

The phylogeny of termites (Dictyoptera: Isoptera) based on mitochondrial and nuclear markers: Implications for the evolution of the worker and pseudergate castes, and foraging behaviors

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ABSTRACT

A phylogenetic hypothesis of termite relationships was inferred from DNA sequence data. Seven gene fragments (12S rDNA, 16S rDNA, 18S rDNA, 28S rDNA, cytochrome oxidase I, cytochrome oxidase II and cytochrome b) were sequenced for 40 termite exemplars, representing all termite families and 14 outgroups. Termites were found to be monophyletic with *Mastotermes darwiniensis* (Mastotermitidae) as sister group to the remainder of the termites. In this remainder, the family Kalotermitidae was sister group to other families. The families Kalotermitidae, Hodotermitidae and Termitidae were retrieved as monophyletic whereas the Termopsidae and Rhinotermitidae appeared paraphyletic. All of these results were very stable and supported with high bootstrap and Bremer values. The evolution of worker caste and foraging behavior were discussed according to the phylogenetic hypothesis. Our analyses suggested that both true workers and pseudergates (“false workers”) were the result of at least two different origins. Our data support a traditional hypothesis of foraging behavior, in which the evolutionary transition from a one-piece type to a separate life type occurred through an intermediate behavioral form.

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1. Introduction

Termites are eusocial insects, with differentiated castes and complex, coordinated group behaviors of nest construction and foraging, the evolution of which are poorly known compared with other eusocial groups. As reviewed by Eggleton (2001), phylogenetic investigation has been very limited, and a dearth of knowledge on phylogeny has hindered the study of their macroevolution.

All termite species are known to be eusocial (with castes), but many modalities of their social behavior vary within a continuum (Shellman-Reeve, 1997; Sherman et al., 1995). A diversity of behaviors is shown by termites from a mere excavation within their food resource (i.e., one-piece type *sensu* Abe, 1987) to the building of sophisticated nest constructions such as clay cathedrals, with different food resources from wood to humus (Noirot, 1992), and with different symbioses from intestinal protista (flagellates) to fungi cultivation (Bignell, 2000). Given this wide range of behavioral diversity, termites are an important group to under-

stand the evolution of eusociality in diploid organisms. A phylogenetic framework is essential for deciphering the origin and evolution of this behavioral diversity, especially with respect to the so-called “lower termites” which presumably show the first steps of this evolution. Unfortunately, the relationships among these so-called “lower” termites are still controversial (Kambhampati and Eggleton, 2000) notably between Hodotermitidae, Kalotermitidae, Mastotermitidae and Termopsidae.

As reviewed by Grassé (1986) and Donovan et al. (2000), taxonomic bases for termites were laid by Holmgren (1909, 1911, 1912). According to later studies by Snyder (1949), Grassé (1949) and Emerson (1965), seven families within Isoptera were defined and this constitutes the classification which is most widely accepted at the present day. The families Mastotermitidae, Kalotermitidae, Termopsidae, Hodotermitidae, Rhinotermitidae and Serritermitidae include termites with hindgut protozoa (the so-called “lower” termites), whereas the family Termitidae contains protozoa-independent species (the so-called “higher” termites—Krishna and Weesner, 1969, 1970). “Lower” and “higher” terms refer to a gradistic classification which is not really informative (Shellman-Reeve, 1997) and will not be used hereafter.

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This taxonomic scheme was built with a constant reference to different kinds of phylogenetic hypotheses. The most well-known hypotheses (Ahmad, 1950; Emerson and Krishna, 1975; Krishna, 1970) were proposed without explicit phylogenetic methodology but they shaped evolutionary hypotheses about termites during several decades (Grassé, 1986; Grassé and Noirot, 1959; Krishna and Weesner, 1970; Noirot, 1992; Noirot and Pasteels, 1988; Weidner, 1966). Recently, several studies attempted to reconstruct phylogeny using a modern phylogenetic methodology, based either on morphological characters (Donovan et al., 2000) or on molecular ones (Kambhampati et al., 1996; Kambhampati and Eggleton, 2000; Thompson et al., 2000). Most of these analyses included representatives from all termite families but recovered different tree topologies (Fig. 1). Moreover, except for the morphological study of Donovan et al. (2000) which included large taxon and character samples (49 taxa and 196 characters), all other studies were more exploratory since they considered one or two portions of genes and less than 20 taxa. Some recent studies focused on more restricted groups, in an attempt to reconstruct relationships within the families Rhinotermitidae or Termitidae (Aanen et al., 2002; Aanen and Eggleton, 2005; Austin et al., 2004; Bitsch and Noirot, 2002; Lo et al., 2004; Miura et al., 1998; Noirot, 2001; Ohkuma et al., 2004), but they were not designed to robustly resolve phylogenetic relationships throughout Isoptera. Therefore, apart from the monophyly of some well-characterized

families (Kalotermitidae, Hodotermitidae, Termitidae), phylogenetic relationships within termites are still highly controversial and need to be investigated.

The purpose of the present study is to reconstruct a robust phylogeny for Isoptera by using multiple molecular markers and a wide range of taxa sampled among the seven widely recognized family groups and appropriate outgroups (Orthoptera, Blattaria and Mantodea; Grandcolas and Deleporte, 1996; Grandcolas and D'Haese, 2004; Lo et al., 2000). The basal relationships within termites, and especially among Kalotermitidae, Hodotermitidae and Termopsidae, constitute the main phylogenetic challenge of this study. This topology is then used to explore the evolution of social behavior throughout the group, in an attempt to bring new information about the controversial evolution of the worker caste and of the foraging behavior (Abe, 1987; Grandcolas and D'Haese, 2002; Higashi et al., 1991; Noirot, 1982, 1985a,b; Thompson et al., 2000, 2004; Watson and Sewell, 1985).

2. Materials and methods

2.1. Taxon sampling

The taxon sample comprised 40 species, and as many genera, belonging to the seven termites families currently recognized (Table 1). These 40 species are distributed as follows: two Hod-

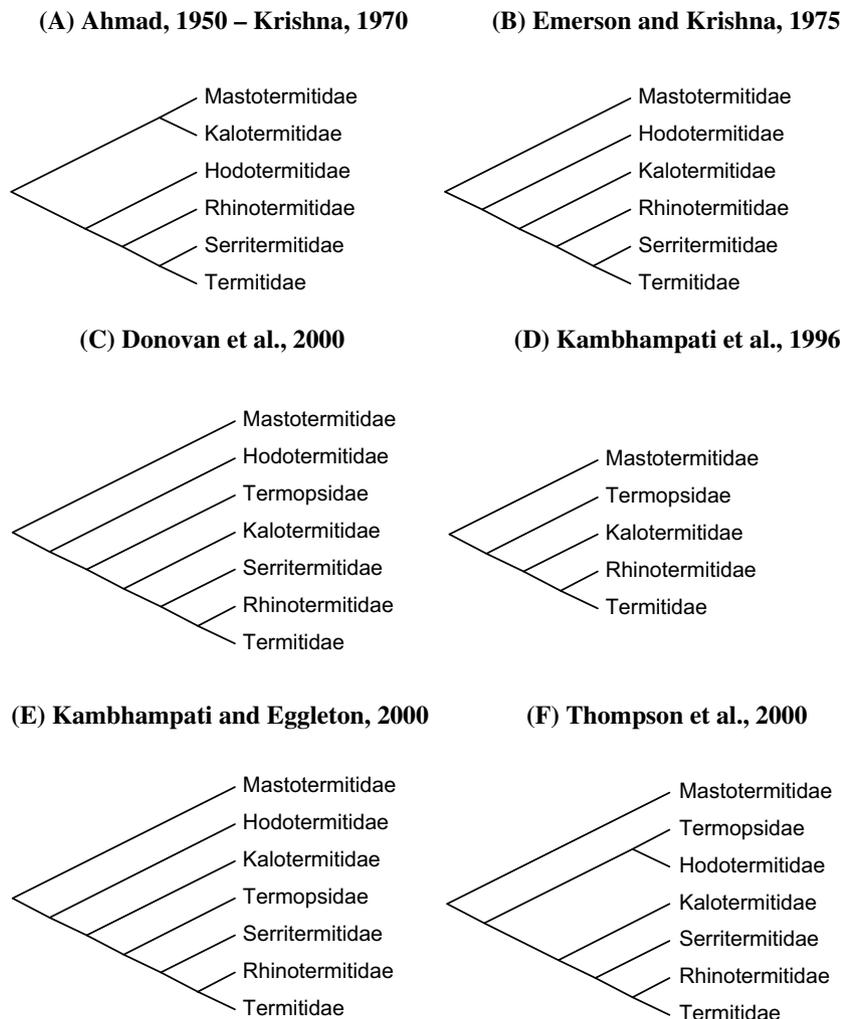


Fig. 1. Main hypotheses of phylogenetic relationships within termites (note that in A and B Hodotermitidae includes Termopsidae).

