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Historical biogeography of *Reticulitermes* termites (Isoptera: Rhinotermitidae) inferred from analyses of mitochondrial and nuclear loci [☆]

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ABSTRACT

Termites of the genus *Reticulitermes* are ecologically and economically important wood feeding social insects that are widespread in the Holarctic region. Despite their importance, no study has yet attempted to reconstruct a global time scaled phylogeny of *Reticulitermes* termites. In this study, we sequenced mitochondrial (2096 bp) and nuclear (829 bp) loci from 61 *Reticulitermes* specimens, collected across the genus' entire range, and one specimen of *Coptotermes formosanus*, which served as an outgroup. Bayesian and Maximum likelihood analyses conducted on the mitochondrial and nuclear sequences support the existence of four main lineages that span four global geographical regions: North America (NA lineage), western Europe (WE lineage), a region including eastern Europe and western Asia (EA + WA lineage), and eastern Asia (EA lineage). The mitochondrial data allowed us to clarify the phylogenetic relationships among these lineages. They were also used to infer a chronogram that was time scaled based on age estimates for termite fossils (including the oldest *Reticulitermes* fossils, which date back to the late Eocene early Oligocene). Our results support the hypothesis that the extant *Reticulitermes* lineage first differentiated in North America. The first divergence event in the ancestral lineage of *Reticulitermes* occurred in the early Miocene and separated the Nearctic lineages (i.e., the NA lineages) from the Palearctic lineages (i.e., WE, EE + WA, and EA lineages). Our analyses revealed that the main lineages of *Reticulitermes* diversified because of vicariance and migration events, which were probably induced by major paleogeographic and paleoclimatic changes that occurred during the Cenozoic era. This is the first global and comprehensive phylogenetic study of *Reticulitermes* termites, and it provides a crucial foundation for studying the evolution of phenotypic and life history traits in *Reticulitermes*. For instance, the phylogeny we obtained suggested that 'asexual queen succession', a unique reproductive system, independently evolved at least three times during the diversification of the genus.

1. Introduction

Reticulitermes Holmgren, 1913 is a Holarctic genus of subterranean termites (Isoptera: Rhinotermitidae) that is widespread and abundant in temperate regions where their biomass can approach that of many termite taxa living in tropical regions (Bignell and Eggleton, 2000; Gentry and Whitford, 1982). These eusocial wood feeding insects are ecologically important in their habitats because they participate in the decomposition of large quantities of organic matter (Bignell and Eggleton, 2000). However, *Reticulitermes* termites also frequently attack wooden structures

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and therefore have major economic impacts. Damage caused by and pest control efforts aimed at *Reticulitermes* and its closest living relative, *Coptotermes*, are estimated to cost nearly \$11 billion per year in the United States alone (Su, 2002). The ecological and economic importance of *Reticulitermes* has spurred a substantial amount of research on various subjects, including termite phylogeography and population genetics (Kutnik et al., 2004; Lefebvre et al., 2008; Leniaud et al., 2010; Luchetti et al., 2007, 2013a; Perdereau et al., 2013, 2015; Uva et al., 2004; Vargo and Husseneder, 2009; Velonà et al., 2010; Ye et al., 2004), caste differentiation and reproductive systems (Matsuura, 2011; Matsuura et al., 2009; Miura and Scharf, 2011), behavioral ecology and chemical communication (Bagnères and Hanus, 2015), as well as gut microbial ecology and nutritional physiology (Bignell, 2011; Brune, 2014; Ohkuma and Brune, 2011).

The genus *Reticulitermes* belongs to the Rhinotermitidae family; its closest living relatives are the genera *Coptotermes* and *Heterotermes* (Bourguignon et al., 2015; Cameron et al., 2012; Inward et al., 2007; Lo et al., 2004; Thompson et al., 2000). To date, 138 extant *Reticulitermes* species have been described (Krishna et al., 2013). The oldest *Reticulitermes* fossils were found in Baltic amber deposits and have an estimated age of ~38 million years, which means they date back to the late Eocene (Emerson, 1971; Engel et al., 2007, 2009). The vast majority of contemporary species identified so far have been collected in western Europe, North America (in the USA), or in eastern Asia (mostly in Japan and China). Almost all phylogenetic and phylogeographic studies have dealt with taxa collected in one of these geographic regions and have focused on either European (Austin et al., 2007, 2002; Jenkins et al., 2001; Kutnik et al., 2004; Lefebvre et al., 2008; Leniaud et al., 2010; Luchetti et al., 2007, 2013a, 2004; Marini and Mantovani, 2002; Uva et al., 2004; Velonà et al., 2010), North American (Austin et al., 2007; Copren et al., 2005; Ye et al., 2004), or Japanese taxa (Kim et al., 2012; Park et al., 2006). Only a few studies have investigated the phylogenetic relationships among European, North American and Asian taxa in targeted analyses (Austin et al., 2004, 2002). However, these previous studies examined a single mitochondrial region (cytochrome oxidase II), which yielded insufficient phylogenetic information. Despite the importance, diversity, and wide distribution of *Reticulitermes* termites, phylogenetic relationships among the taxa composing the genus are poorly characterized. Furthermore, to date, no study has attempted to analyze the global timing of diversification.

To study the evolutionary changes that occurred during the diversification of this lineage, it is crucial to obtain a reliable phylogeny for *Reticulitermes*. Many phenotypic and life history traits are known to vary among *Reticulitermes* species. These traits include characteristics related to social organization, such as reproductive systems (Matsuura, 2011) and colony breeding structures (Vargo and Husseneder, 2009); chemical cues involved in inter-individual recognition (Bagnères and Hanus, 2015); or assemblages of gut microorganisms that are essential for lignocellulose digestion (Hongoh et al., 2005). A better understanding of the phylogenetic relationships among *Reticulitermes* species will make it easier to assess this variation from an evolutionary perspective. For instance, some *Reticulitermes* species employ a unique reproductive system called asexual queen succession (AQS). In species displaying AQS, queens produce neotenic replacement queens by parthenogenesis but use normal sexual reproduction to produce other colony members (Matsuura et al., 2009). Although it has been argued that AQS may provide selective advantages over non AQS forms at both the colony and individual levels (Matsuura, 2011), this phenotype does not occur in all *Reticulitermes* species. AQS has been observed in three (*R. speratus*, *R. virginicus* and *R. lucifugus*) of the nine *Reticulitermes* species examined for this system to date (Huang et al., 2013; Kobayashi et al., 2013; Luchetti et al., 2013b;

Matsuura et al., 2009; Vargo et al., 2012; Wu et al., 2013). The fact that species exhibiting AQS do not seem to be closely related suggests that this reproductive system has been independently acquired (or lost) several times during the diversification of *Reticulitermes*. Detailed information on the phylogenetic relationships among species in this genus could clarify this issue, and therefore provide crucial knowledge for studying the conditions under which AQS evolved, persisted or disappeared.

The first goal of this study was to use DNA sequence data obtained from two coding mitochondrial regions (cytochrome oxidases I and II [COI and COII]), one non coding mitochondrial region (16S rRNA) and two non coding nuclear regions (ribosomal DNA Internal Transcribed Spacer 1 and 2 [ITS1 and ITS2]) to resolve the phylogenetic relationships among the main lineages and species of *Reticulitermes*. The second goal was to estimate the time frame in which the genus diversified, in order to correlate cladogenic events with known palaeogeographic and palaeoclimatic events. This study provides the first comprehensive phylogenetic analysis of *Reticulitermes* termites. Results are discussed in the context of termite historical biogeography and taxonomy, and the evolution of AQS is also addressed.

