

# Molecular Phylogenetics and Evolution

## Molecular phylogeny and historical biogeography of Apicotermitinae (Blattodea: Termitidae) --Manuscript Draft--

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<b>Abstract:</b>	<p>Soil-feeding termites are abundant in tropical regions and play an important role in soil bioturbation and in the organic matter cycle. The Apicotermitinae are arguably the most diverse lineage of soil-feeding termites, but they are also the most understudied, probably because many species are soldierless, which makes identification difficult, and because their cryptic lifestyle prevents easy sampling. Although the backbone of the termite phylogenetic tree is now well-resolved, the relationships among representatives of Apicotermitinae are still largely unknown. Here, we present phylogenetic trees inferred from 113 mitochondrial genomes of Apicotermitinae representative of the group diversity. Our analyses confirm the monophyly of the Apicotermitinae and the basal position of soldiered taxa, within which two lineages of soldierless species are nested. We resolved, with high support, the position of Asian genera as sister group of a clade comprising the monophyletic neotropical Anoplotermes -group and a small African clade including Adaiphrotermes and an undescribed genus. Our trees cast light on the intergeneric and interspecific relationships within Apicotermitinae and reveal the polyphyly of several genera, including Ruptitermes, Astalotermes and Anoplotermes. Biogeographic reconstructions revealed two dispersal events out of Africa, one to the Oriental realm and one to the Neotropical realm. Overall, the timing of Apicotermitinae diversification and dispersal, following the Eocene-Oligocene boundary, matches that found for other groups of Neoisoptera.</p>
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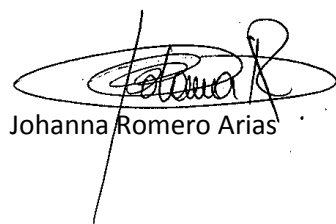
Dear Dr. Elizabeth Zimmer,

We have uploaded a research paper entitled “Molecular phylogeny and historical biogeography of Apicotermatinae (Blattodea: Termitidae)” to be considered for publication in *Molecular Phylogenetics and Evolution*. Our study reconstructed the phylogenetic relationships of the termite subfamily Apicotermatinae and revealed the polyphyly of several genera that should be taxonomically revised. These soil-feeding termites have spread from Africa to the Neotropical and Oriental regions. Our study resolved with great support the close relationship between the Asian soldiered species and the African and Neotropical soldierless sister groups. We believe that our results are of general interest, especially due to the extensive and pioneer phylogeny of this well widespread group.

This manuscript is original and not under consideration for publication elsewhere. All authors contributed and approved the manuscript and this submission. We have no conflicts of interest to declare.

Thank you for your time and consideration. We look forward to hearing from you.

Sincerely, also on behalf of my co-authors,



Johanna Romero Arias

## Highlights

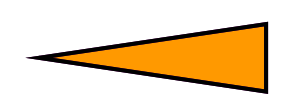
- Monophyly in Apicotermitinae was supported by mitochondrial genome phylogenetics.
- Apicotermitinae diverged about 45 Ma during Eocene in Africa and dispersed twice.
- Soldiered taxa are more closely related to other termitids than soldierless species.
- *Adaiphrotermes* + a new genus are close relatives of neotropical soldierless taxa.
- Taxonomic revisions of *Anoplotermes*, *Ruptitermes*, *Astalotermes* are required.



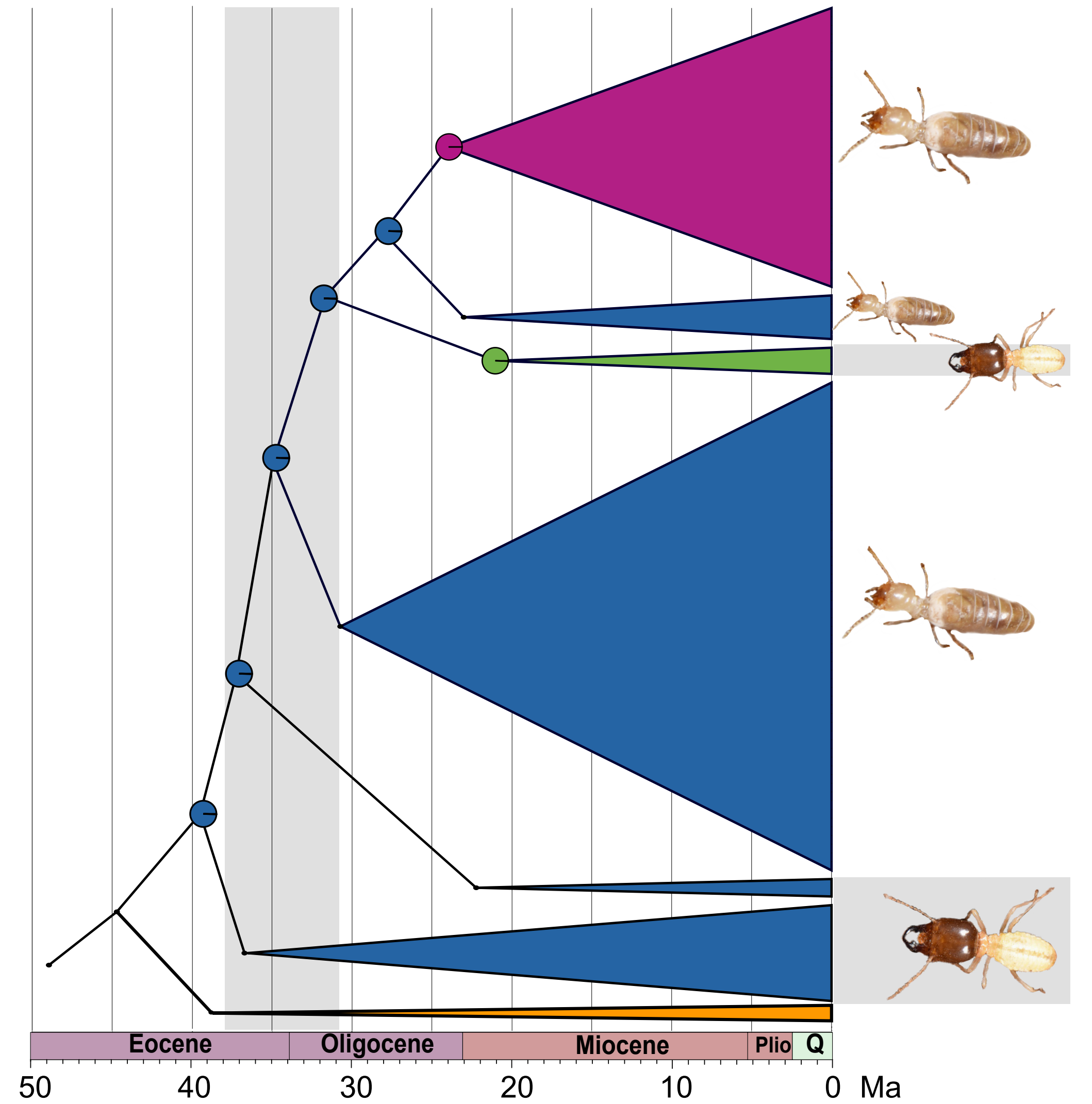
soldiered species



soldierless species



other termitids



1 **Molecular phylogeny and historical biogeography of Apicotermitinae (Blattodea:**  
2 **Termitidae)**