Table 1

List of the 54 taxa used in this phylogenetic analysis with their assignments to families and subfamilies (according to Grassé, 1949 and Emerson, 1965), and GenBank accession numbers (n.d. for no data)

Genus	Species	Family	Subfamily	12S/16S/18S/28S/COI/COII/Cytb
<i>Locusta</i>	<i>migratoria</i>	Acrididae	Oedipodinae	X80245.1/X80245.1/AF370793/AF370809.1/X80245.1/X80245.1/X80245.1
<i>Mantoida</i>	<i>schraderi</i>	Mantoididae		EF383155/EF383315/EF383475/EF383637/EF383799/AY491275/n.d.
<i>Metilia</i>	<i>brunnerii</i>	Acanthopidae	Acanthopinae	EF383232/EF383392/ EF383552/EF383715/EF383874/EF384002/n.d.
<i>Pseudocreobotra</i>	<i>occellata</i>	Hymenopodidae	Hymenopodinae	EF383280/EF383438/EF383599/EF383765/EF383919/EF384049/n.d.
<i>Epilampra</i>	sp.	Blaberidae	Epilamprinae	EU253698/EU253737/EU253775/EU253815/EU253831/EU253871 /n.d.
<i>Parasphaeria</i>	<i>boleiriana</i>	Blaberidae	Zetoborinae	EU253699/EU253738/EU253776/EU253817/EU253832/EU253872/EU253909
<i>Calolampra</i>	sp.	Blaberidae	Diplopterinae	n.d./ EU253739/EU253774/EU253816/EU253829 /n.d./n.d.
<i>Blattella</i>	<i>germanica</i>	Blattellidae	Blattellinae	EF363293/EF363265/EF363236/ EU253813/EU253828 /EF363216/n.d.
<i>Blatta</i>	<i>orientalis</i>	Blattidae	Blattinae	EF363292/EF363264/EF363235/ EU253820/EU253827/EU253869/EU253907
<i>Pelmatosilpha</i>	<i>guyanae</i>	Blattidae	Polyzosterinae	EU253697/EU253734/EU253773/EU253819/EU253833 /EU253873/EU253910
Unidentified genus		Nocticolidae	Nocticolinae	n.d./ EU253736/EU253695/EU253660 /n.d./n.d./n.d.
<i>Cryptocercus</i>	sp.	Cryptocercidae	Cryptocercinae	n.d./ EU253735/EU253777/EU253659/EU253830/EU253870/EU253908
<i>Therea</i>	<i>petiveriana</i>	Polyphagidae	Polyphaginae	EF363310/EF363282/EF363253/ EU253818/EU253835/EU253874/EU253912
<i>Supella</i>	<i>longipalpa</i>	Pseudophyllodromidae	Pseudophyllodromiinae	EF363309/EF363281/EF363252/ EU253814/EU253834/EF363224/EU253911
<i>Hodotermes</i>	<i>mossambicus</i>	Hodotermitidae	Hodotermitinae	EU253700 /n.d./ EU253779/EU253661/EU253836/EU253875/EU253913
<i>Microhodotermes</i>	<i>viator</i>	Hodotermitidae	Hodotermitinae	EU253701/EU253741/EU253780/EU253662/EU253837/EU253876/EU253914
<i>Calcaritermes</i>	<i>temnocephalus</i>	Kalotermitidae	Kalotermitinae	EU253702/EU253743/EU253781/EU253663/EU253838/EU253877/EU253915
<i>Comatermes</i>	<i>perfectus</i>	Kalotermitidae	Kalotermitinae	EU253703 /n.d./ EU253782/EU253664/EU253839/EU253878/EU253916
<i>Cryptotermes</i>	<i>brevis</i>	Kalotermitidae	Kalotermitinae	EU253704/EU253744/EU253783/EU253665/EU253840/EU253879/EU253917
<i>Incisitermes</i>	<i>tabogae</i>	Kalotermitidae	Kalotermitinae	EU253705/EU253745/EU253784/EU253822/EU253841/EU253880/EU253918
<i>Kalotermes</i>	<i>flavicollis</i>	Kalotermitidae	Kalotermitinae	EU253706/EU253746/EU253785/EU253666/EU253842/EU253881/EU253919
<i>Neotermes</i>	<i>holmgreni</i>	Kalotermitidae	Kalotermitinae	EU253707/EU253747/EU253786/EU253667/EU253843/EU253882/EU253920
<i>Postelectrotermes</i>	<i>howa</i>	Kalotermitidae	Kalotermitinae	EU253708/EU253748/EU253787/EU253823/EU253844/EU253883/EU253921
<i>Procryptotermes</i>	<i>leewardensis</i>	Kalotermitidae	Kalotermitinae	EU253709/EU253749/EU253788/EU253668/EU253845/EU253884/EU253922
<i>Bifiditermes</i>	<i>improbis</i>	Kalotermitidae	Kalotermitinae	n.d./ EU253742/EU253693/EU253821 /n.d./AF189080/AF189110
<i>Mastotermes</i>	<i>darwiniensis</i>	Mastotermitidae		EU253710/EU253740/EU253778/EU253669 and AY125281/ EU253846 /EU253885/EU253923
<i>Coptotermes</i>	<i>lacteus</i>	Rhinotermitidae	Coptotermitinae	EU253711/EU253750/EU253789/EU253670/EU253847/EU253886/EU253924
<i>Heterotermes</i>	<i>vagus</i>	Rhinotermitidae	Heterotermitinae	EU253712/EU253751/EU253790/EU253671/EU253848/EU253887 /n.d.
<i>Prorhinotermes</i>	<i>canalifrons</i>	Rhinotermitidae	Prorhinotermitinae	EU253713/EU253752/EU253791/EU253672/EU253849/EU253888/EU253925
<i>Reticulitermes</i>	<i>santonensis</i>	Rhinotermitidae	Heterotermitinae	EU253714/EU253753/EU253792/EU253673/EU253850/EU253889 /n.d.
<i>Rhinotermes</i>	<i>marginalis</i>	Rhinotermitidae	Rhinotermitinae	EU253715/EU253754/EU253793/EU253674/EU253851/EU253890 /n.d.
<i>Schedorhinotermes</i>	sp.	Rhinotermitidae	Rhinotermitinae	EU253716/EU253755/EU253794/EU253675/EU253852/EU253891/EU253926
<i>Termitogeton</i>	sp.	Rhinotermitidae	Termitogetoninae	n.d./ EU253772/EU253812/EU253692 /n.d./ EU253906/EU253941
<i>Serritermes</i>	<i>serrifer</i>	Serritermitidae		n.d./AF262577/AF220565/n.d./n.d./AF220598/n.d.
<i>Macrotermes</i>	<i>subhyalinus</i>	Termitidae	Macrotermitinae	EU253722/EU253759/EU253800/EU253681/EU253856/EU253895/EU253932
<i>Odontotermes</i>	<i>hainanensis</i>	Termitidae	Macrotermitinae	EU253723/EU253760/EU253801/EU253682/EU253857/EU253896/EU253933
<i>Pseudacanthotermes</i>	<i>spiniger</i>	Termitidae	Macrotermitinae	EU253724/EU253761/EU253802/EU253824/EU253858/EU253897/EU253934
<i>Sphaerotermes</i>	<i>sphaerotherax</i>	Termitidae	Macrotermitinae	AF475056/n.d./n.d./n.d./AY127725/n.d./n.d.
<i>Constrictotermes</i>	<i>cyphergaster</i>	Termitidae	Nasutitermitinae	n.d./ EU253762/EU253803/EU253683/EU253859/EU253898/EU253935
<i>Cornitermes</i>	<i>cumulans</i>	Termitidae	Nasutitermitinae	EU253725/EU253763/EU253804/EU253825/EU253860/EU253899/EU253936
<i>Diwaitermes</i>	<i>kanehirae</i>	Termitidae	Nasutitermitinae	EU253726/EU253764/EU253805/EU253684/EU253861/EU253900/EU253937
<i>Nasutitermes</i>	<i>voeltzkowi</i>	Termitidae	Nasutitermitinae	EU253727/EU253765/EU253806/EU253685 /n.d./ EU253901 /n.d.
<i>Procornitermes</i>	<i>araujo</i>	Termitidae	Nasutitermitinae	EU253728/EU253766/EU253807/EU253686/EU253862/EU253902 /n.d.
<i>Syntermes</i>	<i>grandis</i>	Termitidae	Nasutitermitinae	n.d./ EU253767/EU253808/EU253687/EU253863/EU253903 /n.d.
<i>Velocitermes</i>	sp.	Termitidae	Nasutitermitinae	EU253729/EU253768/EU253696/EU253826/EU253864 /n.d./ EU253938
<i>Cubitermes</i>	sp.	Termitidae	Termitinae	EU253730 /n.d./ EU253694/EU253688/EU253865 /n.d./n.d.
<i>Inquilinitermes</i>	sp.	Termitidae	Termitinae	EU253731/EU253769/EU253809/EU253809/EU253866/EU253904/EU253939
<i>Microcerotermes</i>	sp.	Termitidae	Termitinae	EU253732/EU253770/EU253810/EU253690/EU253867/EU253905 /n.d.
<i>Termes</i>	<i>hispaniolae</i>	Termitidae	Termitinae	EU253733/EU253771/EU253811/EU253691/EU253868 /n.d./ EU253940
<i>Archotermopsis</i>	<i>wroughtoni</i>	Termopsidae	Termopsinae	EU253717/EU253756/EU253795/EU253676/EU253853/EU253892/EU253927
<i>Hodotermopsis</i>	<i>sjustedti</i>	Termopsidae	Termopsinae	EU253718 /n.d./ EU253796/EU253677 /n.d./ EU253893/EU253928
<i>Porotermes</i>	sp.	Termopsidae	Porotermitinae	EU253719/EU253757/EU253797/EU253678/EU253854 /n.d./ EU253929
<i>Zootermopsis</i>	<i>nevadensis</i>	Termopsidae	Termopsinae	EU253721/EU253758/EU253799/EU253680/EU253855/EU253894/EU253931
<i>Stolotermes</i>	<i>brunneicornis</i>	Termopsidae	Stolotermitinae	EU253720 /n.d./ EU253798/EU253679 /n.d./n.d./ EU253930