2. Materials and methods

2.1. Taxon sampling

Sixty one colonies of *Reticulitermes* species and one colony of *Coptotermes formosanus* Shiraki were sampled for this study. Samples were collected between 1998 and 2013 in five major geographic regions of the world: North America (five US states: Louisiana, Florida, Georgia, California, and Mississippi), western Europe (France, Spain, and Italy), eastern Europe (Greece, Crete, and the Cyclades), western Asia (Israel, Turkey, Cyprus and Iran), and eastern Asia (China and Japan) (Table 1). Sampled individuals were preserved in 96% or absolute ethanol and stored at 4 °C or room temperature until DNA extraction. Specimens of described species were identified using morphological, chemical or molecular criteria (Clément et al., 2001; Ye et al., 2004). Non identified specimens were designated as “*R. sp.*”, followed by a reference number. In this study, eight samples could not be identified (*R. sp.* 1 through *R. sp.* 8; Table 1).

2.2. Molecular procedures

Genomic DNA was extracted from one worker per colony using standard phenol-chloroform purification (Kutnik et al., 2004) or the Wizard® Genomic DNA Purification Kit (Promega). Three mitochondrial loci, COI (723 bp), COII (658 bp), and 16S rRNA (715 bp), and two nuclear loci, ITS1 (471 bp) and ITS2 (358 bp), were amplified using PCR. Amplifications were performed using DreamTaq® PCR Master Mix (Thermo Scientific). An initial denaturing step at 95 °C (2 min) was followed by 35 iterations of the following cycles: 94 °C (30 s), annealing temperature (30 s), and 72 °C (1 min). The extension step took place at 72 °C (10 min). Information on the primers and annealing temperatures used is provided in Table 2. ITS1 primers were designed using Primer 3 (V0.9) for the 18S (ITS1F) and the 5.8S (ITS1R) portions of the nuclear ribosomal RNA gene of *Blattella germanica* (Genbank accession number: AF321214) (Mukha et al., 2002). Similarly, the reverse primer for the 16S gene (16SR1) was designed using the 16S mitochondrial ribosomal RNA gene of *Reticulitermes santonensis* (synonymous with *R. flavipes*; Genbank accession number: AF262589) (Thompson et al., 2000). The PCR products were purified and sequenced using Big Dye 3.1 and a 96 capillary ABI 3730xl sequencing system (Applied Biosystems). Sequences were inspected, corrected and aligned using the

Table 1
Geographical regions, localities, and taxonomic information for the *Reticulitermes* samples used in the phylogenetic analyses, and the GenBank accession numbers for sequences obtained in this study. Samples *R. sp. 1* to *R. sp. 8* are unidentified species.

Geographical region	Taxon	Sampling location	Code	16S	COI	COII	ITS1/ITS2
<i>North America (NA)</i>							
	<i>R. flavipes</i>	New Orleans, Louisiana, USA	NEO	KM245639	KM245702	KM245765	KM245828
	<i>R. flavipes</i>	Gainesville, Florida, USA	GAI	KM245640	KM245703	KM245766	KM245829
	<i>R. flavipes</i>	Ile d'Oléron, France ^a	OLE	KM245641	KM245704	KM245767	KM245830
	<i>R. hageni</i>	Athens, Georgia, USA	ATH	KM245642	KM245705	KM245768	KM245831
	<i>R. hesperus</i>	Placerville, California, USA	PVI	KM245643	KM245706	KM245769	KM245832
	<i>R. hesperus</i>	Novato, California, USA	NOV	KM245644	KM245707	KM245770	KM245833
	<i>R. mallei</i>	Picayune, Mississippi, USA	PIC	KM245645	KM245708	KM245771	KM245834
	<i>R. nelsonae</i>	Fontainebleau Park Forest, Louisiana, USA	FON	KM245646	KM245709	KM245772	KM245835
	<i>R. nelsonae</i>	Paynes Prairies State Park, Florida, USA	PAY	KM245647	KM245710	KM245773	KM245836
	<i>R. virginicus</i>	New Orleans, Louisiana, USA	NEO	KM245648	KM245711	KM245774	KM245837
	<i>R. virginicus</i>	Wakulla Spring State Park, Florida, USA	WAK	KM245649	KM245712	KM245775	KM245838
	<i>R. sp. 1^b</i>	Placerville, California, USA	PVI	KM245650	KM245713	KM245776	KM245839
	<i>R. sp. 2</i>	Athens, Georgia, USA	ATH	KM245651	KM245714	KM245777	KM245840
<i>Western Europe (WE)</i>							
	<i>R. banyulensis</i>	Cassis, France	CAS	KM245652	KM245715	KM245778	KM245841
	<i>R. banyulensis</i>	Teruel, Spain	TER	KM245653	KM245716	KM245779	KM245842
	<i>R. grassei</i>	Oléron, France	OLE	KM245654	KM245717	KM245780	KM245843
	<i>R. lucifugus corsicus</i>	Feniglia Natural Reserve, Italy	FEN	KM245655	KM245718	KM245781	KM245844
	<i>R. lucifugus lucifugus</i>	Marseille, France	MAR	KM245656	KM245719	KM245782	KM245845
<i>Eastern Europe (EE)</i>							
	<i>R. balkanensis</i>	Schinias, Greece	SCH	KM245657	KM245720	KM245783	KM245846
	<i>R. balkanensis</i>	Nea Makri, Greece	NEM	KM245658	KM245721	KM245784	KM245847
	<i>R. urbis</i>	La Ciotat, France	LAC	KM245659	KM245722	KM245785	KM245848
	<i>R. urbis</i>	Sivota, Greece	SIV	KM245660	KM245723	KM245786	KM245849
	<i>R. urbis</i>	Areopolis, Greece	ARE	KM245661	KM245724	KM245787	KM245850
	<i>R. sp. 3^c</i>	Georgioupoli, Crete, Greece	GEO	KM245662	KM245725	KM245788	KM245851
	<i>R. sp. 3^d</i>	Kokkino Pyrgos, Crete, Greece	KOK	KM245663	KM245726	KM245789	KM245852
	<i>R. sp. 4</i>	Amorgos, Cyclades Islands, Greece	AMO	KM245664	KM245727	KM245790	KM245853
<i>Western Asia (WA)</i>							
	<i>R. clypeatus</i>	Ben Shemenn, Israel	BES	KM245665	KM245728	KM245791	KM245854
	<i>R. lucifugus</i> Turkey	Ankara, Turkey	ANK	KM245666	KM245729	KM245792	KM245855
	<i>R. lucifugus</i> Turkey	Konya, Turkey	KON	KM245667	KM245730	KM245793	KM245856
	<i>R. lucifugus</i> Turkey	Mugla, Turkey	MUG	KM245668	KM245731	KM245794	KM245857
	<i>R. sp. 5</i>	Limassal, Cyprus	CYP	KM245669	KM245732	KM245795	KM245858
	<i>R. sp. 6</i>	Shahrekordeh, Iran	IRA	KM245670	KM245733	KM245796	KM245859
<i>Eastern Asia (EA)</i>							
	<i>R. chinensis</i>	Jingmen, Hubei, China	JIN-1	KM245671	KM245734	KM245797	KM245860
	<i>R. chinensis</i>	Jingmen, Hubei, China	JIN-2	KM245672	KM245735	KM245798	KM245861
	<i>R. guangzhouensis</i>	Guangzhou, Guangdong, China	GUA	KM245673	KM245736	KM245799	KM245862
	<i>R. kanmonensis</i>	Nanchang, Jiangxi, China	NAC	KM245674	KM245737	KM245800	KM245863
	<i>R. kanmonensis</i>	Yichang, Hubei, China	YIC	KM245675	KM245738	KM245801	KM245864
	<i>R. khaoyaiensis</i>	Nanchang, Jiangxi, China	NAC	KM245676	KM245739	KM245802	KM245865
	<i>R. khaoyaiensis</i>	Nanling, Wuhu, Anhui, China	NAN	KM245677	KM245740	KM245803	KM245866
	<i>R. leptomandibularis</i>	Wanzai, Yichun, Jiangxi, China	WAN	KM245678	KM245741	KM245804	KM245867
	<i>R. sp. 7</i>	Nanling, Wuhu, Anhui, China	NAN	KM245679	KM245742	KM245805	KM245868
	<i>R. sp. 8</i>	Guangzhou, Guangdong, China	GUA-1	KM245680	KM245743	KM245806	KM245869
	<i>R. sp. 8</i>	Guangzhou, Guangdong, China	GUA-2	KM245681	KM245744	KM245807	KM245870
	<i>R. amamianus</i>	Yuidake, Amami, Japan	AMA-1	KM245682	KM245745	KM245808	KM245871
	<i>R. amamianus</i>	Yuidake, Amami, Japan	AMA-2	KM245683	KM245746	KM245809	KM245872
	<i>R. amamianus</i>	Kosyuku, Amami, Japan	KOS	KM245684	KM245747	KM245810	KM245873
	<i>R. kanmonensis</i>	Ejio Park, Yamaguchi, Japan	YAM-1	KM245685	KM245748	KM245811	KM245874
	<i>R. kanmonensis</i>	Ejio Park, Yamaguchi, Japan	YAM-2	KM245686	KM245749	KM245812	KM245875
	<i>R. miyatakei</i>	Yuwandake, Amami, Japan	AMA-1	KM245687	KM245750	KM245813	KM245876
	<i>R. miyatakei</i>	Yuwandake, Amami, Japan	AMA-2	KM245688	KM245751	KM245814	KM245877
	<i>R. okinawanus</i>	Yona, Kunikami, Okinawa, Japan	OKI-1	KM245689	KM245752	KM245815	KM245878
	<i>R. okinawanus</i>	Hedomisaki, Okinawa, Japan	OKI-2	KM245690	KM245753	KM245816	KM245879
	<i>R. speratus</i>	Ejio Park, Yamaguchi, Japan	YAM	KM245691	KM245754	KM245817	KM245880
	<i>R. speratus</i>	Handayama, Okayama, Japan	OKA	KM245692	KM245755	KM245818	KM245881
	<i>R. speratus</i>	Motoyama, Kochi, Japan	KOC	KM245693	KM245756	KM245819	KM245882
	<i>R. speratus</i>	Jigenji, Kagoshima, Japan	KAG	KM245694	KM245757	KM245820	KM245883
	<i>R. speratus</i>	Kamigamo, Kyoto, Japan	KYO	KM245695	KM245758	KM245821	KM245884
	<i>R. speratus</i>	Sapporo, Hokkaido, Japan	HOK	KM245696	KM245759	KM245822	KM245885
	<i>R. speratus</i>	Tsukuba, Ibaraki, Japan	IBA	KM245697	KM245760	KM245823	KM245886
	<i>R. speratus</i>	Shibukawa, Gunma, Japan	GUN	KM245698	KM245761	KM245824	KM245887
	<i>R. yaeyamanus</i>	Iriomote, Okinawa, Japan	OKI	KM245699	KM245762	KM245825	KM245888
<i>Outgroup</i>	<i>C. formosanus</i>	St Gabriel, Louisiana, USA	-	KM245700	KM245763	KM245826	KM245889