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16

17 **Abstract**

18 Soil-feeding termites are abundant in tropical regions and play an important role in soil bioturbation and  
19 in the organic matter cycle. The Apicotermitinae are arguably the most diverse lineage of soil-feeding  
20 termites, but they are also the most understudied, probably because many species are soldierless, which  
21 makes identification difficult, and because their cryptic lifestyle prevents easy sampling. Although the  
22 backbone of the termite phylogenetic tree is now well-resolved, the relationships among representatives  
23 of Apicotermitinae are still largely unknown. Here, we present phylogenetic trees inferred from 113  
24 mitochondrial genomes of Apicotermitinae representative of the group diversity. Our analyses confirm  
25 the monophyly of the Apicotermitinae and the basal position of soldiered taxa, within which two lineages  
26 of soldierless species are nested. We resolved, with high support, the position of Asian genera as sister  
27 group of a clade comprising the monophyletic neotropical *Anoplotermes*-group and a small African clade  
28 including *Adaiphrotermes* and an undescribed genus. Our trees cast light on the intergeneric and  
29 interspecific relationships within Apicotermitinae and reveal the polyphyly of several genera, including  
30 *Ruptitermes*, *Astalotermes* and *Anoplotermes*. Biogeographic reconstructions revealed two dispersal  
31 events out of Africa, one to the Oriental realm and one to the Neotropical realm. Overall, the timing of  
32 Apicotermitinae diversification and dispersal, following the Eocene-Oligocene boundary, matches that  
33 found for other groups of Neoisoptera.

34

35 **Keywords:** Humivorous, Isoptera, mitochondrial genome, systematics, molecular clock

## 36 1. Introduction

37 The Apicotermittinae are a subfamily of soil-feeding termites that play important roles in soil bioturbation  
38 and organic matter cycling in tropical rainforests and savannas (Jones and Eggleton, 2011, Bourguignon  
39 et al., 2016). Several lineages of Apicotermittinae are characterized by the absence of the soldier caste.  
40 Soldiered species encompass the African *Apicotermes*-group and the Oriental *Speculitermes*-group  
41 (Grassé and Noirot, 1954; Sands, 1972). Soldiers are, however, rare and often unknown in species of the  
42 *Speculitermes*-group. Soldierless species comprise the *Astalotermes*-group in Africa and the  
43 *Anoplotermes*-group in the Neotropics (Sands, 1972), both of which are very abundant and can locally  
44 make up more than 30% of the termite species diversity (Eggleton et al., 1995, 2002; Bourguignon et al.,  
45 2011, 2016; Dahlsjö et al., 2015, 2020; Nduwarugira et al., 2017).

46 The Apicotermittinae are one of the most diverse subfamilies of Termitidae, and the most understudied,  
47 probably because they include many soldierless species that can only be distinguished morphologically by  
48 tedious dissections of the worker digestive tract (Grassé and Noirot, 1954; Sands, 1972, 1998; Noirot,  
49 2001; Bourguignon et al., 2016). To date, 224 species and 52 genera of Apicotermittinae have been  
50 described, with diversity hotspots located in Africa and South America (Krishna et al., 2013;  
51 Bourguignon et al., 2016; Constantino, 2020; Roisin, 2020). However, the actual diversity of the group is  
52 much larger, and many species, still awaiting formal description, have been informally labelled as  
53 morphospecies in ecological surveys (e.g., Eggleton et al. 1995, 2002; Davies, 2002; Bourguignon et al.,  
54 2011; Nduwarugira et al., 2017).

55 The first comprehensive phylogenetic study of termites was based on a combination of morphological  
56 characters and genetic markers, including two mitochondrial genes (COII and 12S) and one nuclear gene  
57 (28S) (Inward et al., 2007). This study supported the monophyly of Apicotermittinae, which were  
58 retrieved as the sister group of a clade composed of all other Termitidae except the fungus-growers  
59 (Macrotermittinae) and the two small subfamilies Sphaerotermittinae and Foraminitermittinae (Inward et  
60 al., 2007). This phylogenetic position was later confirmed by molecular phylogenies inferred from  
61 complete mitochondrial genomes and transcriptomes (Bourguignon et al., 2015, 2017, Bucek et al.,  
62 2019). In addition, the phylogenetic tree of Inward et al. (2007) suggested that (1) the African soldiered  
63 taxa are paraphyletic to a clade composed of the soldierless lineages and the Asian (soldiered)  
64 Apicotermittinae; (2) the Oriental *Speculitermes*-group is monophyletic; (3) the Neotropical  
65 *Anoplotermes*-group is monophyletic; and (4) the Oriental *Speculitermes*-group, the Neotropical  
66 *Anoplotermes*-group and the African soldierless *Adaiphrotermes* form a monophyletic group sister to all  
67 other African soldierless taxa. This tree topology implies two independent losses of soldiers in  
68 Apicotermittinae, and two independent dispersal events between continents, with unclear directionality.  
69 Complete mitochondrial genome phylogenies confirmed that Asian and Neotropical taxa are closer to  
70 each other than to most African soldierless genera (Bourguignon et al., 2017), but, because of their  
71 insufficient sampling, poor characterization of some described genera (e.g., *Astalotermes*,

72 *Anenteotermes*), and uncertain identifications, the history of Apicotermitinae remains unclear. In addition,  
73 most relationships among African and Neotropical soldierless taxa were unresolved by Inward et al.  
74 (2007), and several genera (e.g., *Aderitotermes*, *Astalotermes* or *Anoplotermes*) appeared as polyphyletic  
75 in the phylogenetic trees of Bourguignon et al. (2017).