Genbank accession numbers in bold represent sequences generated for this study.

otermitidae (out of three genera known—i.e., 67% of the genera known are included in this study), nine Kalotermitidae (out of 21 genera—43%), one Mastotermitidae (one genus—100%), seven Rhinotermitidae representing five of the seven subfamilies (13 genera—54%), one Serritermitidae (two genera—50%), 15 Termitidae (241 genera—6%) and five Termopsidae sampling the three subfamilies (five genera—100%). Some recent molecular and morphological papers (e.g., Lo et al., 2000; Inward et al., 2007a; Klass and Meier, 2006) inferred close relationships between Isoptera and Blattaria, with *Cryptocercus* as sister taxon of termites, in

contradiction with previous morphological or peptidic studies (Gäde et al., 1997; Grandcolas, 1996), with *Cryptocercus* nested within the cockroaches. Therefore, 10 Blattaria, three Mantodea and one Orthoptera were added to our sampling (Table 1), the orthopteran *Locusta migratoria* being used as the rooting out-group. These outgroups were selected from previous studies (Grandcolas, 1996; Pellens et al., 2002; Svenson and Whiting, 2004; Pellens et al., 2007) to avoid constraining a close relationship between roaches and termites since other hypotheses exist (e.g., Thorne and Carpenter, 1992).

2.2. Primers, PCR, sequencing

Thoracic muscle tissue was excised from termites and leg muscle tissue was taken from cockroaches specimens preserved in 100% ethanol. DNA was extracted using the QIAGEN DNeasy protocol for animal tissue. The head and abdomen of these exemplars were stored in 100% ethanol as primary voucher; specimens from the same colony, when available, were stored intact in 100% ethanol as secondary voucher. Vouchers and DNAs are deposited in the Insect Genomics Collection, Brigham Young University.

Seven markers were amplified in one or several fragments according to the length of the sequences targeted. Thus 12S rDNA (~360 bp), 16S rDNA (~385 bp), cytochrome b (Cytb, 307 bp) and cytochrome oxidase II (COII, including a portion of Leu tRNA, ~725 bp) were amplified in one fragment; 18S rDNA (~1870 bp) and 28S rDNA (~2200 bp) were sequenced in four fragments; and cytochrome oxidase I (COI, 1179bp) in two or three fragments. The PCR primers and amplification protocols are listed in Table 2. The first PCR profile was used for all the different genes targeted (especially in fresh specimens)

whereas the others were gene-specific and were used for more problematic taxa. PCR amplification was performed on a DNA Engine DYAD™, Peltier Thermal Cycler (Bio-Rad Laboratories). PCR products were fractionated on an agarose gel to check for specificity and to monitor for contamination using a negative control.

PCR products were purified via the Montage PCR₉₆ Cleanup Kit (Millipore®) and sequenced using ABI Big Dye 3.1® with the following sequence profile: 27 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 4 min. Sequencing reactions products were purified with Sephadex™ columns (Amersham Biosciences) and fractionated on an ABI 3730 XL DNA sequencer.

Each sequence was edited using Sequencher® 4.0 (Gene Codes, 1999) after BLAST searches (Altschul et al., 1997) as implemented by the NCBI website (<http://www.ncbi.nlm.nih.gov/>) to check for contamination.

Thirty-nine outgroup sequences and eight termite sequences were taken from GenBank. The sequences generated for this study have been submitted to GenBank under the accession numbers provided in Table 1.

Table 2
Sequences of primers and PCR profiles used

Gene	Primer	Sequence (5' → 3')	Sources			
12S	12Sai	AAA CTA GGA TTA GAT ACC CTA TTA T	Simon et al., 1994			
	12Sbi	AAG AGC GAC GGG CGA TGT GT	Simon et al., 1994			
16S	16SAr	CGC CTG TTT ATC AAA AAC AT	Xiong and Kocher, 1991			
	16SF	TTA CGC TGT TAT CCC TAA	Kambhampati, 1995			
Cytb	cytb612	CCA TCC AAC ATC TCC GCA TGA TGA AA	Kocher et al., 1989			
	cytb920	CCC TCA GAA TGA TAT TTG GCC TCA	Kocher et al., 1989			
COII	Fleu	TCT AAT ATG GCA GAT TAG TGC	Whiting, 2002			
	Rlys	GAG ACC AGT ACT TGC TTT CAG TCA TC	Whiting, 2002			
COI	LCO	GGT CAA CAA ATC ATA AAG ATA TTG G	Folmer et al., 1994			
	HCO	TAA ACT TCA GGG TGA CCA AAA AAT CA	Folmer et al., 1994			
	mtd6	GGA GGA TTT GGA AAT TGA TTA GTT CC	Simon et al., 1994			
	calvin	GGR AAR AAW GTT AAR TTW ACT CC	Whiting lab, BYU ^a			
	Ron	GGA TCA CCT GAT ATA GCA TT CCC	Simon et al., 1994			
	Deep6R2	WCC WAC DGT RAA YAT RTG RTG DGC	Whiting lab, BYU ^a			
	Deep6f2	TTG AYC CWG CWG GDG GDG GNG AYC C	Whiting lab, BYU ^a			
18S	1F	TAC CTG GTT GAT CCT GCC AGT AG	Giribet et al., 1996			
	1.2F	TGC TTG TCT CAA AGA TTA AGC	Whiting, 2002			
	b5.0	TAA CCG CAA CAA CIT TAA T	Whiting et al., 1997			
	2F	AGG GTT CGA TTC CGG AGA GGG AGC	Hillis and Dixon, 1991			
	b2.9	TAT CTG ATC GCC TTC GAA CCT CT	Jarvis et al., 2004			
	a1.0	GGT GAA ATT CTT GGA CCG TC	Whiting et al., 1997			
	7R	GCA TCA CAG ACC TGT TAT TGC	Whiting, 2002			
	a3.5	TTG TGC ATG GCC GYT CTT AGT	Whiting, 2002			
	9R	GAT CCT TCC GCA GGT TCA CCT AC	Giribet et al., 1996			
28S	Rd1.2a	CCC SSG TAA TTT AAG CAT ATT A	Whiting, 2002			
	Rd3b	CCY TGA ACG GTT TCA CGT ACT	Jarvis et al., 2004			
	Rd3.2a	AGT ACG TGA AAC CGT TCA SGG GT	Whiting, 2002			
	Rd4b	CCT TGG TCC GTG TTT CAA GAC	Jarvis et al., 2004			
	Rd4.2b	CCT TGG TCC GTG TTT CAA GAC GG	Whiting, 2002			
	28SA	GAC CCG TCT TGA AGC ACG	Whiting et al., 1997			
	28SB	TCG GAA GGA ACC AGC TAC	Whiting et al., 1997			
	Rd4.5a	AAG TTT CCC TCA GGA TAG CTG	Whiting, 2002			
	Rd7.b1	GAC TTC CCT TAC CTA CAT	Whiting, 2002			
	Gene	Heat	Denaturation	Annealing ^b	Extension	Final extension
All	94 °C (2min)	94 °C (1min)	55 °C (1min)	72 °C (1min 15s)	72 °C (7min)	40
Cytb	94 °C (2min)	94 °C (1min)	51.5 to 59.5 °C (1min)	72 °C (1min 15s)	72 °C (7min)	40
16S	94 °C (2min)	94 °C (1min)	50 °C (1min)	72 °C (1min 15s)	72 °C (7min)	40
COII	94 °C (2min)	94 °C (1min)	45 to 53 °C (1min)	72 °C (1min 15s)	72 °C (7min)	40
^c COIa	94 °C (2min)	94 °C (1min)	49.5 to 57.5 °C (1min)	72 °C (1min 15s)	72 °C (7min)	40
^d COIbc	94 °C (2min)	94 °C (1min)	50 °C (1min)	72 °C (1min 15s)	72 °C (7min)	40
28SB	94 °C (2min)	94 °C (1min)	50 °C (1min)	72 °C (1min 15s)	72 °C (7min)	40

^a BYU = primers developed in the Brigham Young University.