^a Samples of non-native French species (*R. flavipes* and *R. urbis*) introduced to France (Leniaud et al., 2010; Perdereau et al., 2013). ^b *R. sp. CAB* in Copren et al. (2005).

^c Crete – Group 4 in Velonà et al. (2010).

^d Crete – Group 2 in Velonà et al. (2010).

Table 2

PCR primer sequences, annealing temperatures, and the sources used. Refer to Table 3 for more details on individual loci.

Locus	Primer name	Direction	Sequence (5'-3')	Annealing temperature (°C)	Source
COI	C1-J-2195 ^a	Forward	TTGATTCTTTGGTCACCCAGAAGT	50	Simon et al. (1994)
	C1-J-2183 ^a	Forward	CAACACTTATTTTGATTCCTTGG		Simon et al. (1994)
	TL2-N-3014 ^a	Reverse	TCTAATGCATTAATCTGCCATATTA		Simon et al. (1994)
COII	Modified A-tLeu	Forward	CAGATAAGTGCATTGGATT	52	Miura et al.(2000)
	TK-N-3785 ^a	Reverse	GTTAAGAGACCATTACTTA		Simon et al. (1994)
16S	LR-J-13017	Forward	TTACGCTGTTATCCCTAA	50	Kambhampati and Smith (1995) Present study
	16SR1	Reverse	CCCGATTCTAAAAGGGGCTA		
ITS1	ITS1F	Forward	TCGTAACAAGGTTTCCGTAGG	57	Present study
	ITS1R	Reverse	CAATTGCTGCGTTCTTCAT		Present study
ITS2	ITS2F	Forward	TGTGAAGTGCAGGACACAT	50	Jenkins et al. (2001)
	ITS2R	Reverse	GCTTAAATTTAGGGGGTAGTC		Jenkins et al. (2001)

^a These primers correspond to the locust taxa primers given in Simon et al. (1994).

Clustal W algorithm implemented in the MEGA v.6 package. The alignments obtained were further inspected and manually edited. All sequences were deposited in GenBank under the accession numbers provided in Table 1.

2.3. Phylogenetic analyses

To evaluate possible saturation of the phylogenetic signal in each mtDNA marker dataset, the pairwise proportions of transitions (ts) and transversions (tv) were plotted against the pairwise uncorrected *p* distances. For the protein coding sequences (COI and COII), we also plotted the proportions of ts and tv occurring at the third codon position against the pairwise uncorrected *p* distances.

The best substitution model for each of the five loci was determined using MEGA v.6 and employing the Bayesian information criterion (Table 3). Phylogenetic trees were estimated utilizing two datasets: (i) the mitochondrial DNA (mtDNA) sequences (COI, COII, and 16S) and (ii) the nuclear DNA (ncDNA) sequences (ITS1 and ITS2). The congruence between the two datasets was tested using the ILD test (Farris et al., 1995), implemented in PAUP* (Swofford, 2001), with 500 bootstrap replicates. For the two data sets, a Maximum likelihood (ML) search and Bayesian inference (BI) were performed using a partitioned dataset (three and two partitions for mtDNA and ncDNA, respectively). Maximum likelihood trees were calculated using RAxML v. 7.0.3 (Stamatakis, 2006); bootstrap support was estimated using the rapid hill climbing algorithm (Stamatakis et al., 2008) on 500 replicates. Fifty percent majority rule consensus trees were obtained using Phyutility v. 2.2.6 (Smith and Dunn, 2008). Bayesian inference was performed using MrBayes v. 3.2.2 (Ronquist et al., 2012). For both datasets, two searches of four chains each were run for 30,000,000 generations; trees were sampled every 500 generations. Convergence diagnostics were assessed via the variance of split frequencies (<0.01), PSRF (≥ 1.00) and ESS (≥ 200). The first

10% of the sample trees were discarded as *burnin*. The 50% majority rule consensus tree was obtained via the *sumt* command, and the maximum clade credibility (MCC) tree was selected using TreeAnnotator v. 1.8 (Drummond and Rambaut, 2007). For the two datasets, the 50% majority rule consensus tree and the MCC tree topology gave identical results.