76 In this study, we used 113 mitochondrial genomes of Apicotermitinae species to reconstruct robust  
77 phylogenetic trees including most described genera, well-characterized by anatomical features. Using  
78 these trees, we tested previous phylogenetic hypotheses regarding the relationships among major  
79 Apicotermitinae clades and provided a timeframe for their evolution. We also investigated the historical  
80 biogeography of Apicotermitinae and determined the number of independent losses of soldiers. Our  
81 analyses clarify the taxonomy of Apicotermitinae and pave the path to future taxonomic revisions of non-  
82 monophyletic genera, such as *Astalotermes* or *Anoplotermes*, and provide a framework to study the  
83 anatomical evolution of the subfamily.

## 84 **2. Material and methods**

### 85 **2.1. Sampling**

86 Termite sampling was conducted in Burundi (n = 7), Cameroon (n = 28), Ivory Coast (n = 18), Kenya (n  
87 = 1) and French Guiana (n = 13) (Table S1). For each sample, we collected specimens in RNA-later® or  
88 in 100% ethanol for genetic analyses, and in 80% ethanol for morphological analyses. Samples collected  
89 in RNA-later® and 100% ethanol were temporarily stored at a temperature ranging from -20°C to 4°C,  
90 and shipped to the Czech University of Life Sciences or to the Okinawa Institute of Science and  
91 Technology, where they were stored at -80°C until DNA extraction. Samples collected in 80% ethanol are  
92 stored at the Université Libre de Bruxelles and the Czech University of Life Sciences. In addition to the  
93 67 samples collected in this study, we also obtained the full mitochondrial genome sequences of 43  
94 samples of Apicotermitinae from GenBank (Bourguignon et al. 2015, 2017) and reconstructed  
95 mitochondrial genomes from transcriptome sequences of three species (Bucek et al., 2019) (Table S1).

96 Species identifications were based on morphological and anatomical characters, which included the  
97 worker digestive tube configuration, the shape of the gizzard and enteric valve armature, as described in  
98 Romero Arias et al. (2020). We also re-examined the voucher material of samples sequenced in previous  
99 studies and whose phylogenetic position appeared inconsistent. In a few cases, we found that the voucher  
100 samples contained a mixture of two species. We labelled these samples with both species names. Revised  
101 species identifications are detailed in Supplementary Appendix A (see also Table S1).

### 102 **2.2. DNA extraction and sequencing**

103 Whole genomic DNA was extracted from head and thorax of three to five workers using the DNeasy  
104 Blood & Tissue extraction kits (Qiagen). Because DNA extracts were sequenced at different periods of  
105 time, two different approaches were used. For the first approach, the complete mitochondrial genome was  
106 amplified in two long-PCR reactions with the TaKaRa LA Taq polymerase, using primers previously  
107 designed for termites (Bourguignon et al., 2015). The concentration of both long PCR fragments was



108 determined using a Qubit 3.0 fluorometer, and the two fragments were mixed in equimolar concentration.  
109 Libraries were prepared with the NEBNext Ultra II DNA Library Prep Kit (New England Biolabs) and  
110 sequenced with Illumina MiSeq. For the second approach, whole genomic DNA libraries were directly  
111 prepared with the aforementioned NEB kit and sequenced using Illumina HiSeq4000.

### 112 **2.3. Assembly and annotation of mitochondrial genomes**

113 Paired-end reads were quality-assessed using FastQC v0.11.7 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) and adapter sequences were removed with Trim Galore v0.4.5  
114 ([http://www.bioinformatics.babraham.ac.uk/projects/trim\\_galore/](http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/)) using default settings. Mitochondrial  
115 reads were identified using the mitogenome of *Astalotermes murcus* (accession no. KY224676) as a  
116 reference and assembled using GetOrganelle v1.5.1 (Jin et al., 2019). Each resulting assembly graph was  
117 inspected with Bandage v0.8.1 (Wick et al. 2015) and mitochondrial genome sequences were manually  
118 circularized when necessary. Control regions were discarded from the final assemblies as they provide  
119 limited phylogenetic information and are difficult to accurately assemble with short reads. We used the  
120 MITOS2 Webserver with the invertebrate genetic code and the protein prediction method of Donath et al.  
121 (2019) to annotate the two rRNA genes, 22 tRNA genes, and 13 protein-coding genes. Other parameters  
122 were set on default settings. Annotated genomes are deposited in GenBank (accession numbers to come).  
123 In total, we generated 67 new mitochondrial genome sequences, mostly from African species (54). Forty-  
124 eight mitochondrial genomes were complete, and 19 mitochondrial genomes were nearly complete  
125 because of ambiguous circularization. The mitochondrial genomes from Bourguignon et al. (2015), that  
126 included 60 non-Apicotermitinae termites and eight non-termite polyneopteran insects, were used as  
127 outgroups (Table S2). Therefore, the final data set comprised 181 mitochondrial genomes, including 113  
128 genomes of Apicotermitinae.

### 130 **2.4. Sequence alignment**

131 We aligned separately each of the two rRNA genes, 22 tRNA genes, and 13 protein-coding genes using  
132 MAFFT v7.300b (Katoh et al. 2002, 2013) with default settings. Protein-coding genes were aligned as  
133 protein sequences and back-translated into nucleotide sequences using PAL2NAL (Suyama et al. 2006).  
134 rRNAs and tRNAs were aligned as DNA sequences. The 37 aligned genes were concatenated and  
135 partitioned into five partitions: one for each codon position of the combined protein-coding genes; one for  
136 the combined 12S and the 16S rRNA genes; and one for the combined tRNA genes. We found no clear  
137 evidence of mutational saturation for the third codon positions of the protein-coding genes ( $I_{SS}=0.572$ ,  
138  $I_{SS,cSym}=0.809$ ) using the Xia's method implemented in DAMBE (Xia et al. 2003; Xia and Lemey 2009)  
139 and therefore retained the third codon positions in our phylogenetic analyses.

### 140 **2.5. Phylogenetic inference**

141 We used RAxML version 8.2.4 (Stamatakis, 2014) to reconstruct a maximum-likelihood phylogenetic  
142 tree. We used the GTR+G model for each partition. Bootstrap values were estimated from 1000  
143 replicates. We used MrBayes version 3.2 (Ronquist et al. 2012) to reconstruct a Bayesian phylogenetic

144 tree. The analysis was run with four chains (three hot and one cold), and we estimated posterior  
145 distributions using Markov chain Monte Carlo (MCMC) sampling drawn every 5000 steps. The chain  
146 was run for a total of 10 million steps, with the first 1 million steps discarded as burnin, as suggested by  
147 inspection of the trace files using Tracer v1.5 (Rambaut and Drummond 2009). We used a GTR model  
148 with gamma-distributed rate variation across sites (GTR+G) for each partition. The analysis was run in  
149 triplicate to insure convergence of the chains and check for consistency. Node support was estimated  
150 using Bayesian posterior probabilities.