^b Note that PCR where annealing temperature increases were done as 0.2 °C per cycle.

^c COIa = with primers LCO/HCO.

^d COIbc = with primers mtd6/calvin.

2.3. Phylogenetic analyses

The data set was analyzed by direct optimization with parsimony optimality criterion. Direct optimization has the advantage to associate alignment and tree reconstruction steps and to propose sequence alignments implied by the analysis. Therefore, the analyses are not based on a priori alignments arbitrarily chosen among competing and equally justified ones in the case of ribosomal sequences of different lengths (Wheeler, 1996; Wheeler et al., 2006). Events (indels and substitutions) are directly optimized on the topology, maximizing historical continuity. Parsimony was used as an optimality criterion because it maximizes the explanatory power of the data and it decreases the number of *ad hoc* hypotheses implied by the usual evolutionary models of common mechanism among sites and tree branches (Farris, 1983; Steel and Penny, 2000). To show how much our results are sensitive to different methodologies and to bring information for those who preferred other tree reconstruction algorithms, additional analyses used a probabilistic paradigm and a static alignment.

Phylogenetic analyses under direct optimization as implemented in POY 3.0.11a (Wheeler, 1996) were carried out in parallel on a supercomputer (Dell 1855 Blade Cluster, 1260 Pentium EM64T Xeon processors @ 3.6 GHz, 610 compute nodes, 2520 GB total memory, Brigham Young University). The large subunit nuclear ribosomal (28S) sequences were partitioned in four regions (approximately A, B, C and DEF regions) corresponding to the four regions sequenced separately and which were not overlapping. All the ribosomal sequences (12S, 16S, 18S and 28S) were preliminarily aligned with Muscle (Edgar, 2004) and then 16S, 18S, 28SA and 28SDEF were partitioned according to highly conserved regions in order to speed up phylogenetic analyses and to avoid misleading alignments when portions of genes were lacking. The protein coding genes COI, COII and Cytb were aligned based on conservation of codon reading frame in Sequencher 4.0, and treated as prealigned data in POY. The portion of Leu tRNA was neither prealigned nor partitioned.

The POY search parameters included 100 replicates with tree bisection reconnection (TBR) branch swapping, treefusing (Goloboff, 1999) and ratchetting (Nixon, 1999b) algorithms. Details of the commands used are listed in Appendix A1.

A sensitivity analysis was conducted in order to test the robustness of the phylogenetic conclusions to different costs of insertion, transversion and transition events. Ten sets of parameters (gap: transversions: transitions) were tested this way: 111, 222 (with an extension gap cost of 1), 211, 221, 421 and [16]41 with an extension gap cost equal to and half of the opening gap cost for these four last parameters sets (called hereafter 211 \times , 221 \times , 421 \times and [16]41 \times). The tree search approach outlined above was also used for each of these searches, with command *-molecularmatrix* specifying the parameter set. The results are shown using stability plots or “navajo rugs” to summarize whether a monophyletic group was recovered or not at a node over the parameter space (Wheeler, 1995).

Parsimony bootstrap values for 1000 replicates (Felsenstein, 1985, 1988), and Bremer and partitioned Bremer support values (Bremer, 1988, 1994; Baker and DeSalle, 1997) were calculated in PAUP 4.0b10 (Swofford, 1998) and TreeRot.v2b (Sorenson, 1999), respectively, using the implied alignment provided by POY in the analysis 111. The default parameters were used in TreeRot.v2b.

Additional analyses based on a static alignment were done in a Bayesian framework with MrBayes v3.0b4 (Huelsenbeck and Ronquist, 2001), under the likelihood criterion with PHYML v2.4.4 (Guindon and Gascuel, 2003) and under parsimony with PAUP 4.0b10. In all the cases Muscle v3.6 (Edgar, 2004) with default parameters was used to generate alignments of the ribosomal se-

quences. The parsimony analysis consisted in 1000 random addition sequences with TBR branch swapping algorithm. Parsimony bootstrap values for 1000 replicates (Felsenstein, 1985, 1988) were calculated with PAUP 4.0b10. For the probabilistic analyses, Modeltest v3.6 (Posada and Crandall, 1998) was used to determine the model which best fit the data. A General Time Reversible model with a proportion of invariant sites and a gamma distributed rate variation among sites (GTR+I+G) was selected as the best fit of the models investigated, using either the hierarchical likelihood ratio tests or the Akaike Information Criterion, as implemented in Modeltest. In the likelihood analysis, bootstrap values were computed for 1000 replicates with PHYML v2.4.4. In the Bayesian analysis, the parameters of the model GTR+I+G were estimated independently during the tree reconstruction for the different partitions, i.e., the genes, using the command ‘unlink’. Four chains were run for 1,000,000 generations and sampled every 100 generations after an initial burn-in period of 50,000 generations. Details of the commands for these three analyses are provided in Appendix A1.

2.4. Attributes optimization

Three attributes were optimized a posteriori of the phylogenetic analyses using the parsimony criterion as implemented in WinClada v1.00.08 (Nixon, 1999a): the presence of pseudergates, the presence of true workers and the foraging behavior (Table 3). The evolutionary history of these attributes and their ancestral states were reconstructed considering only unambiguous transformations (command ‘*optimizations/unambig changes only*’). They were optimized on the preferred phylogenetic hypothesis (i.e., POY analysis under equal weighting) to infer caste evolution.

True workers and pseudergates evolved from different ontogenetic pathways (Noirot and Pasteels, 1987; Fig. 2) and can coexist in a same colony (e.g., Buchli, 1958). True workers separate early and irreversibly from the imaginal line. They present morphological specialization such as the development of the head and of mandibular muscles, and have rudimentary sex organs and no wings. They constitute the main working force in their colony. On the contrary, pseudergates separate late from the imaginal development. They are not specialized morphologically; they have short wing buds or no wings at all, and their genital organs are similar to those of the nymphs of the same size. Finally, they are not more active than nymphs or older larvae and do not constitute the main working force in their natural society (Noirot and Pasteels, 1987). Therefore, pseudergates and true workers were optimized on the phylogenetic tree as two different binary attributes (pseudergates: present/absent and true workers: present/absent). As explained in Grandcolas and D’Haese (2004), coding worker caste in a single multistate character has not been employed since it would incorrectly imply a homology statement between pseudergates and true workers. Mantids, cockroaches and *Locusta migratoria* do not exhibit developmental pathways leading to pseudergates or true workers and both attributes were coded absent in outgroups.

The foraging behavior was split into three large categories according to Abe’s work (1987): one-piece type, intermediate type and separate type. One-piece type termites spend their life in a single piece of wood which serves as both shelter and food. Their nests occupy the space resulting from wood consumption. Intermediate type and separate type species have uncoupled feeding from nesting sites but this uncoupling is more partial in the former. Those species build various kinds of nests and galleries (Abe, 1987). One-piece, intermediate and separate types are discrete states of the ecological character “foraging behaviour” and were considered as unordered states.