Dating of cladogenetic events was performed using BEAST software v. 1.8 (Drummond and Rambaut, 2007) on the mtDNA dataset. The software calculated the tree topology, as well as the posterior probability support and a divergence time estimate for each node. The algorithm was setup to use an uncorrelated, log normal relaxed clock and the birth death speciation process. Two MCMC chains were run for 100,000,000 generations, starting from a random tree; sampling took place every 500 generations. As the loci examined are all part of the mitochondrial genome, data partitioning was the same as in the BI analysis (unlinked site models and a linked clock model). Chain stationarity and convergence were reached when ESS ≥ 200 . The MCC tree was determined after discarding the first 10% of the trees obtained (*burnin*).

For calibration purposes, the following outgroup taxa were added to the dataset: *Coptotermes michaelsoni*, *C. lacteus*, *C. acinaciformis*, *Heterotermes tenuis*, *H. tenuior*, *H. longiceps*, *Heterotermes* sp., and *Schedorhinotermes breinli* (Genbank accession numbers are provided in Supplementary Table S1). Two calibration points based on fossil records were applied to the analysis: the oldest known *Reticulitermes* fossils, *R. minimus*, *R. fossarum*, *R. antiquus* and *R. sp.* (minimum age = 38 Ma; Scudder, 1883; Snyder, 1928; Jarzembowski, 1980; Engel et al., 2007), calibrating the node *Reticulitermes* + *Heterotermes* + *Coptotermes* and the oldest *Heterotermes* fossil, *H. eocenicus* (minimum age = 38 Ma; Engel, 2008) calibrating the node *Heterotermes* + *Coptotermes*. Prior probability distribution for calibration points was set as exponential with a soft maximum bound of 100 Ma (97.5% of probability distribution, as in Bourguignon et al., 2015).

Table 3

Summary of information on the individual loci and the relative substitution models used in the phylogenetic analyses.

Locus	No. of sites	No. of variable sites (%)	No. of parsimony-informative sites (%)	No. of unique sequences	Substitution model
COI	723	214 (29.6)	175 (24.2)	51	TN93+G+I
COII	658	232 (35.2)	169 (25.7)	58	HKY+G+I
16S	715	187 (26.1)	116 (16.2)	58	HKY+G
ITS1	471	110 (23.3)	37 (7.8)	32	T92+G
ITS2	358	71 (19.8)	25 (6.9)	30	JC+G
mtDNA dataset	2096	633 (30.2)	460 (21.9)	62	n.a. ^a
ncDNA dataset	829	181 (21.8)	62 (7.5)	37	n.a. ^a

^a Not applicable.

3. Results

3.1. Sequence characteristics and nucleotide variation

The five target loci were sequenced for all of the 62 termite samples. When the outgroup sample (i.e., *Coptotermes formosanus*) was excluded, the percentage of variable sites ranged from 19.8% for ITS2 to 35.2% for COII (Table 3). For the two protein coding genes, COI and COII, the percentage of variable sites at the third codon position was 87.8% and 76.7%, respectively. None of the loci analyzed showed transitional or transversional saturation, even when the third codon positions were considered (Supplementary Fig. S1).

3.2. Phylogenetic analyses

ML analysis using the mtDNA sequences (Fig. 1) revealed two main lineages of *Reticulitermes* termites. The first lineage included all the western European (WE) taxa, and the second lineage was divided in two sublineages. The first sublineage grouped together taxa from eastern European (EE) and western Asian (WA) whereas the second sublineage comprised all the taxa from eastern Asian (EA). In this analysis, North American taxa did not group together but instead clustered in multiple lineages at basal positions in the tree. With some exceptions, the BI tree (Fig. 1) was congruent with the ML tree and robust posterior probabilities provided support for the WE, EE + WA, and EA lineages.

The ML and BI analyses conducted on the ncDNA dataset were less informative than those conducted on the mtDNA dataset. The ncDNA based phylogenetic trees indeed contained numerous deep nodes that were poorly supported or not supported at all (Fig. 2). For instances, these analyses did not support the sister relationship between the EE + WA and EA lineages. They also failed to support the WE lineage and the phylogenetic subdivision within the WA lineage. In addition, the topologies of these trees were not congruent with trees obtained on the mtDNA dataset (ILD test $p < 0.01$). For this reason, analyses on the full dataset combining mtDNA and ncDNA sequences were not conducted. Interestingly, however, analyses performed on the ncDNA dataset clarified the phylogenetic status of the North American taxa, which all grouped together in a well supported lineage (NA lineage) (Fig. 2). Altogether, analyses conducted separately with the mtDNA and the ncDNA datasets suggested that NA lineage was the first to diverge in *Reticulitermes* genus followed by WE lineage before EE + WA and EA lineages. Such relationships between the main lineages of *Reticulitermes* termites were also supported by the BEAST analysis conducted with the mtDNA dataset (Fig. 3).

The topology within the WE lineage was very well supported and in perfect agreement with previous studies (Clément et al., 2001) (Figs. 1 and 3). Similarly, the topology within the EE + WA lineage was similar to that found in previous studies (Ghesini and Marini, 2012; Luchetti et al., 2007; Uva et al., 2004; Velonà et al., 2010). In this lineage, a well supported group composed of the three *R. urbis* samples had a sister relationship with the WA lineage, and the later was further subdivided in two groups: WA.1 and WA.2. The WA.1 group provided confirmation that a close relationship exists between the northern samples previously called “*R. lucifugus* Turkey” and samples from the Aegean Islands (Cyclades, Crete, and Cypriot), which were represented in this study by individuals taken from the Cyclades, Crete and Cypriot (Ghesini and Marini, 2012; Velonà et al., 2010). The WA.2 group brought together widely distributed taxa, whose range spanned from northern Iran (*R. sp. 6*, IRA) to eastern Greece (*R. balkanensis*); it also included taxa from the eastern Mediterranean such as “*R. lucifugus* Turkey” and *R. clypeatus* from Israel.

The EA lineage was divided into two groups (Figs. 1–3). The first group, EA.1, included several Japanese taxa collected in southern Japan (*R. kanmonensis*) and the Ryukyu Islands (*R. amaianus*, *R. miyatakei*, *R. okinawanus*, and *R. yaeyamanus*) as well as some Chinese taxa (*R. khaoyaiensis*, *R. sp. 6*, and *R. sp. 7*). The ML and BI tree topologies for the EA.1 group were highly congruent. The EA.2 group comprised the Japanese taxon *R. speratus*, whose haplotypes formed two clusters, and the Chinese taxa *R. chinensis* and *R. leptomandibularis*. The internal topology of EA.2 was supported by significant ML bootstrap values. However, the BI posterior probability was not significant for the node linking the sample of *R. leptomandibularis* with those of *R. chinensis*.