## 151 **2.6. *Molecular dating***

152 We estimated time-calibrated trees using BEAST2 version 2.4.4 (Bouckaert et al. 2014). We performed  
153 the analyses with and without third codon positions to assess the influence of third codon positions on  
154 time estimates. The trees were reconstructed using an uncorrelated lognormal relaxed clock to model rate  
155 variation among branches, with single model for each partition, allowing different relative rates. A Yule  
156 speciation model was used as tree prior. We used a GTR+G model of nucleotide substitution for each  
157 partition. The chains were run for 500 million steps and were sampled every 10,000 generations to  
158 estimate the posterior distribution. We discarded the first 50 million steps as burn-in, as suggested by  
159 inspection of the trace files using Tracer v1.5 (Rambaut and Drummond 2009). A total of 13 fossils were  
160 used as minimum age constraints (see Table S3). We determined soft upper bounds using phylogenetic  
161 bracketing (Ho and Phillips 2009). Each calibration was implemented as exponential priors of node time.  
162 The analyses were run in triplicate to insure convergence of the chains and check for consistency.

## 163 **2.7. *Reconstruction of ancestral distribution***

164 The ancestral distribution of Apicotermitinae was reconstructed using the ace function of the R package  
165 APE version 5.0 (Paradis and Schliep 2018). We used the Maximum Likelihood model described by  
166 Pagel (1994) and an equal-rates of transition. Sampling locations were used to assign each tip to one  
167 biogeographic realm. Apicotermitinae are distributed across three biogeographic realms, as described by  
168 Holt et al. (2013): Afrotropical, Neotropical, and Oriental. We reconstructed ancestral distribution on the  
169 maximum-likelihood tree, the Bayesian tree, and the two time-calibrated trees.

# 170 **3. Results**

## 171 **3.1. *Molecular phylogeny***

172 Our phylogenetic trees fully supported the monophyly of Apicotermitinae (Figs 1 and S1-S3). African  
173 soldiered taxa (the *Apicotermes*-group) formed a paraphyletic assemblage, composed of two or three  
174 lineages, within which a clade composed of Asian genera and African and Neotropical soldierless taxa  
175 was nested (Figs 1 and S2-S3). This latter clade was divided into four lineages, fully supported in all  
176 analyses: (I) the African soldierless species, with the exclusion of *Adaiphrotermes* and Genus F, was  
177 retrieved as sister to the other three lineages, (II) the Asian *Speculitermes*-group was sister to the last two  
178 lineages, (III) the African genera *Adaiphrotermes* and Genus F, and their sister group, (IV) the  
179 Neotropical soldierless *Anoplotermes*-group.

180 Discrepancies among analyses were found for the position of soldiered lineages. More precisely, the  
181 position of the clade including Genus C + (*Hoplognathotermes* + *Labidotermes*) was variable among  
182 analyses (Figs 1 and S1-S3). Similarly, the position of species within the *Astalotermes*-group and the  
183 *Anoplotermes*-group was variable. The relationships among taxa of *Astalotermes*-group were often  
184 weakly supported, and several genera were retrieved as polyphyletic, i.e. *Astalotermes*, *Anenteotermes*  
185 and *Astratotermes*. Within the neotropical *Anoplotermes*-group (clade IV), the relationships among  
186 genera were weakly supported, and many species, referred to as *Anoplotermes*-group sp., lie on long  
187 branches and belong to undescribed genera (Fig. 1 and S1-S3). The genus *Ruptitermes* appears  
188 polyphyletic, the arboreal *R. arboreus* being broadly separated from the other species of the genus.

### 189 **3.2. Divergence time estimation**

190 The time-tree reconstructed with third codon positions included yielded older age estimates (Fig. S1),  
191 up to 10.9 million years (My) older than the analysis with third codon positions excluded (Fig. 1). The  
192 ranges given hereafter encompass the results of both analyses, with and without third codon positions. We  
193 estimated that the most recent common ancestor of Apicotermitinae lived 39.5–48.6 million years ago  
194 (hereafter Ma) (95% HPD: 34.7–53.2 Ma), during the middle Eocene. The most recent common ancestor  
195 of the soldierless Apicotermitinae + *Speculitermes*-group was estimated at 34.9–44.2 Ma (95% HPD:  
196 30.8–48.5 Ma). The split between the *Speculitermes*-group and their sister group was dated at 31.7–41.6  
197 Ma (95% HPD: 27.7–45.9 Ma), during the early Oligocene. The Neotropical *Anoplotermes*-group  
198 diverged from its African sister lineage (*Adaiphrotermes* + Genus F) 28.0–38.0 Ma (95% HPD: 24.4–42.0  
199 Ma). The age estimates of cladogenesis for the current taxonomic groups are summarized in Table 1.

### 200 **3.3. Ancestral distribution**

201 We reconstructed the ancestral distribution of Apicotermitinae on the four phylogenetic trees generated in  
202 this study and found entirely congruent results (Figs S4-S7). We found that the Apicotermitinae  
203 originated in the African realm, and dispersed from there twice: once to the Oriental realm, where they  
204 gave rise to the *Speculitermes*-group, and once to the Neotropical realm, where they gave rise to the  
205 *Anoplotermes*-group.

## 206 **4. Discussion**

### 207 **4.1. Phylogenetic relationships and systematics**

208 Our findings are in partial agreement with those of Inward et al. (2007) and Bourguignon et al. (2017).  
209 For instance, we confirm the paraphyly of the *Apicotermes*-group, which is composed of several basal  
210 lineages, closely matching the subgroups proposed by Noirot (2001) on the basis of digestive anatomy:  
211 the *Labidotermes* subgroup, comprising also *Hoplognathotermes* (+ *Acutidentitermes*, not sequenced), is  
212 characterized by a simple enteric valve armature wholly enclosed within the P2 section of the hindgut,  
213 which probably represents an ancestral condition; the *Apicotermes* subgroup, including also  
214 *Allognathotermes* + *Duplidentitermes* and *Coxotermes* + *Heimitermes*, possesses very sophisticated  
215 enteric valve armatures protruding into the paunch; and the *Trichotermes* subgroup, including

216 *Jugositermes* and *Phoxotermes* (+ *Rostrotermes*, not sequenced), displays an enteric valve with six  
217 sclerotized plates bearing numerous, variously developed spines, which also penetrate into the paunch.  
218 Noirot (2001) made a fourth subgroup for *Eburnitermes* and *Machadotermes*, which were not sequenced.  
219 These two genera possibly constitute another basal lineage. Finally, some new taxa are known from the  
220 worker only, although their anatomy places them in the *Apicotermes*-group: this is the case of the new  
221 Genus C, whose mt-DNA confirmed distant affinities with *Labidotermes* and *Hoplognathotermes*.  
222 Another such taxon is the new genus labelled "*Kaktotermes*" (*nomen nudum*) by Donovan (2002), which  
223 still awaits sequencing.

