genera *Dihoplotermes*, *Neocapritermes* and *Planicapritermes* which have very similar asymmetrical mandibles but which are on three distinctly different parts of the termitid tree.

A closely parallel example of such morphological plasticity is evident in the Nasutitermitinae, which we found, as proposed by Bitsch and Noirot (2002), to be polyphyletic, with a phylogenetically separate *Syntermes*-group (*Syntermitinae sensu* Engel and Krishna) and *Nasutitermes*-group (*Nasutitermitinae sensu* Engel and Krishna). Both groups have a highly distinctive nasus used to spray defensive chemical secretions on to predators (primarily ants). However, while the *Syntermes* soldiers retain large sickle-shaped piercing mandibles to immobilise an attacker (Prestwich, 1984), the mandibles of the ‘true’ nasute species are vestigial. Previous workers have quite understandably considered the mandibulate nasutes to be ancestral to the rest of the nasute termites, with a single origin of the nasus and subsequently a single (or possibly diphyletic; Miller, 1986) loss of defensive mandibles. By deliberately excluding some of the more traditional but apparently convergent morphological characters, we have now demonstrated the diphyletic origin of this highly derived defensive strategy (indicated by orange arrows on Fig. 3), which would appear to have proven extremely successful in the true nasutes, since they form the most species-rich and biogeographically widespread group of the termites (Eggleton, 2000). In fact, a diphyletic origin was previously suggested for the nasus by Ahmad (1950); (although within a monophyletic *Nasutitermitinae*



including Syntermitinae), but until now has been untested and deemed unlikely.

A number of putative clades are poorly supported on our tree. This includes a number of rhinotermitid groups (e.g. *Psammotermes*/*Prorhinotermes*, *Serritermes*/*Termitogiton*) where taxon sampling is less complete, either due to a lack of suitable material or because the extant species are sparsely distributed across those parts of the tree. Other parts of the tree are comprehensively sampled but still weakly supported. This is particularly so in the part of the tree spanned in Fig. 3 by *Planicapritermes* to *Microcerotermes*.

There are also a small number of our recovered clades that seem very unlikely to be truly monophyletic. The most striking of these is the proposed monophyly of *Verrucositermes* (Nasutitermitinae) plus *Protohamitermes*/*Orientotermes* (Termitinae). *Verrucositermes* appears morphologically to belong to a group of African soil-feeding nasutes that probably includes *Postsubulitermes* (Sands, 1965). In contrast, *Protohamitermes* and *Orientotermes* are systematic oddities, both having no soldier caste, and having previously thought to be close to *Amitermes* (Ahmad, 1976). It may be that long branch attraction is grouping the three taxa together. Additional taxon sampling within the *Postsubulitermes*-group may be required to tackle this problem.

There are a number of very species-rich pan-tropical genera presently recognised within the Isoptera: *Coptotermes*, *Heterotermes*, *Microcerotermes*, *Amitermes*, *Termes*, and *Nasutitermes*. Our analysis suggests that the first three of these may be monophyletic but that the last three are not. *Amitermes* is made paraphyletic by *Drepanotermes*, an Australian grass-feeding genus already thought to be very close to *Amitermes*. *Termes* is similarly made paraphyletic by a group of Australian genera described by Miller (1987). In the case of *Nasutitermes* we have sequenced representatives of 17 presently described genera that are nested within a paraphyletic *Nasutitermes*. If we are to have monophyletic genera across the whole termite tree then the number of genera will have either to increase greatly (to represent multiple monophyletic sub-groups) or be drastically cut. Our view is that *Drepanotermes* and the Australian *Termes*-group genera will probably have to be sunk within their nesting genera (*Amitermes* and *Termes*, respectively), but that the *Nasutitermes* problem is too extensive to allow all the existing genera to be included in *Nasutitermes* sensu lato, and that inevitably a large number of new genera will have to be erected within the Nasutitermitinae. However, especially in the case of *Nasutitermes*, more extensive taxon sampling will be required before a formal reclassification will be either possible or desirable.