Caste evolution was also evaluated in a likelihood framework (Pagel, 1999) using the command *Multistate* in the program

Table 3
Characteristics of the worker caste (with references) and life type within the different termite families

Taxon	Worker caste	References	Life types (modified after Abe, 1987)
Hodotermitidae	True workers	Watson (1973)	Separate type
Kalotermitidae	Pseudergates	Grassé and Noirot (1947), Noirot and Pasteels (1987), Nagin (1972)	One-piece type
Mastotermitidae	True workers	Watson et al. (1977)	Intermediate type
Other Rhinotermitidae	True workers	Renoux (1976)	Intermediate type
<i>Coptotermes</i>	True workers	Bordereau (pers. obs.)	Separate type
<i>Schedorhinotermes</i>	True workers	Bordereau (pers. obs.)	Separate type
<i>Prorhinotermes</i>	Pseudergates	Miller (1942), Roisin (1988)	One-piece type
<i>Reticulitermes</i>	Pseudergates and true workers	Buchli (1958), Noirot and Pasteels (1987), Lainé and Wright (2003)	Intermediate type
<i>Termitogeton</i>	Pseudergates	Parmentier and Roisin (2003)	One-piece type
<i>Heterotermes</i>	True workers		Intermediate type
Serritermitidae	True workers	Cancello and DeSouza (2004)	Missing data
Termitidae	True workers	Noirot (1955)	Separate type
Termopsidae	Pseudergates	Heath (1927), Imms (1919), Mensa-Bonsu (1976), Morgan (1959)	One-piece type

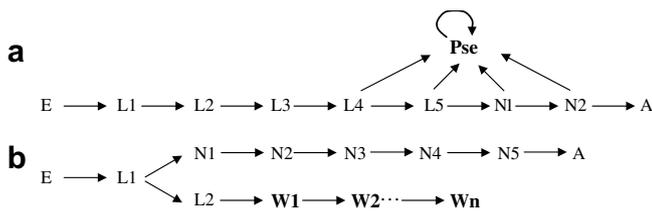


Fig. 2. Developmental pathways of (a) *Kalotermitidae* (simplified after Grassé and Noirot, 1947) and of (b) *Microcerotermes* sp. (after Noirot, 1985a). Soldiers, pre-soldiers and neotenic have been omitted for a better visualization of the castes of interest (in bold). E, egg; L, larvae; N, nymphs; A, alates; Pse, pseudergates; W, workers. Each arrow symbolizes a molt.

BayesTraits (by Pagel and Meade—available at www.evolution.rdg.ac.uk). It was run using the topology and branch lengths generated by means of standard likelihood method. Following Pagel (1999), a difference of two log units was taken as an evidence of statistical significance.

3. Results

3.1. Relationships among termite families

The POY analysis based on equally weighted gaps, transitions and transversions resulted in one most parsimonious tree which is depicted in Fig. 3 ($L = 17,347$, $CI = 0.28$, $RI = 0.44$) and in an implied alignment of 8381 characters. Our analysis supports the monophyly of Mantodea and the paraphyly of Blattaria with respect to termites, with *Cryptocercus* as sister taxon of termites. Termites are monophyletic with *Mastotermes darwiniensis* as sister group to the remainder of the termites. In this remainder, Kalotermitidae is sister group to others termites, a position which has never been postulated before (see Fig. 1). The families Kalotermitidae, Hodotermitidae and Termitidae are supported as monophyletic. Termopsidae and Rhinotermitidae are paraphyletic with respect to Hodotermitidae and Termitidae, respectively. Within Termitidae, Macrotermitinae and Nasutitermitinae are paraphyletic as well. Within Rhinotermitidae, the monophyly of two subfamilies was examined. Rhinotermitinae was strongly supported as a monophyletic subfamily whereas the monophyly of Heterotermitinae was rejected. A close relationship between *Prorhinotermes* and *Termitogeton* is also well supported. The majority of nodes had bootstrap support >50% and high Bremer support values (Fig. 3). Only two nodes had bootstrap support <50%, and 29 nodes (i.e., 57%) had Bremer support values ≥ 15 , including the nodes supporting the monophyly of Isoptera, Kalotermitidae and Hodotermitidae (Bremer values of 55, 27 and 73, respectively). The deep nodes 14, 23, 24, 30 and 31 have also high Bremer support values

(52, 22, 25, 45 and 15, respectively). Partitioned Bremer support values (PBS) are listed in Table 4. Except for cytochrome b, each gene supports some deep and apical nodes, but not all of them. For instance, COII supports the node 13 (PBS = 13) but does not support the node 14 (PBS = -12). Those results speak for total evidence approaches. Finally, 12S and 28S bring more than 40% of the signal ($(316.3 + 295) * 100/1381 = 44.3$), but when the support of each gene is normalized by the number of informative characters, 12S, 16S and 18S appear as the most informative genes whereas COI and COII are the less informative markers for our data set.

Sensitivity analyses were performed in order to test the stability of the phylogenetic hypothesis. The results are shown in Fig. 3 in the form of stability plots or “navajo rugs”. The results at the family level and below appeared very stable. The monophyly of Isoptera, Kalotermitidae, Hodotermitidae and Termitidae is found in at least eight of the 10 parameters sets. The clade (Termopsidae + Hodotermitidae) is also extremely stable as well as the one comprising *Serritermes*, Rhinotermitidae and Termitidae. *Mastotermes darwiniensis* and Kalotermitidae as the first and second diverging lineage, respectively, are also stable results. The deeper nodes are not supported in the two analyses with an opening gap cost of 16 because of the placement of the termite *Sphaerotermes sphaerotherax* within the Mantodea. This spurious position is certainly due to missing data, *Sphaerotermes sphaerotherax* being only represented in the matrix by a portion of the COI and by the fast evolving 12S. Within termites, two positions for *Serritermes* are found among the different analyses: as sister taxon of the clade (Rhinotermitidae + Termitidae) and nested within Rhinotermitidae as sister taxon of *Termitogeton* (parameters sets 111, 222 \times , 211 \times , 421, [16]41 \times and 211, 221, 221 \times , 421 \times , [16]41, respectively). The relationships within the Termopsidae are not so clear in that five different clades are hypothesized as sister group of Hodotermitidae. However, Termopsidae always appeared paraphyletic except in one of the two most parsimonious trees in the analysis 221 \times , where they are monophyletic. From this point of view, Termopsidae paraphyly is also a stable result.

The Muscle v3.6 aligned data set had 7606 characters and the analyses based on this static alignment gave a slightly different topology. The main difference is localized in the second diverging lineage within the termites (i.e., after *Mastotermes darwiniensis*). The optimal topology found in the maximum likelihood analysis is provided in Appendix A11 ($\ln L = -80987.79847$). Again, Isoptera and the families Kalotermitidae, Hodotermitidae and Termitidae are supported as monophyletic, Termopsidae and Rhinotermitidae are paraphyletic, and *Mastotermes darwiniensis* is sister group to the remainder of termites. In this remainder, the clade (Hodotermitidae + Termopsidae) is sister group to others termites with a rather low bootstrap support value (72). The Bayesian topology obtained ($\ln L = -77482.157$; data not shown) was similar to the one