224 The phylogeny of Inward et al. (2007) featured a large clade comprising, on the one hand, the  
225 *Astalotermes*-group (including all African soldierless taxa except *Adaiphrotermes*, without deeper  
226 resolution), and on the other hand, an unresolved clade including *Adaiphrotermes*, which appeared  
227 paraphyletic, the Asian taxa (with soldiers known in all genera, but often very rare) and the neotropical  
228 taxa (all soldierless, not further resolved). Here, we confirm the *Astalotermes*-group as monophyletic and  
229 resolve its sister clade with a strong support: Asian genera (*Indotermes* + *Euhamitermes*) now branch out  
230 first, as sister group to a soldierless clade including the neotropical taxa, confirmed as monophyletic (=   
231 the *Anoplotermes*-group clade), and an African branch composed of *Adaiphrotermes* plus a distinctive  
232 new African genus here called Genus F (*Adaiphrotermes*-group clade).

233 Inward et al. (2007) suggested that the loss of the soldier caste occurred only once in the evolution of the  
234 Apicotermitinae, but in view of the best supported phylogeny, this hypothesis cannot account for the  
235 presence of soldiers in Asian taxa. Unless soldiers have been reacquired in Asian taxa, which seems  
236 unlikely, their loss must have occurred at least twice: once at the origin of the *Astalotermes*-group (clade I  
237 on Fig. 1), and once at the origin of the clade composed of the *Adaiphrotermes*-group and the  
238 *Anoplotermes*-group (clades III+IV on Fig. 1). Note that missing taxa might in the future cast additional  
239 light on soldier loss events, when their phylogenetic position is ascertained: according to Noirot (2001),  
240 the soldiered genus *Firmitermes* possesses a digestive anatomy reminiscent of soldierless species,  
241 whereas the soldierless genus *Skatitermes* anatomically matches the *Apicotermes*-group. In addition,  
242 soldiers are very rare or even unknown in some Asian species (especially in the genus *Speculitermes*),  
243 which suggests that they may have rarefied to the point of disappearing completely several times.

244 Thus far, most generic descriptions of Apicotermitinae have been written in the absence of a solid  
245 phylogenetic background. Some genera are characterized by conspicuous apomorphies, such as the  
246 hypertrophied sclerotization of cushion 1 of the enteric valve in *Ateuchotermes* (Sands, 1972), whereas  
247 others mostly accommodate species that do not display particular diagnostic features. For instance, as  
248 Sands (1972: 51) himself admitted, *Astalotermes* was difficult to define because this genus "occupies a  
249 transitional position between others with more primitive and more specialized characters". Not  
250 surprisingly, this genus came out of our study as polyphyletic. Likewise, *Astratotermes* —basically,  
251 *Astalotermes* with enteric valve scales ending in tiny points— was defined on characters of poor

252 phylogenetic significance and ended up polyphyletic as well. The situation is even more caricatural in the  
253 Neotropics, where the genus *Anoplotermes* lumps all soldierless species that have so far not been  
254 considered eccentric enough to deserve a transfer to another genus. All those genera are now in need of an  
255 in-depth revision. The present phylogeny will constitute a useful framework to revise the whole subfamily  
256 and identify characters of phylogenetic interest.

257 This work also yielded less intuitive results. For instance, the tiny *Anenteotermes nanus* now appears  
258 distant from the equally tiny *An. polyscolus* and other species with a bilateral enteric valve armature —  
259 *An. cnaphorus*, *An. sp. A* (CIVT120), and probably *An. cherubimi*, recently described (Scheffrahn and  
260 Roisin, 2018) and awaiting sequencing. In the Neotropics, the arboreal open-air forager *Ruptitermes*  
261 *arboreus* was known to be slightly different, on anatomical grounds, from other species of the genus  
262 which are ground-dwelling litter feeders (Acioli and Constantino 2015). Our results now show that *R.*  
263 *arboreus* has been wrongly assigned to this genus, being closer to *Tetimatermes* than to other *Ruptitermes*  
264 species.

#### 265 **4.2. Time frame of Apicotermitinae evolution**

266 As suggested by Inward et al. (2007) and Bourguignon et al. (2017), our results support the African origin  
267 of Apicotermitinae. The molecular dating analyses with and without third codon positions yield age  
268 estimates diverging by up to 10.9 My. Likely, this difference is caused by the high base compositional  
269 heterogeneity at third codon positions which can influence the estimation of divergence times (Shong et  
270 al., 2010; Zheng et al., 2011). However, time estimates of our tree with the third codon position excluded  
271 are similar to those of other phylogenetic trees (Bourguignon et al., 2015, 2017; Bucek et al., 2019). For  
272 instance, our estimation of the most recent ancestor of Apicotermitinae diverged by less than 5 My from  
273 those time-trees (Bourguignon et al., 2015, 2017; Bucek et al., 2019). According to both molecular  
274 clocks, Apicotermitinae cladogenesis was initiated during the Eocene 39.5–48.6 Ma (95% HPD: 34.7–  
275 53.2 Ma) when rainforests were more extended than nowadays. Nevertheless, most clades originated after  
276 the Eocene-Oligocene transition (about 34 Ma). This event may be compatible with a timeframe in which  
277 the atmospheric concentration of carbon dioxide dropped (Pagani et al., 2005), global temperatures  
278 decreased, and the megathermal rainforests retracted to low latitudes (Morley, 2011). Thus, this climate  
279 change led to some species extinctions and created refuges in relicts of equatorial forests that could have  
280 driven speciation events. The age estimates of our molecular clock analysis without the third codon  
281 positions match with past climatic transitions and forest distributions that may have led to the  
282 diversification of the Apicotermitinae lineage. According to our results, migratory movements of the  
283 ancestors of the *Speculitermes* and *Anoplotermes* groups occurred in two separate occasions out of the  
284 Afrotropical realm. The first dispersal event occurred 21.0–31.7 Ma (95% HPD: 16.2–35.9 Ma, without  
285 3<sup>rd</sup> codon position) or 29.6–41.6 (95% HPD: 23.6–45.9 Ma, with 3<sup>rd</sup> codon position) and gave rise to the  
286 Oriental soldiered species while the sister lineage remained in the Afrotropical realm. Following the  
287 second dispersal event, 24.0–28.1 Ma (95% HPD: 20.8–31.9 Ma, without 3<sup>rd</sup> codon position) or 34.2–