3.3. Dating cladogenetic events

A chronogram was built solely using the mtDNA dataset (Fig. 3). Fossil based calibration points allowed to retrieve the time estimates of cladogenetic events that mostly occurred between late Paleocene and late Miocene, although a few events also occurred during the Pliocene (Fig. 3). Interestingly, our time estimates were consistent with those obtained by other phylogenetic studies in termites (Bourguignon et al., 2015; Lee et al., 2015). For instance, the divergence of the lineages leading to *Reticulitermes* genus and to the group comprising both *Heterotermes* and *Coptotermes* genera was estimated to have occurred 66.6 Ma (51.2–87.0 Ma 95% high posterior density [HPD]) in the present study, whereas the same event has been estimated to have occurred about 60 Ma by Bourguignon et al. (2015) and about 50 Ma by Lee et al. (2015). Similarly, the origin of the genus *Coptotermes* was estimated to have occurred about 20.9 Ma (15.4–28.2 Ma 95% HPD) in the present study, a time estimates that fully overlapped those found by Lee et al. (2015). These convergent results suggest that the chronogram computed in this study is reliable. This time scaled phylogeny indicated that the *Reticulitermes* genus started to diversify about 18.4 Ma, a geological period corresponding to the early Miocene (Fig. 3). The NA lineages started to diverge during the same time whereas the WE, EE + WA, and EA lineages started their diversification during the mid or late Miocene.

3.4. Taxonomy and systematics of *Reticulitermes* species

All the *Reticulitermes* species included in this study, whether described or not, formed well supported lineages in the phylogenetic trees (Figs. 1–3). None of *Reticulitermes* taxa for which several samples were analyzed ($N = 17$) demonstrated polyphyly or paraphyly. One possible exception was the taxonomic group containing the *R. nelsonae* (FON and PAY) and *R. sp. 2* (ATH) samples; however, we cannot exclude the possibility that *R. sp. 2* was, in fact, a *R. nelsonae* sample.

4. Discussion

4.1. Origin and historical biogeography of the *Reticulitermes* genus

The oldest *Reticulitermes* fossils described so far, namely *R. antiquus*, *R. minimus*, *R. sp.*, and *R. fossarum*, date back to the late Eocene (about 38 Ma; Figs. 3 and 4a). With the exception of *R. fossarum*, which was discovered in North America (Colorado, USA), most of these fossils were found in Europe (i.e., the Baltics, Russia, Poland, and the United Kingdom), which led Emerson (1971) to propose that the *Reticulitermes* genus originated in the northern part of the West European platform (Meulenkamp et al., 2000). This hypothesis appears plausible from a climatological perspective because, in the Eocene, the climate at the northern latitudes was tropical (Tertiary Thermal Maximum) (Zachos et al., 2001)

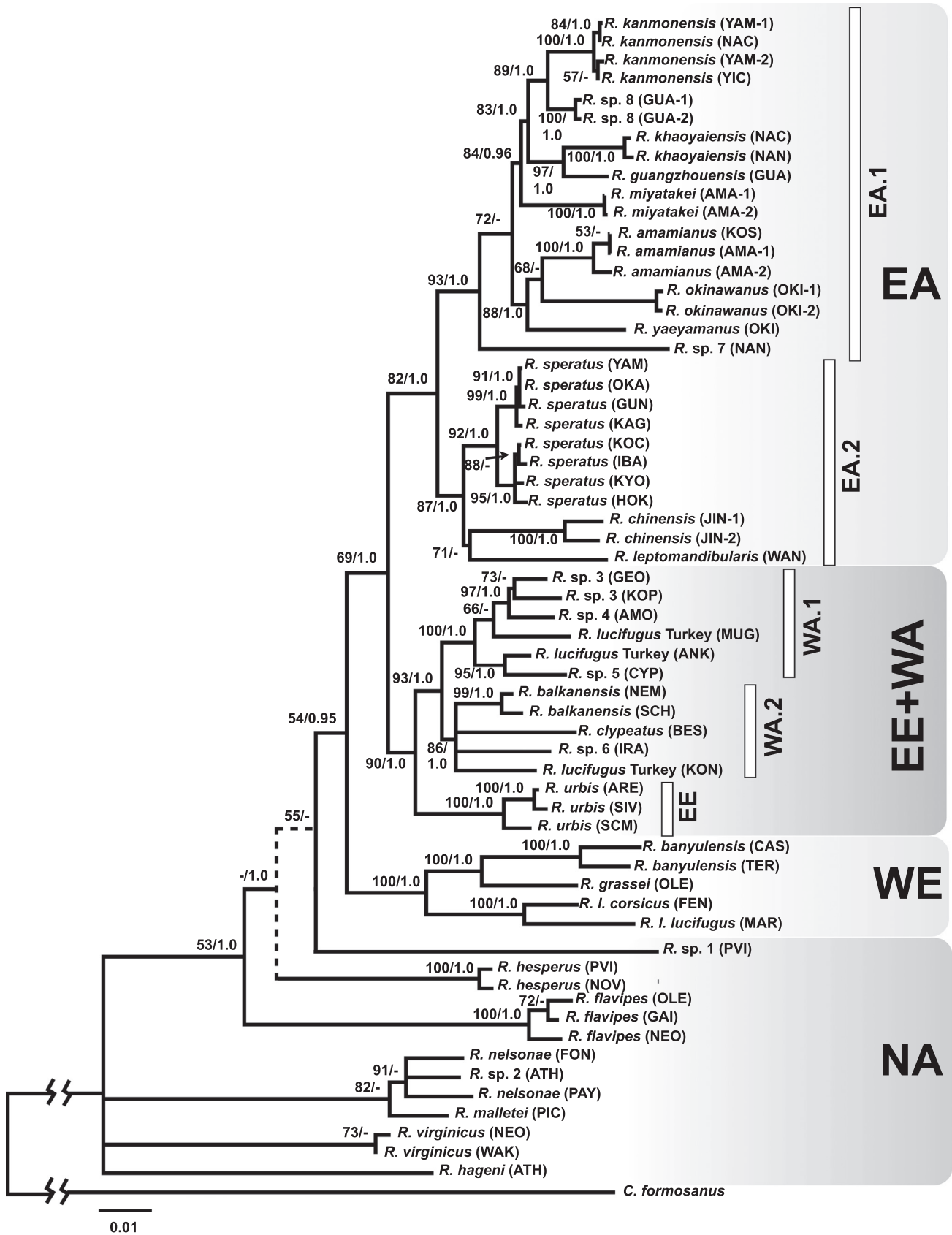


Fig. 1. Schematic drawing of the ML ($-\ln L = 10402.44$) and BI ($-\ln L = 12003.33$) analyses using the mtDNA dataset. The numbers at the nodes indicate bootstrap support/posterior probability. Only bootstrap values ≥ 50 and posterior probabilities ≥ 0.90 were considered. The dashed lines indicate that the branches were supported only by the BI analysis. Clade names are as in Table 1.