Fig. 5. Tree with characters mapped on using maximum parsimony, showing feeding groups. White branches are equivocal. Lower termites are shown as monophyletic merely for graphical convenience. (For interpretation of the references to colors in this figure legend, the reader is referred to the web version of this paper.)

Table 4

Comparisons of recent phylogenetic reconstructions compared with the tree presented in this paper

Present classification	Our findings (2006)	Donovan et al. (2001b)	Noirot & Bitsch (2002)	Family consensus (Eggleton, 2001)
Mastotermitidae	M [all other I]	✓	—	✓
Hodotermitidae	M [part Te]	S [Te, K, Rh, Se, Tm]	—	S [all rem I]
Termopsidae	Pa	M [K, Rh, Se, Tm]	—	✓
Kalotermitidae	M [Tm, Rh, Se]	Pa	—	✓
Rhinotermitidae	Pa	✓	—	S [Tm]
Rhinotermitinae	M [part Rh, Tm]	S [Co, Tm]	—	—
Stylotermitinae	—	—	—	—
Psammotermitinae	S [Pr]	—	—	—
Termitogetoninae	S [Se]	—	—	—
Prorhinotermitinae	S [Ps]	—	—	—
Coptotermitinae	M [part He]	S [Tm]	—	—
Heterotermitinae	Pa	—	S [Tm]	—
Serritermitidae	S [part Rh]	S [Co, He, Tm]	—	S [Rh, Tm]
Termitidae	M	✓	✓	S
Macrotermitinae	M [all other Tm]	Pa	✓	—
Sphaerotermitinae	S [Fo]	S [Fo, Ap, Sy, Tn, Na]	—	—
Foraminitermitinae	M [Sp]	Po	✓	—
Apicotermitinae	M [Sy, Tn, Na]	Pa	✓	—
Syntermitinae	M [part Tn]	Pa	✓	—
Termitinae	Po	Po	Po	—
Nasutitermitinae	M, a [without Sy*]	M	Pa [without Sy]	—

Taxa as in Table 1 abbreviations as in Table 3, with S, singleton in analysis; ✓, agrees with our findings. *Position of *Verrucositermes* is probably an artefact (see text). Taxa in square brackets are sister groups on the relevant tree: I, Isoptera; Te, Termopsidae; K, Kalotermitidae; Rh, Rhinotermitidae; Se, Serritermitidae; Tm, Termitidae; Pr, *Prorhinotermes*; Ps, *Psammotermes*; Co, Coptotermitinae; He, Heterotermitinae; Fo, Foraminitermitinae; Sp, *Sphaerotermes*; Ap, Apicotermitinae; Sy, Syntermitinae; Tn, Termitinae; Na, Nasutitermitinae.

4.2. The evolution of true workers and the relationship with nesting strategy

Our cladogram suggests three independent origins of the true worker caste, supporting a suggestion first made by Noirot and Pasteels (1988) that workers have independently evolved in the Mastotermitidae, Hodotermitidae, and at least once in the Rhinotermitidae + Termitidae. This conflicts with the single basal origination suggested by Thompson et al. (2000), primarily because we have found the Termopsidae to be paraphyletic, so that multiple acquisitions or losses are required on our tree. It is perhaps more significant that all taxa without true workers are single-piece nesters, including *Prorhinotermes* which appears to have undergone a reversal in nesting strategy and subsequently lost its worker caste. It seems likely that the need for true workers is linked to the more complex social organisation necessary in intermediate or separate piece nesting and probably more explicitly to the need for foraging outside the nest. The single-piece feeding strategy does not require workers to forage for food beyond the nest, and presumably an absolute division of labour by different castes becomes less critical to colony success. Consequently, mapping the trait by parsimony may be misleading if the cost of unnecessary caste differentiation violates the key parsimony assumption that change is rare. Since *Mastotermes* exhibits both true workers and the intermediate nesting behaviour, it seems that these key ecological strategies evolved very basally in the Isoptera, and lends support to the alternative hypothesis of multiple subse-

quent losses. This highlights the difficulty of estimating the ancestral biological conditions of clades when rates of biological change are apparently high and when there may be many extinct species without which tree topologies cannot be fully resolved.