288 38.0 (95% HPD: 30.8–41.9 Ma, with 3<sup>rd</sup> codon position), the African ancestor diverged into Neotropical  
289 *Anoplotermes* soldierless species, sister of the African *Adaiphrotermes*-group. This first important  
290 migratory event coincides with a generalized trend of species dispersion and diversification in other  
291 insects and plants which followed the extensive extinction during the Eocene–Oligocene transition  
292 (Maley 1996; Engel, 2001; Morley, 2011). As suggested by Bourguignon et al. (2015), we confirm the  
293 single dispersion to the Neotropical region. Since no land connection between the Neotropics and the  
294 Afrotropics existed during the Tertiary, the ancestor of the *Anoplotermes*-group must have reached the  
295 Neotropics over the ocean, possibly by rafting in wood (Bourguignon et al., 2017). However, soil feeding  
296 behavior was recently suggested as the ancestral state in the major clade of Termitidae, after the  
297 Macrotermitinae and Sphaerotermitinae branched off (Bucek et al., 2019), and certainly at the origin of  
298 the Apicotermitinae. Soil-feeding termites are especially poor dispersers, but a possible explanation for  
299 their overseas dispersal is through nests attached to floating logs (Bourguignon et al., 2017). Another  
300 possibility is the colonization by a soil-wood interface feeder settled in a decayed part of a log. The  
301 closest African living relative of the Neotropical *Anoplotermes*-group, the *Adaiphrotermes*-group, is not  
302 known to build cohesive nests, but *Adaiphrotermes* species have been found on wood baits (Sands, 1972).  
303 Nowadays, several neotropical species are known to build nests, often attached to tree trunks or roots, and  
304 some commonly occur under bark or in decayed logs (Bourguignon et al., 2015).

305 A second important lapse of time in the evolutionary history of the Apicotermitinae is largely congruent  
306 with the return of greenhouse climates during the early-middle Miocene (Morley, 2011). With the  
307 stabilized carbon dioxide levels, increasing temperatures, and the rapid expansion of megathermal forests  
308 between 10 to 20 Ma, the Apicotermitinae underwent an important diversification. For the African  
309 *Astalotermes*-group, the colonization of forests by Angiosperms and the expansion of savannas with  
310 grasses as its key element (Maley, 1996; Linder, 2014) suggest an alteration of the organic proportion of  
311 soil as well as new opportunities to exploit the soil resources in savannas. For the *Anoplotermes*-group,  
312 the wet tropical monsoon climate (Kaandorp et al., 2005), the expansion of the Andean mountains during  
313 the mid-Miocene and the draining of wetlands during the late Miocene provided favorable environmental  
314 conditions for a particularly rapid diversification of species (Hoorn et al 2010, van der Hammen et al  
315 2000).

316 The large number of species that recently diverged (Pliocene to present) sheds new light on the previously  
317 underestimated diversity of Apicotermitinae and highlights the need for efficient taxa sampling. Today,  
318 the known diversity of Apicotermitinae species continues to increase regularly, in particular in the  
319 Neotropical clade. In this study, we established the phylogenetic relationships of the Oriental  
320 *Speculitermes* and the Neotropical *Anoplotermes* groups by determining the position of the African  
321 soldierless genus *Adaiphrotermes* (+ genus F) as a sister group of the neotropical soldierless species. A  
322 denser sampling of individual subclades in *Astalotermes* and *Anoplotermes* groups is necessary to resolve  
323 their intra-group relationships and understand their diversification processes.

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469  
470

Table 1. Estimation dates for the major and basal Apicotermitinae clades (Ma) with all sites included and without third codon positions. The differences ( $\delta$ ) of node ages are included.

crown clade	description	without Third codon positions		with Third codon positions		$\delta$ node ages
		node ages	95% HPD	node ages	95% HPD	
<b>I</b>	<i>Astalotermes</i> -group	30.7	27.0-34.8	40.5	36.8-44.8	9.8
<b>II</b>	<i>Speculitermes</i> -group	21.0	16.2-25.6	29.6	23.6-34.8	8.6
<b>III</b>	<i>Adaiphrotermes</i> + genus F	23.0	18.4-27.7	32.2	27.5-37.3	9.2
<b>IV</b>	<i>Anoplotermes</i> -group	24.0	20.8-27.2	34.2	30.8-37.9	10.2