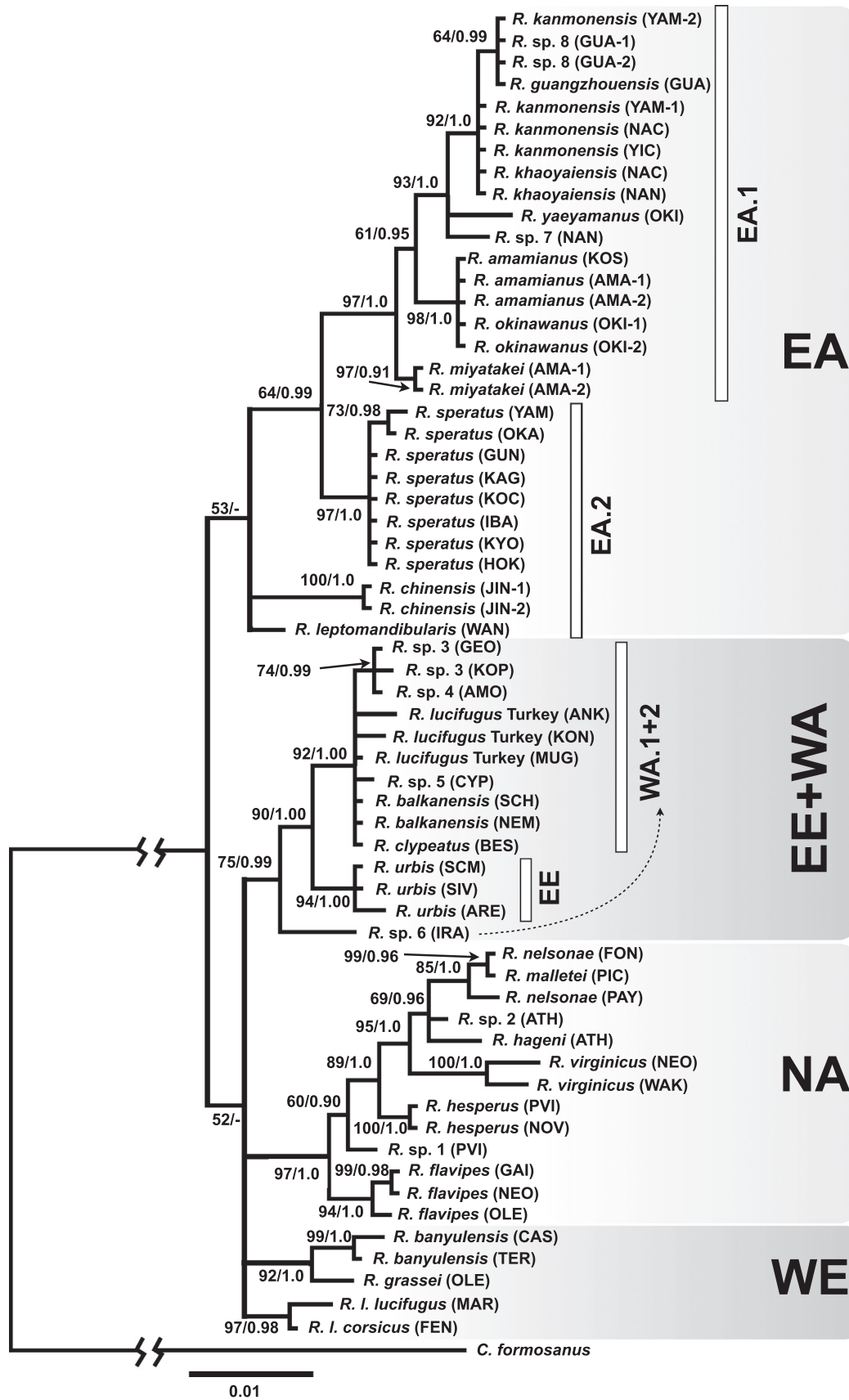


Fig. 2. Schematic drawing of the ML ($-\ln L = 2505.30$) and BI ($-\ln L = 2610.80$) analyses using the ncDNA dataset. The numbers at the nodes indicate bootstrap support/posterior probability. Only bootstrap values ≥ 50 and posterior probabilities ≥ 0.90 were considered. Clade names are as in Table 1.

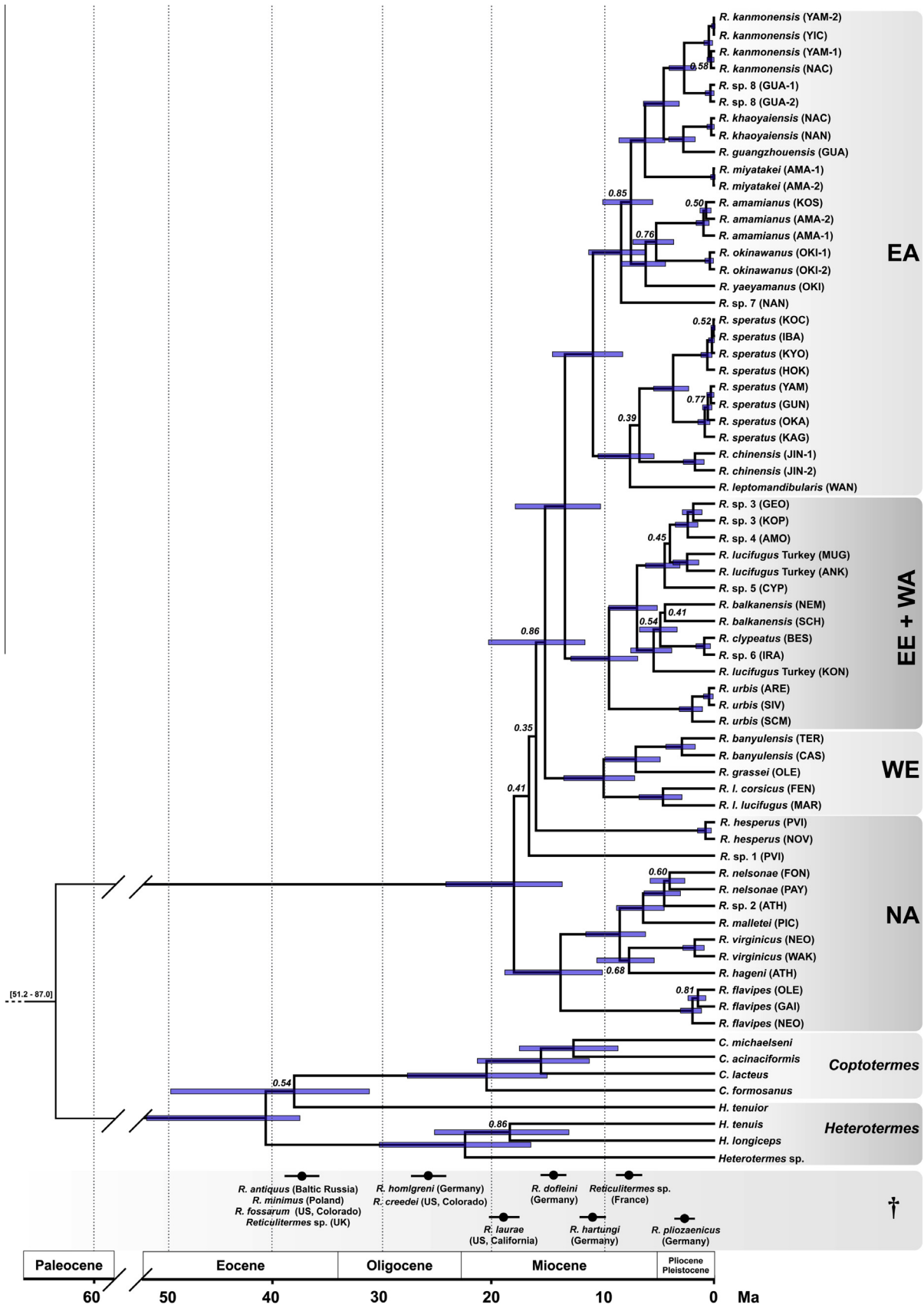


Fig. 3. Estimates of cladogenetic events time based on the mtDNA dataset. Chronogram with time scale (million years ago, Ma) and geological epochs. The bars at the nodes indicate the 95% high posterior density (HPD) intervals for the ages. It should be noted that, for graphical purposes, the outgroup *Schedorhinotermes breinli* has been omitted and for the first node (the *Reticulitermes* + *Coptotermites* + *Heterotermites* clade) the 95% HPD interval is numerically represented. Posterior probabilities ≥ 0.90 were omitted; other values are reported at the appropriate node. Clade names are as in Table 1; †: panel showing the temporal distribution of extinct *Reticulitermes* species, as estimated from the fossil collections (see Section 2.3 and Krishna et al. (2013) for references).