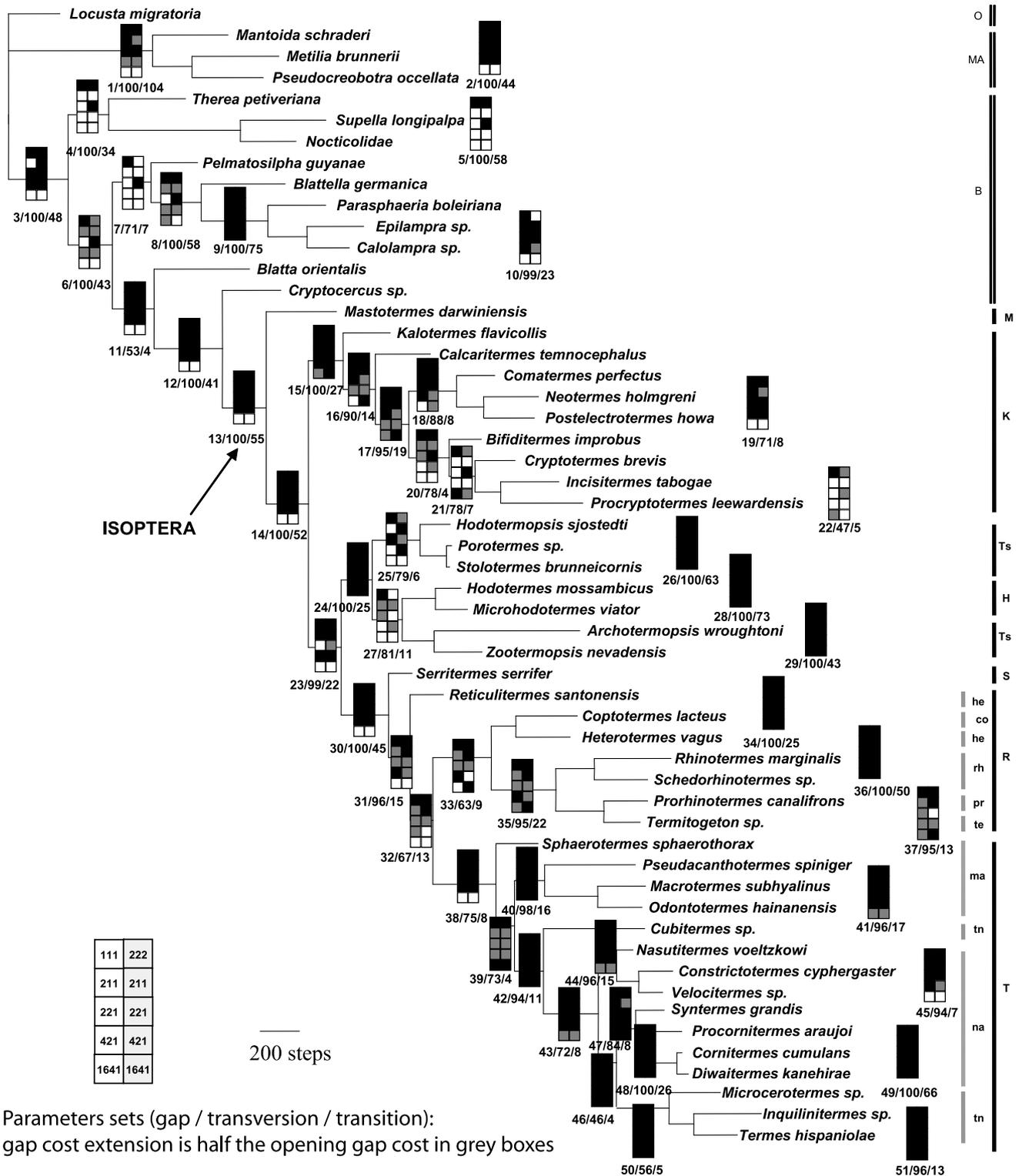


Fig. 3. Most optimal topology found in the direct optimization analysis with the parameter set 111 ($L = 17,347$, $CI = 0.28$, $RI = 0.44$). The results of the sensitivity analysis are depicted at each node with the following notation: monophyly in black, paraphyly in grey and polyphyly in white. Numbers below branches are nodes number/bootstrap values/Bremer support values. O, Orthoptera; MA, Mantodea; B, Blattaria; M, Mastotermitidae; K, Kalotermitidae; Ts, Termopsidae; H, Hodotermitidae; S, Serritermitidae; R, Rhinotermitidae; T, Termitidae; co, Coptotermitinae; he, Heterotermitinae; pr, Prorhinotermitinae; rh, Rhinotermitinae; te, Termitogetoninae; ma, Macrotermitinae; tn, Termitinae; na, Nasutitermitinae.

depicted in Appendix All except for the pattern relationships with in outgroups and Termitidae. Finally, the parsimony analysis conducted in PAUP 4.0b10 resulted in one most parsimonious tree ($L = 18,936$, data not shown) with sister group relationship be-

tween Kalotermitidae and the clade (Hodotermitidae + Termopsidae) being the main difference with POY results. This sister group relationship shows very low support (bootstrap value calculated with PAUP 4.0b10 for 1000 replicates = 160).

Table 4
Partitioned Bremer support values

Char./nodes	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	∑	%	
12S	25	22	2	4	0	3	6	14	9	0	3	0	0	25	23	2	-3	0	-9	0	0	0	-2	6	12	7	28	7	24	17	0	0	10	3	13	15	38	0	3	-1	11	2	3	6	6	0	0	0	0	-7	0	10	316.3	8
16S	24	16	9	3	17	5	4	11	14	10	-1	7	9	0	9	5	6	2	-3	2	6	-3	6	0	0	0	0	5	15	9	6	0	0	7	0	5	0	0	7	0	0	0	7	0	0	0	1	3	5.5	8	2	0	238.5	5.6
18S	13	1	6	4	7	5	-5	27	4	3	-1	23	14	8	-1	1	0	0	0	0	0	0	-1	4	-1	2	1	9	3	7	0	0	0	0	-2	0	2	0	0	0	2	0	-0.5	-6	0	1	0	-1	1	-1	128.5	5.2		
28S	8	12	5	10	34	25	-2	1	50	7	4	5	20	31	6.5	4	0.5	0	2	0	2	-3	13	-2	9	-2	10	4	0	0	0	0	0	7	0	1	0	0	0	4	2	2	6	0	2	2	4	4	5	295	2.9			
COI	22	-5	8	11	0	2	6	4	-3	3	-3	7	-9	0	-4	-3	1	0	3	0	0	4	9	0	0	0	0	0	15	-1	0	3	6	12	2	12	0	5	5	-1	6	0.5	9	0	-5	5	13	35	-1	-1	177.5	2.5		
COII	12	-2	18	-1	0	0	-9	1	-6	0	0	-7	13	-12	13	2	11.5	1	10	-1	5	4	2	-5	0	0	3	0	0	29	9	0	0	-3	0	4	0	0	1	0	0	0	0	0	4	-3	5.5	25	-1	0	123	2.1		
CYTB	0	0	0	3	0	3	7	0	7	0	2	6	8	0	1	3	3	5	5	3	-4	0	3	1	2	24	2	10	5	0	0	0	0	-4	0	1	0	0	0	2	0	0	0	0	0	2	0	0	0	102	4.7			
TOT	104	44	48	34	58	43	7	58	75	23	4	41	55	52	27	14	19	8	4	7	5	22	25	6	63	11	73	43	45	15	13	9	25	22	50	13	8	4	16	17	11	8	15	7	4	8	26	66	5	13	1381			

Nodes correspond to the nodes numbers figuring in the Fig. 3. "∑" is the total Bremer support for each gene and "%" is the percentage of Bremer values that each gene supports normalized by the number of parsimony informative characters as provided by PAUP 4.0b10 (data not shown). Then, for each gene i , $\%_i = ((\sum_i / \sum_{TOT} \times 100) / N_i) \times 100$; with N_i the number of informative character for the gene i .

3.2. Attributes optimization

Caste evolution was studied on every different topology reconstructed, but only shown for the topology of the most parsimonious tree obtained under direct optimization with equal weighting (Fig. 4). Pseudergates and true workers castes were considered as two binary characters, both coded present versus absent, and were optimized using the parsimony criterion. Concerning the evolution of the pseudergates, several equiparsimonious scenarios implying from two different origins and three losses to five different origins can be postulated (Fig. 4 in grey). The most parsimonious topology based on a static alignment give similar optimal scenarios from three different origins and two losses, to four different origins and one loss of the pseudergates. In a maximum likelihood framework (Appendix All), two different origins and six loss of the pseudergate caste are inferred. To sum up, the pseudergate caste evolved convergently at least twice in the termites. As for the true worker caste evolution (Fig. 4 in black), the phylogenetic result implied three origins and one loss for the clade (*Prorethotermitidae*–*Termitogeton*). The same scenario is found in the probabilistic framework, and the optimization of this attribute is ambiguous with the topology based on parsimony with a static alignment involving from two origins (and three losses) to five different origins. Therefore, similarly to the pseudergate caste, multiple origins of the true worker caste are inferred. The presence of the true worker and pseudergate castes in the ancestor of termites is not supported using the parsimony criterion. In the maximum likelihood framework, presence of the pseudergate caste in this ancestor is supported, contrary to the presence of the true worker caste.

Three kinds of foraging behavior were considered: one-piece, intermediate and separate types (Fig. 5). Xylophagous roaches such as *Parasphaeria* and *Cryptocercus* were considered one-piece type. This coding—mainly used here to refer to all recent phylogenetic studies on the subject—is debatable since the foraging in cockroaches is individual-centered and not displayed by a specific caste contrary to termites and may not be homologous to the foraging behavior of termites. Nevertheless, the optimization of this behavioral character suggested that the ancestor of termites was one-piece type. If, in a more conservative way, cockroaches are not considered one-piece type, this foraging behavior still appears deeply in the tree in the sister group of Mastotermitidae. In any case, one transition towards a separate type and two towards an intermediate type were inferred for Hodotermitidae and for *Mastotermes* and some Rhinotermitidae, respectively. Three transitions from an intermediate way of life towards a separate one were postulated within the clade (Rhinotermitidae + Termitidae). Finally, one transition from an intermediate type toward a one-piece type is inferred for the clade (*Prorethotermitidae* + *Termitogeton*).