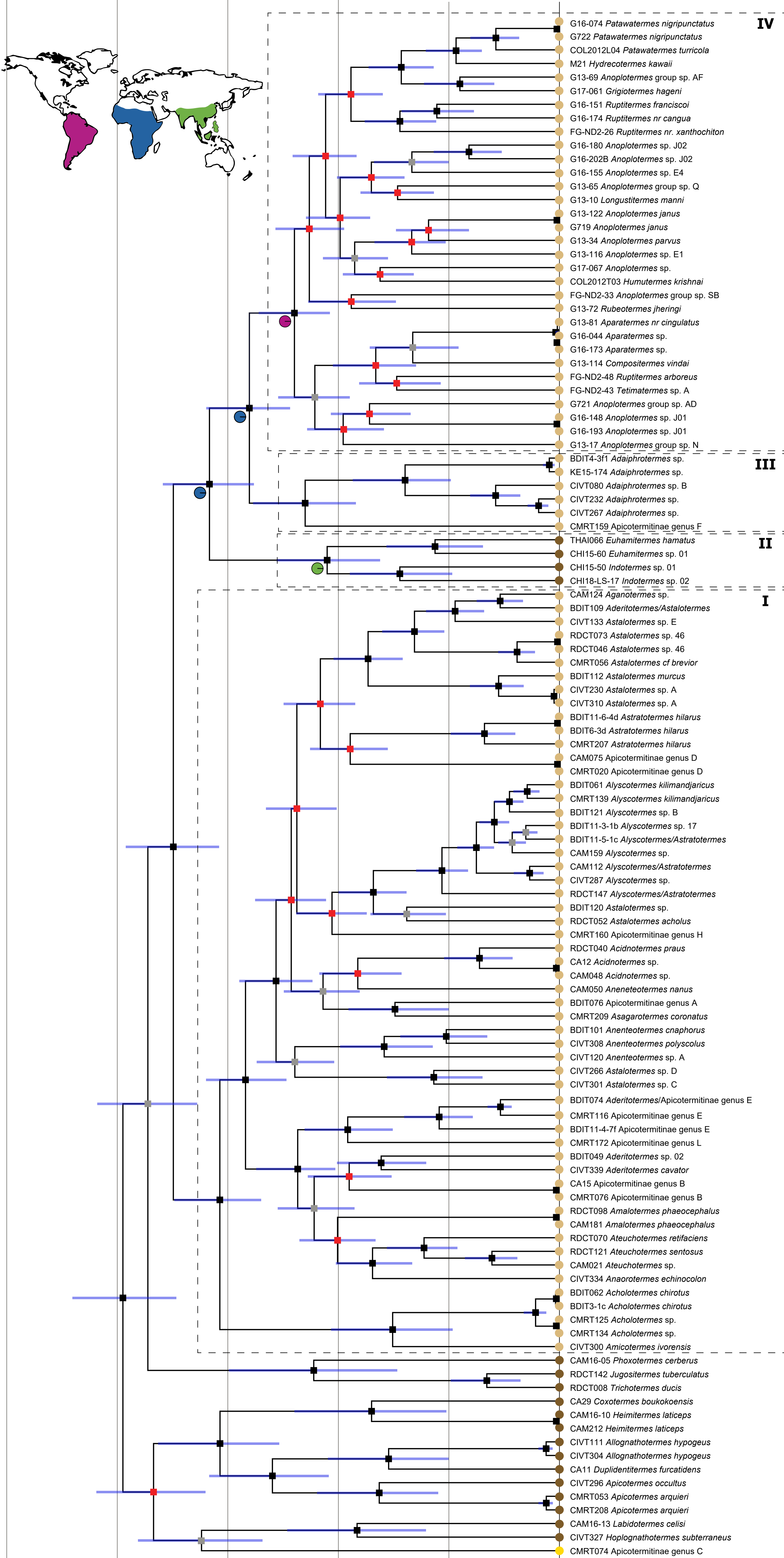
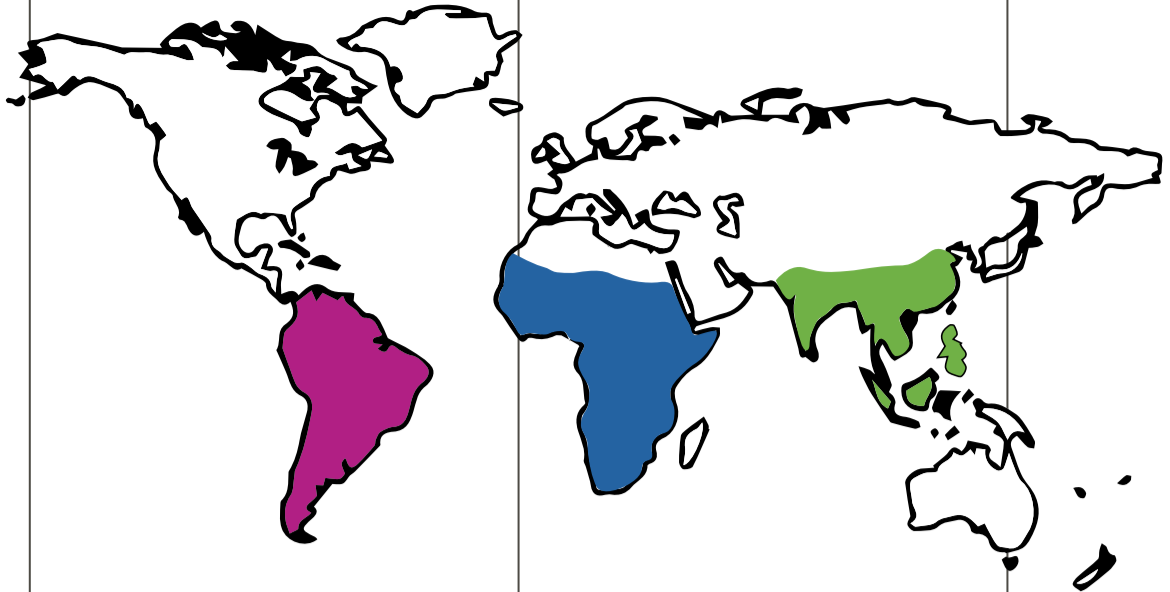
471

472 **Figure caption**

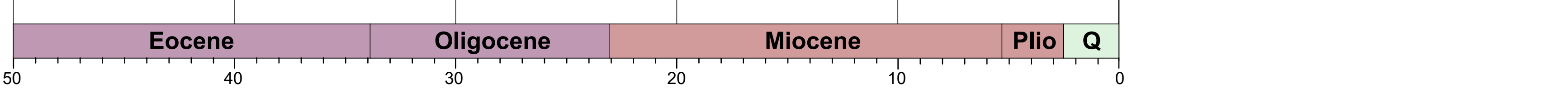
473

474 **Fig. 1.** Bayesian phylogenetic chronogram of Apicotermitinae inferred from mitochondrial genomes, with  
475 third codon positions excluded. The scale bar is given in millions of years. Node bars represent the 95%  
476 HPD intervals for the ages. Nodes are labelled with symbols representing posterior probabilities and  
477 bootstrap support for all analyses (1/100% = black; <1/100%= gray) and with red squares when the  
478 topology differ among analyses. Pie charts close to the nodes show the inferred relevant ancestral shifts of  
479 biogeographic distributions on the map: Afrotropical, Oriental and Neotropical realms. Wide bars indicate  
480 current distribution of species. Dotted boxes with roman numbers indicate the crown clades: I  
481 *Astalotermes*-group, II *Speculitermes*-group, III *Adaiphrotermes* + genus F and IV *Anoplotermes*-group.  
482 Tip circles represent soldiered (dark brown), soldierless (light brown) species and unknown soldier caste  
483 presence (yellow). Names of species include colony code and scientific name, respectively.