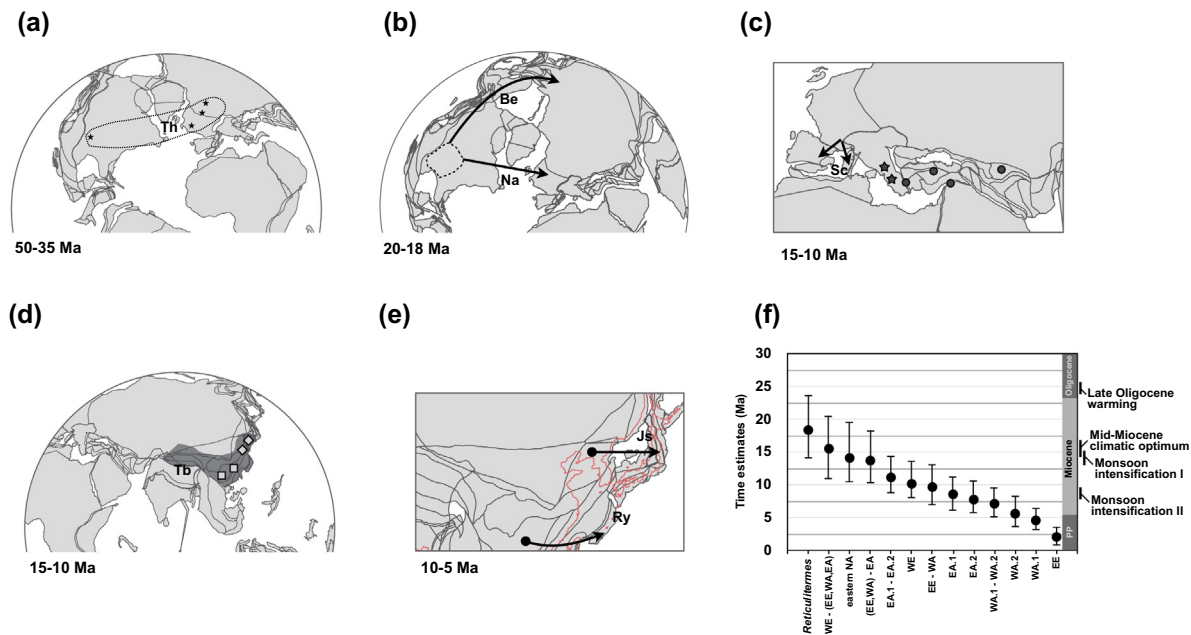


Fig. 4. Historical biogeography of *Reticulitermes* taxa. Panels (a)–(e) represent a time frame depicting the main events that shaped the genus' present-day diversity and distribution. (a) Connection between North America and Europe via the Thulean Bridge (Th) during the Eocene. Black stars indicate the approximate position of the oldest *Reticulitermes* fossil records; the dotted line delimits the putative ancestral distribution. (b) After the retraction of the ancestral lineage into the North American plate, the following expansion led to the Palearctic recolonization through the Bering (Be) or the North Atlantic (Na) land bridge. (c) About 15–10 Ma, ancestors of Iberian and Italian *Reticulitermes* diverged after migration into the Iberian Peninsula and into the Sardo-Corsican microplate (Sc), respectively. During the same time period, ancestors of the EE and WA clades dispersed into the eastern European plate (gray stars) and across the Anatolian plate, spanning from the present-day eastern Greece to North Iran (gray dots). (d) At the end of Oligocene, the uplift of the Tibetan plateau (Tb) and the emergence and intensification of the monsoon system changed the climate of the central-eastern China, giving rise to a large warm and humid zone (dark gray shadowing); this may have promoted the colonization and diversification of the eastern Asian taxa (EA.1: diamonds; EA.2: squares). (e) During the mid-late Miocene, the final opening of the Sea of Japan (Js) may have promoted the separation of *R. speratus* from its Chinese relatives. In the late Miocene, the Ryukyu Islands were connected to the mainland: a wave of migration from southern China may have given rise to the extant Ryukyu taxa (the light gray lines indicate present-day coastlines). (f) Graph indicating the correspondence between time estimates for the main cladogenetic events and paleoclimatic history. Errors bars represent the 95% high posterior density for each node reported (PP: Pliocene–Pleistocene). The maps were downloaded from <http://www.odsn.de/odsn/services/paleomap/paleomap.html> (Hay et al., 1999) and modified.

and therefore favorable for termites. The distribution of coeval Eocene *Reticulitermes* fossils across the Laurasian supercontinent (Fig. 4a) suggests that these termites differentiated well before the separation of North America and Eurasian plates, about 50 Ma, when the Thulean land bridge finally disappeared (Sanmartin et al., 2001). In the present analysis, the origin of extant *Reticulitermes* lineages was however estimated to have occurred during the early Miocene, about 18.4 Ma, definitely later than the minimum age of the oldest fossils. Considering the diversity and distribution of Eocene taxa and the late Oligocene fossils *R. creedei* (Colorado) and *R. holmgreni* (Germany) (Fig. 3), this temporal shift could be explained by an extensive lineages extinction. This could be indeed related to a major paleoclimatic change at the transition between Eocene and Oligocene when a sudden and deep temperature decline occurred (*Oi 1* glaciation, about 34 Ma; Zachos et al., 2001; Eldrett et al., 2009) and could have caused the extinction of numerous *Reticulitermes* lineages.

ML, BI and BEAST analyses indicated that the NA taxa do not form a monophyletic group but instead constitute an assemblage of different lineages at the most basal positions of the tree (Figs. 1 and 3). Moreover, beside the ancestral node of all *Reticulitermes* taxa, the first nodes within the clade are not supported, thus evidencing a polytomic topology. Although the analyses conducted in this study did not allow the distinction between a hard or a soft polytomy, they nevertheless suggest that all of the extant *Reticulitermes* taxa used in this study have a North American origin. One plausible hypothesis is that the ancestral lineage retracted into the North America during the *Oi 1* glaciation before to start an expansion when global temperatures increased in Oligocene until Mid Miocene, 18.4 and 15.6 Ma (Fig. 4f; Zachos et al., 2001). These

events would have driven the diversification of the NA lineages with the eastern taxa (*R. flavipes*, *R. virginicus*, *R. hageni*, *R. malletei*, *R. nelsonae* and *R. sp. 2*) diverging from the western taxa (*R. hesperus* and *R. sp. 1*), and to the re-colonization of the Palearctic.

The obtained tree topologies did not allow to identify the routes that have been followed during the Palearctic re-colonization. Two hypotheses can be nevertheless proposed (Fig. 4b). The Bering land bridge, connecting the North America and Asia, is considered to have constituted the main route for Holarctic intercontinental dispersals since the early Miocene (Sanmartin et al., 2001). In alternative to the trans-beringian route, the *Reticulitermes* lineage may have experienced a trans-Atlantic dispersal (Fig. 4b). The main North Atlantic land bridges, the Thulean bridge (through the British Islands and Greenland) and the De Geer route (through Fennoscandia, Greenland and Canadian Islands) are indeed known to have disappeared in the Mid Eocene (Sanmartin et al., 2001; Brikiatis, 2014). However, recent geological surveys have suggested the existence of another North Atlantic land connections during Miocene that lasted until 20 Ma (Ellis and Stoker, 2014). Recent palynological, fossil and molecular phylogenetic analyses on plants (Grimsson and Denk, 2005; Tiffney, 2008; Denk et al., 2010, 2015) and on freshwater fishes (i.e., *Perca* and *Sander*) (Haponski and Stepien, 2013; Stepien et al., 2015) concluded that a land bridge, or at least a stepping stone islands path, connecting North America and Western Europe still existed during Miocene.