4. Discussion

4.1. Relationships among termite families

The phylogeny of termites has always been controversial, as reviewed by Kambhampati and Eggleton (2000), and Eggleton (2001). Except the monophyly of some well-characterized families, every other feature has been questioned or doubted, even the position of *Mastotermes* as the first diverging lineage (Thorne and Carpenter, 1992; but see Deitz et al., 2003). This was the reason why we designed the present study with particular attention paid to large taxon and character samples representative of the real taxonomic and behavioral diversity of termites and their Dictyopteran relatives, the cockroaches and the mantids (Grandcolas and D'Hase, 2004). The results strongly supported the monophyly of

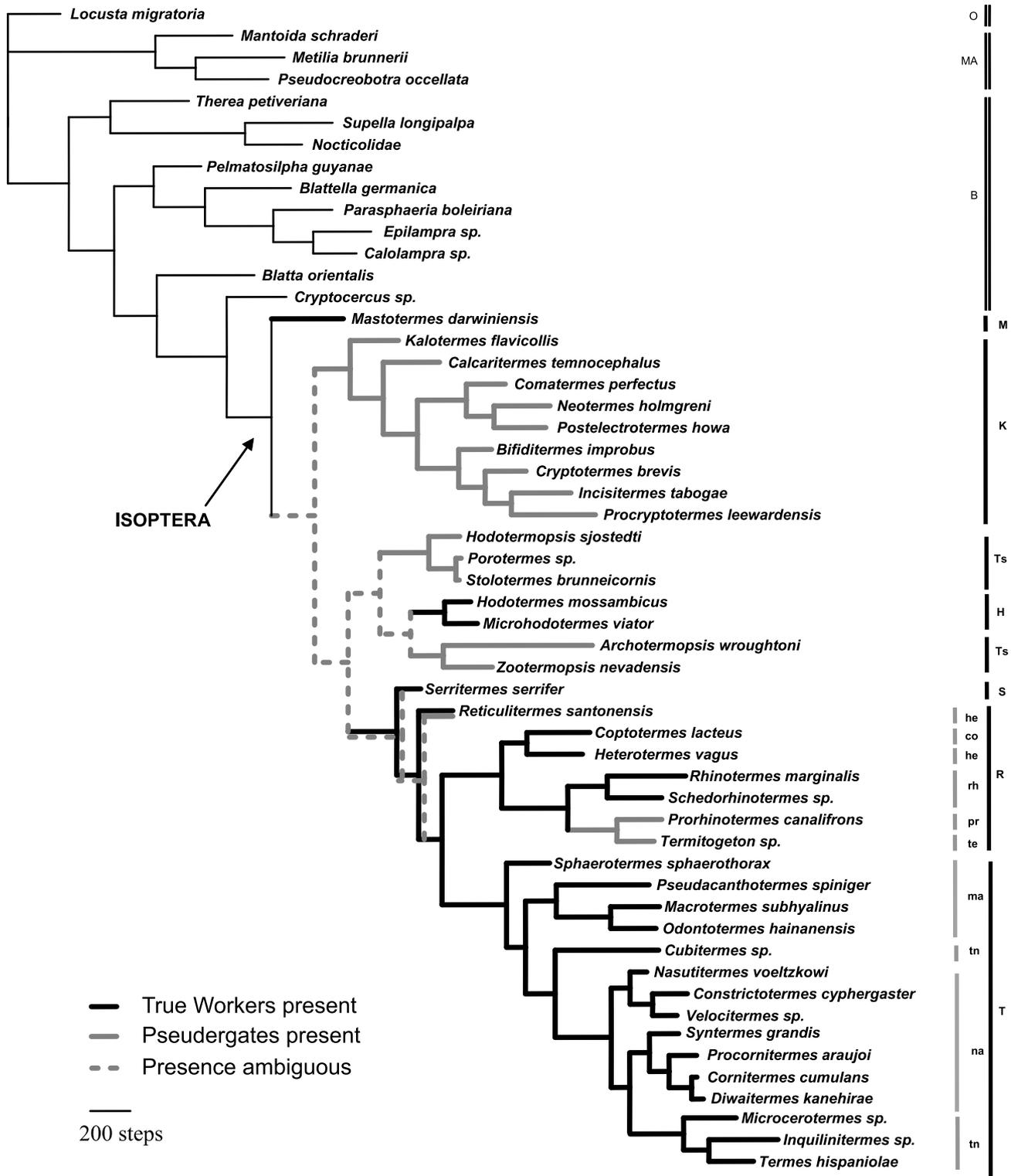


Fig. 4. Most parsimonious optimization of the attribute “pseudergates” (in grey) and “true workers” (in black) according to the most parsimonious tree in the direct optimization analysis with the parameter set 111. Thin lines = absence of the attribute; thick lines = presence of the attribute; dashed lines = ambiguous optimizations. Labeled bars at right margin as in Fig. 3.

termites with the woodroach genus *Cryptocercus* as sister taxon. In this respect, this agrees with the results of previous studies such as Klass (1997), Lo et al. (2000), Terry and Whiting (2005), Kjer et al. (2006), Klass and Meier (2006) and Inward et al. (2007a) but contrasts with Grandcolas (1994, 1996) or Gäde et al. (1997). *Mastotermes* appeared as the first diverging lineage within the termites.

Others features which were not really controversial are strongly supported by these analyses such as the monophyly of Kalotermitidae, Hodotermitidae and Termitidae. This latter result confirmed the hypothesis of a single event of loss of gut flagellate symbionts. In the same way, the paraphyly of Macrotermitinae within Termitidae is compatible with a single origin of symbiosis with fungi and

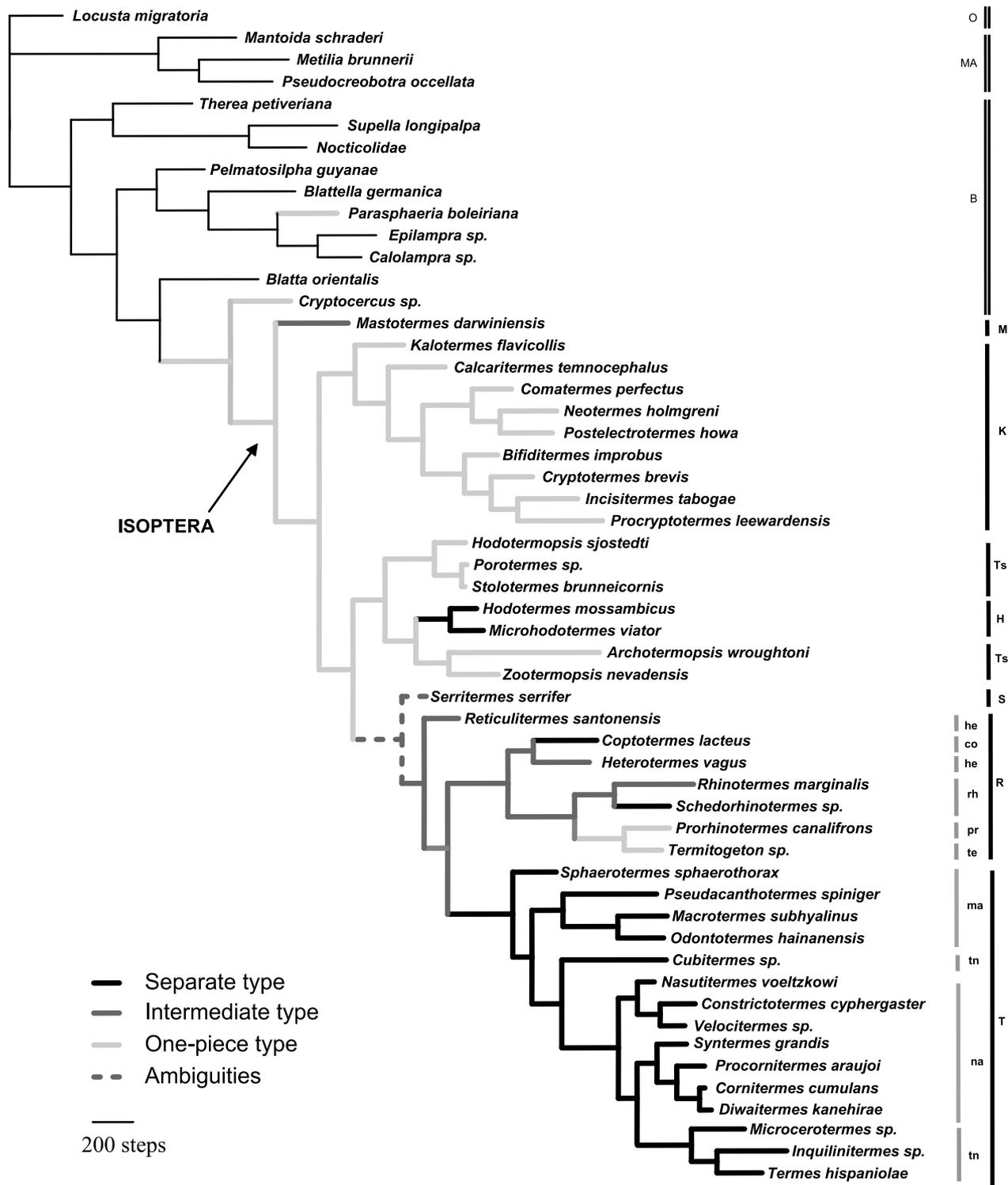


Fig. 5. Most parsimonious optimization of the attribute “foraging behavior” according to the most parsimonious tree in the direct optimization analysis with the parameter set 111. Thin lines = inapplicable character; pale grey lines = one-piece type; dark grey lines = intermediate type; black lines = separate type; dashed lines = ambiguous optimization or missing data. Labelled bars at right margin as in Fig. 3.