4.3. Evolution of feeding groups

Our trees suggests that after the loss of the endosymbiont gut flagellates, which characterise the lower termites and *Cryptocercus*, there is rapid diversification into numerous different feeding types in the Termitidae, including group II fungus-feeding and both group III and IV soil-feeding. *Sphaerotermes* is therefore anatomically the closest to the ancestral termitid condition: that is a non-fungus-growing wood-feeding termite having lost the gut flagellates, prior to the development of soil-feeding.

The reconstruction of feeding group shifts at the base of the Termitidae is ambiguous under a simple parsimony model. All four possible shifts from group I are equally plausible and so it seems on the face of it to be impossible to reconstruct this key evolutionary transition (i.e. to establish which trophic shift is associated with the loss of the flagellates). However, we believe that there is a biologically most plausible hypothesis that is consistent with the overall trophic shift reconstructions. This hypothesis is essentially the same as that proposed in Donovan et al. (2001b). The argument centres on the physical process that would allow the loss of the flagellates. Of the available shifts only one (II to II_f) leads instantly to flagellate loss. Fungus feeding termites

have externalised the gut so that a significant part of the degradation of organic matter occurs in the fungus-comb rather than in the gut. Wood is masticated and passes through the worker gut essentially unchanged before being defecated on to the fungus comb. Much of the digestive burden carried originally by the flagellates is therefore passed on to the fungus (Rouland-Lefèvre, 2000) and the flagellates are no longer required. One additional consequence of the need to plaster all woody faecal material onto a fungus comb is that faecal material is no longer available as a building material ('carton') for nests and so fungus-growing termites build their colony structures out of soil, specifically clay-rich sub-surface soil (Noirot and Darlington, 2000). In line with this reasoning, previous research has shown that Macrotermitinae workers have substantial amounts of soil in their guts (Donovan et al., 2001a), presumably because they (intentionally or unintentionally) ingest soil particles during mound building. This might pre-adapt fungus-growing termites to soil-feeding on clay-rich soil, which has predominantly highly recalcitrant soil organic matter (SOM) that is very hard to digest. This postulated shift from fungus-growing to group III/IV soil-feeding is a profound one in physiological and microbiological terms, requiring a change from a predominantly external gut to an internal one. It also requires a shift from a predominantly acetate-producing gut biota to a predominantly methane-producing one. This hypothesis would be supported, of course, by evidence that the Macrotermitinae can digest at least some of the soil in their guts in a similar way to soil-feeders proper.

Reconstructing the tree with fungus-growing as the ancestral termitid condition gives us three non-fungus-growing lineages that together form the sister group of the Macrotermitinae. These are: (1) the Sphaerotermitinae (wood-feeders that have lost the fungal symbiosis), (2) the Foraminitermitinae (group III soil-feeders with a unique gut configuration), and (3) the crown-group Termitidae (plesiomorphically group IV soil-feeders).

We can postulate that the early origin of soil-feeding in the termitids contributed significantly to the radiation and speciation of that group. Soil is an almost limitless resource, much less patchily distributed than wood, and there are few invertebrate competitors in tropical rain forest soils as soil-feeding earthworms are found at very high biomass densities only in tropical grasslands. The rapid diversification of the soil-feeding termitids makes it difficult to unambiguously map the development of feeding behaviour onto the backbone of the tree between the group II fungus-feeders and the Nasutitermitinae (Fig. 3), and the backbone of the tree at those points remains equivocal between group III and IV soil-feeding.

It seems likely given the predominance of group IV soil-feeding at the base of the Nasutitermitinae, that that clade has reverted to group II wood-feeding. In addition, the nasutes seem to have retained the ability to switch from wood-feeding to group III soil-feeding. This appears to have occurred several times after colonization events by wood-feeding taxa (e.g. in Madagascar, Eggleton and Davies,

2003). This may be of critical importance in explaining the extensive radiation of this subfamily, which presently makes up some 550 species worldwide, or 30% of all Termitidae, and is an ecologically important group. These biogeographical considerations will be discussed more fully in a later paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.ympev.2007.05.014](https://doi.org/10.1016/j.ympev.2007.05.014).

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