484



- G16-074 *Patawatermes nigripunctatus*
- G722 *Patawatermes nigripunctatus*
- COL2012L04 *Patawatermes turricola*
- M21 *Hydrocotermes kawaii*
- G13-69 *Anoplotermes* group sp. AF
- G17-061 *Grigiotermes hageni*
- G16-151 *Ruptitermes franciscoi*
- G16-174 *Ruptitermes nr cangua*
- FG-ND2-26 *Ruptitermes nr. xanthochiton*
- G16-180 *Anoplotermes* sp. J02
- G16-202B *Anoplotermes* sp. J02
- G16-155 *Anoplotermes* sp. E4
- G13-65 *Anoplotermes* group sp. Q
- G13-10 *Longustitermes manni*
- G13-122 *Anoplotermes janus*
- G719 *Anoplotermes janus*
- G13-34 *Anoplotermes parvus*
- G13-116 *Anoplotermes* sp. E1
- G17-067 *Anoplotermes* sp.
- COL2012T03 *Humutermes krishnai*
- FG-ND2-33 *Anoplotermes* group sp. SB
- G13-72 *Rubeotermes jheringi*
- G13-81 *Aparatermes nr cingulatus*
- G16-044 *Aparatermes* sp.
- G16-173 *Aparatermes* sp.
- G13-114 *Compositermes vindai*
- FG-ND2-48 *Ruptitermes arboreus*
- FG-ND2-43 *Tetimatermes* sp. A
- G721 *Anoplotermes* group sp. AD
- G16-148 *Anoplotermes* sp. J01
- G16-193 *Anoplotermes* sp. J01
- G13-17 *Anoplotermes* group sp. N
- BDIT4-3f1 *Adaiphrotermes* sp.
- KE15-174 *Adaiphrotermes* sp.
- CIVT080 *Adaiphrotermes* sp. B
- CIVT232 *Adaiphrotermes* sp.
- CIVT267 *Adaiphrotermes* sp.
- CMRT159 *Apicotermiinae* genus F
- THAI066 *Euhamitermes hamatus*
- CHI15-60 *Euhamitermes* sp. 01
- CHI15-50 *Indotermes* sp. 01
- CHI18-LS-17 *Indotermes* sp. 02
- CAM124 *Aganotermes* sp.
- BDIT109 *Aderitotermes/Astalotermes*
- CIVT133 *Astalotermes* sp. E
- RDCT073 *Astalotermes* sp. 46
- RDCT046 *Astalotermes* sp. 46
- CMRT056 *Astalotermes cf brevior*
- BDIT112 *Astalotermes murcus*
- CIVT230 *Astalotermes* sp. A
- CIVT310 *Astalotermes* sp. A
- BDIT11-6-4d *Astratotermes hilarus*
- BDIT6-3d *Astratotermes hilarus*
- CMRT207 *Astratotermes hilarus*
- CAM075 *Apicotermiinae* genus D
- CMRT020 *Apicotermiinae* genus D
- BDIT061 *Alyscotermes kilimandjaricus*
- CMRT139 *Alyscotermes kilimandjaricus*
- BDIT121 *Alyscotermes* sp. B
- BDIT11-3-1b *Alyscotermes* sp. 17
- BDIT11-5-1c *Alyscotermes/Astratotermes*
- CAM159 *Alyscotermes* sp.
- CAM112 *Alyscotermes/Astratotermes*
- CIVT287 *Alyscotermes* sp.
- RDCT147 *Alyscotermes/Astratotermes*
- BDIT120 *Astalotermes* sp.
- RDCT052 *Astalotermes acholus*
- CMRT160 *Apicotermiinae* genus H
- RDCT040 *Acidnotermes praus*
- CA12 *Acidnotermes* sp.
- CAM048 *Acidnotermes* sp.
- CAM050 *Aneneteotermes nanus*
- BDIT076 *Apicotermiinae* genus A
- CMRT209 *Asagarotermes coronatus*
- BDIT101 *Aneneteotermes cnaphorus*
- CIVT308 *Aneneteotermes polyscolus*
- CIVT120 *Aneneteotermes* sp. A
- CIVT266 *Astalotermes* sp. D
- CIVT301 *Astalotermes* sp. C
- BDIT074 *Aderitotermes/Apicotermiinae* genus E
- CMRT116 *Apicotermiinae* genus E
- BDIT11-4-7f *Apicotermiinae* genus E
- CMRT172 *Apicotermiinae* genus L
- BDIT049 *Aderitotermes* sp. 02
- CIVT339 *Aderitotermes cavator*
- CA15 *Apicotermiinae* genus B
- CMRT076 *Apicotermiinae* genus B
- RDCT098 *Amalotermes phaeocephalus*
- CAM181 *Amalotermes phaeocephalus*
- RDCT070 *Ateuchotermes retifaciens*
- RDCT121 *Ateuchotermes sentosus*
- CAM021 *Ateuchotermes* sp.
- CIVT334 *Anaorotermes echinocolon*
- BDIT062 *Acholotermes chirotus*
- BDIT3-1c *Acholotermes chirotus*
- CMRT125 *Acholotermes* sp.
- CMRT134 *Acholotermes* sp.
- CIVT300 *Amicotermes ivorensis*
- CAM16-05 *Phoxotermes cerberus*
- RDCT142 *Jugositermes tuberculatus*
- RDCT008 *Trichotermes ducis*
- CA29 *Coxotermes boukokoensis*
- CAM16-10 *Heimitermes laticeps*
- CAM212 *Heimitermes laticeps*
- CIVT111 *Allognathotermes hypogaeus*
- CIVT304 *Allognathotermes hypogaeus*
- CA11 *Duplidentitermes furcatidens*
- CIVT296 *Apicotermes occultus*
- CMRT053 *Apicotermes arquieri*
- CMRT208 *Apicotermes arquieri*
- CAM16-13 *Labidotermes celisi*
- CIVT327 *Hoplognathotermes subterraneus*
- CMRT074 *Apicotermiinae* genus C



## **Author statement**

**Johanna Romero Arias:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Validation, Visualization, Writing-original draft, Writing-review & editing. **Arthur Boom:** Data curation, Formal analysis, Methodology, Validation, Writing-original draft. **Menglin Wang:** Data curation, Investigation. **Crystal Clitheroe:** Investigation. **Jan Šobotník:** Resources. **Petr Stiblik:** Resources. **Thomas Bourguignon:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Resources, Writing-original draft, Writing - review & editing. **Yves Roisin:** Conceptualization, Data curation, Funding acquisition, Resources, Writing-original draft, Writing-review & editing, Supervision.

All authors read and approved the final version of the manuscript.