of fungus combs construction, as emphasized by Grassé (1949) and Aanen et al. (2002). *Sphaerotermes sphaerotherax* is a unique Macrotermitinae since it builds fungus combs but does not cultivate fungi. Fungi cultivation could have been lost secondarily in *Sphaerotermes* or gained in other Macrotermitinae. None of these hypotheses could be preferred, according to the present phylogenetic tree. The phylogenetic position of *Sphaerotermes* is congruent

with the proposition of Engel and Krishna (2004) who excluded this taxon from the Macrotermitinae and created a new subfamily, Sphaerotermatinae. However, the taxon sampling for the Termitidae was limited (about 6% of the genera known are represented) and then the relationships within this particular family cannot be fully assessed from this sample. Another expected result was the close relationships between Termitidae and Rhinotermitidae, the

latter being paraphyletic (e.g., Donovan et al., 2000; Lo et al., 2004). The non-monophyly of Rhinotermitidae was long hypothesized due to the high behavioral, developmental and morphological diversity of this group (e.g., Grassé, 1986).

The most problematic relationships within the termites were those involving Kalotermitidae, Hodotermitidae and Termopsidae. Our results support the position of the monophyletic Kalotermitidae as the second diverging lineage within the termites after *Mastotermites*, and this presents a new hypothesis for the placement of this family (Fig. 1). This result is strongly supported and stable in the parsimony analyses under direct optimization (Fig. 3) even if probabilistic analyses give another result. Ahmad (1950) said about the Kalotermitidae that “the imago-worker mandibles are essentially the same as in the Mastotermitidae” and he clustered Kalotermitidae and *Mastotermites* on the basis of those mandibles. According to our study, this similarity between imago-worker mandibles of Kalotermitidae and Mastotermitidae could be symplesiomorphic or convergent but not synapomorphic. A second interesting point dealt with the families Termopsidae and Hodotermitidae. A close relationship between these two taxa has always been assumed, except in Kambhampati and Eggleton’s hypothesis (2000) (Fig. 1). These two families were grouped in a single family Hodotermitidae before Grassé suggested in 1949 to elevate Termopsinae, Porotermitinae and Stolotermitinae to a family rank, Termopsidae (Krishna, 1970). That single-family hypothesis was strongly supported by our data and by morphological characters such as the absence of ocelli (Emerson and Krishna, 1975) and the imago-worker mandibles shape (Ahmad, 1950).

Our results were also congruent with other published concepts regarding family or subfamily groups, such as the Protermitinae (Quennedey and Deligne, 1975) and Serritermitidae (Cancello and DeSouza, 2004), even if their monophyly were not tested here. Within the family Rhinotermitidae, our preferred topology did not reconstruct the subfamily Heterotermitinae as monophyletic. *Heterotermites* and *Reticulitermes* were not closest relatives (see also Lo et al., 2004). Nevertheless, whatever the optimality criterion, relationships within the Rhinotermitidae are the least stable and certainly a larger sampling effort is needed in this family. Fortunately, this instability has no consequence on the study of caste evolution. A close relationship between *Protermitinae* and *Termitogeton* is consistent with their similar social organization, as noted by Parmentier and Roisin (2003). Inward et al. (2007b) recently published a parallel but independent study on termite phylogeny based on 3 molecular markers and 40 morphological characters in approximately 250 taxa. Our results overall agreed with theirs, except that the second diverging lineage within termites in their study is not the Kalotermitidae as in ours, but a paraphyletic assemblage of Termopsidae + Hodotermitidae. Moreover, our study retrieved the clade (Termopsidae + Hodotermitidae) as monophyletic. Our work focused on the evolution of pseudergate and true worker castes and of foraging behavior, whereas theirs focused on true workers, nesting types and feeding groups. According to their results, it was not possible to reconstruct the evolution of true workers unambiguously, whereas our results unequivocally support multiple origins of this caste (see below).

4.2. Evolution of worker castes and foraging behavior

Beyond classification issues of termites, the present phylogenetic tree also brought some new and decisive information regarding the controversy over the evolution of the worker caste and of foraging behavior. Contrary to the soldier caste in which individuals share the same development, passing through a pre-soldier or white soldier stage, a high diversity of developmental pathways leads to termite workers, especially within the termites with hindgut protozoa (Noirot, 1985b). Two different kinds of worker castes

were defined on this basis. True workers were defined functionally, morphologically and ontogenetically by Noirot and Pasteels (1987). These authors emphasized the importance of the ontogenetic criterion and defined true workers “as individuals diverging early and irreversibly from the imaginal development” (Fig. 2). In the same way, pseudergates (or false workers) were defined as “individuals separating late from the imaginal line” following regressive and stationary moults and therefore ontogenetically versatile (Grassé and Noirot, 1947; Noirot and Pasteels, 1987, Fig. 2). Two opposed evolutionary hypotheses were proposed with either pseudergates or true workers as an ancestral condition, respectively (Noirot, 1985b; Watson and Sewell, 1985). More recently, Thompson et al. (2000, 2004) studied the evolution of the worker caste within a phylogenetic framework, but they presented phylogenetic evidence which was actually not decisive (Grandcolas and D’Haese, 2002). They also coded inadequately the worker caste as one character with two states (true worker and pseudergates) to study its evolution (Grandcolas and D’Haese, 2004). It has been shown that pseudergates and true workers could coexist in a same colony in *Reticulitermes* (Buchli, 1958; Noirot, 1985b; Noirot and Pasteels, 1987; Lainé and Wright, 2003). More importantly, true workers and pseudergates do evolve, by definition, from different ontogenetic pathways (Fig. 2). Then, pseudergates and true workers should not be optimized on a phylogenetic tree as a single homologous attribute but two binary attributes should be preferred (pseudergates: present/absent and true workers: present/absent).

Mapping these two attributes on the presently obtained phylogenetic tree showed multiple origins of the true worker caste as previously hypothesized by Noirot and Pasteels (1988) and that both castes are ancestrally absent in the Isoptera. The origin of pseudergates is reconstructed as ambiguous in most of basal branches (Fig. 4) so that it cannot be assessed whether pseudergates have come before true worker caste or not. From this point of view, Noirot’s hypothesis (1985b) where true workers evolved from pseudergates can be neither supported nor rejected. Watson and Sewell (1985) assumed the opposite change—from true workers to pseudergates—a scenario lacking support in this study except for the clade (*Protermitinae* + *Termitogeton*). According to the present study, a multiple origin for the true worker caste is now clearly assessed and well-supported.

Foraging behavior has been assumed to constrain the evolution of worker castes and the simplest behavior, which consists in feeding within the food-nest (“one-piece”), was assumed to be ancestrally related to the supposedly primitive and still ontogenetically versatile pseudergate caste (Abe, 1987). It was supposed also to evolve toward the so-called “separate type” where true workers forage outside the nest to get back their food, with the possible evolutionary intermediate, the “intermediate type”. The present reconstruction validated most of these views: the “one-piece type” evolved early in termites evolution, and changed mostly toward “intermediate” and then to “separate” types, but also once directly to “separate type” (Fig. 5). Even if the evolution of foraging behavior is consistent with Abe’s proposal (1987), we cannot assess whether it evolved congruently with the worker castes the evolution of which is more ambiguous. According to some caste optimization patterns, congruent evolution between caste and foraging is strongly corroborated. According to some other equally parsimonious patterns, changes in foraging behaviour have not been simultaneous with caste evolution.

In conclusion, our study has produced the most-encompassing DNA data set on the phylogenetic relationships among all major groups of termites. The results showed that the relationships among the most basal families were far from well-understood. They also shed light on one of the major evolutionary issues in termites, the evolution of the worker caste. The most traditional

scenario by Noirot (1985b), which assumed multiple and convergent origins for the “advanced” true worker caste, was corroborated by our reconstruction. However, the most basal part of that reconstruction remained ambiguous and it was not possible to assess whether pseudergates preceded true workers. Even though the classical definitions of true worker versus pseudergate castes were quite clear, they were not precise enough for unambiguous optimizations on our phylogenetic trees. Such a lack of accuracy could be resolved in the future by designing more detailed studies on the developmental pathways which led to different castes (Parmentier and Roisin, 2003). The present study has provided a basis and could be an impetus for such future comparative studies concerning which was until now the most poorly known case of social behavior evolution.

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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.2008.04.017.

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