During the Mid Miocene, approximately 15.5 Ma, the Palearctic lineage split into the western European lineage and the eastern Palearctic lineages (Fig. 3). In the case of the western European lineage, we can speculate that some populations migrated toward the southern West European plate (Meulenkamp and Sissingh, 2003;

Meulenkamp et al., 2000), subsequently diverging into the Iberian (i.e., *R. grassei* and *R. banyulensis*) (Kutnik et al., 2004) and the Italian (i.e., *R. l. lucifugus* and *R. l. corsicus*) taxa. In this study, the divergence between Iberian and Italian taxa was estimated to have occurred about 10.2 Ma, much later than previously thought. In this context, a recent study based on both phylogenetic relationships and biogeographic data, proposed that the ancestor of the Italian lineage diverged when the Sardo Corsican microplate detached from the Iberian Peninsula, about 25 Ma (Luchetti et al., 2013a). Because biogeographic based divergence times are usually much less precise than fossil calibrated time trees, however, the time estimates obtained in the present study are probably more reliable. Considering the closer relationship between the Iberian *R. grassei* and the Sardo Corsican Italian subspecies *R. l. corsicus* (Luchetti et al., 2013), the following scenario can be suggested. During the anti clockwise rotation, the Sardo Corsican microplate remained connected to the mainland through a land bridge until the late Miocene – early Pliocene (Meulenkamp and Sissingh, 2003). During the southward migration of the West European lineage, it is likely that some populations migrated within the Sardo-Corsican microplate through the land bridge and constituted the ancestor populations of the Italian *R. lucifugus* complex (Fig. 4c). A similar scenario involving the Sardo Corsican connection with the mainland has been proposed for land snail species of the genus *Solatopupa* (Ketmaier et al., 2006).

Our results indicate that the eastern European and western Asian lineages (EE and WA) may have diverged from the eastern Asian lineage (EA) around 13.7 Ma. During this time, the climate reached a warm optimum around 16–14 Ma (Fig. 4f) while the future Mediterranean area underwent to great tectonic movements (Zachos et al., 2001; Meulenkamp and Sissingh, 2003). These new paleoclimatic and paleogeographic conditions possibly favored the colonization of the eastern European landmasses and the Asian plate. During these migration events, two main lineages may have differentiated: one lineage splitting into the eastern European and western Asian lineages (EE and WA) and the other becoming the eastern Asian lineage (EA).

The chronogram indicates that the eastern European and western Asian (EE + WA) lineage diversified 9.7 Ma (Fig. 3). Some populations of the ancestral lineage likely differentiated into *R. urbis*, whereas other populations would have dispersed between North Iran and East Greece, across the Anatolian plate, and formed several western Asian taxa, including *R. balkanensis*, *R. clypeatus* and at least four other unidentified species (Figs. 3 and 4c). This scenario is consistent with the paleogeographic and biogeographic data. First, the future Balkan peninsula was connected to the Anatolian plate (separating the Tethys and the Peritethys) until 9.0 Ma (i.e., upon the final opening of the mid Aegean trench) (Meulenkamp, 1985; Meulenkamp and Sissingh, 2003; Rögl, 1999; Velonà et al., 2010). Second, the current natural distribution of *R. urbis* ranges from Peloponnisos to the Slovenian coasts (Luchetti et al., 2007). Therefore, this taxon is the westernmost representative of the EE + WA lineage.

The radiation of the EA lineage started around 11.2 Ma (Fig. 3). The first divergence event involved the EA.1 lineage, which includes termites found in China and on the Ryukyu Islands, and the EA.2 lineage, which includes the Japanese *R. speratus* and the Chinese *R. chinensis* and *R. leptomandibularis*. Based on present day biogeographic data, it is possible that EA.1 and EA.2 represent southern and northern lineages, respectively. Interestingly, palynological evidence indicates that, until the late Oligocene, an arid area stretched from western to eastern China, which created separate warm and humid regions in the north and south. Later, at the Oligocene-Miocene boundary, a monsoon system formed, which intensified two times around 15 Ma and 8 Ma (Fig. 4f), and the humid zone widened, spanning from southern to northeastern

China (Sun and Wang, 2005; Wang, 2013). These paleoclimatic and paleoecological changes would have promoted colonization by termite populations; the evolutionary divergence of the EA.1 and EA.2 lineages may have then followed (Fig. 4d). Furthermore, during the same time period (around 15 Ma), the western margin of the Asian plate finally broke up and the Japanese sea opened (Jolivet et al., 1994; Maruyama et al., 1997). This event could have started to a vicariant separation between populations, which may have resulted, finally, in the divergence of *R. speratus* from *R. chinensis* and *R. leptomandibularis* around 8–7 Ma (Fig. 4e).

The diversification pattern of the EA.1 lineage appears to be more complex, especially that of the Ryukyu species *R. amamianus*, *R. miyatakei*, *R. okinawanus* and *R. yaeyamanus*. The paleogeography of the Ryukyu islands is complicated, but the general consensus is that the islands originated in the late Miocene and were connected to the mainland at some point during the Mio-Pliocene and again during the Pleistocene (Kizaki, 1986; Ota, 1998). Many animal taxa underwent differentiation in this area during the Pliocenic fragmentation of the Ryukyus, well after the submergence of the Mio-Pliocenic land bridge (Grismer et al., 1994; Maekawa et al., 1999; Maekawa and Matsumoto, 2003; Ota, 1998). However, our analyses point to an earlier origin for the Ryukyu taxa, suggesting that their diversification took place before the separation of the island arc from the mainland (Fig. 4e). This fits with a polyphyletic origin for the Ryukyu *Reticulitermes*: *R. miyatakei* is more closely related to the Chinese taxa than to the other Ryukyu or Japanese taxa. Interestingly, a recent study on geckos from the central Ryukyu islands also found evidence for a Mid-Miocene lineage splitting that occurred well before land fragmentation (Honda et al., 2014).

It is worth noting that, in general, most cladogenetic events within the main *Reticulitermes* lineages occurred in the Miocene (Fig. 3) and have mostly been driven by paleoclimatic and paleogeographic events. After the Late Oligocene warming (~25 Ma) and following the Mid-Miocene climatic optimum (~15 Ma) (Zachos et al., 2001), global temperatures started to fall again. Therefore, this global cooling could have caused populations to migrate to warmer regions, thus further promoting recent diversification events.

4.2. Considerations on taxonomy and systematics

Our findings largely fit with what is already known about the taxonomy and systematics of *Reticulitermes*, especially for the North American (Copren et al., 2005; Ye et al., 2004) and western European (Clément et al., 2001; Luchetti et al., 2013a; Velonà et al., 2010) taxa. In addition, some of our results provide support for hypotheses regarding the taxonomic status of certain Asian taxa. For instance, our results revealed that *R. chinensis* and *R. leptomandibularis* are closely related, which supports their previous classification as subspecies (Krishna et al., 2013). Our results also confirmed that the Japanese species, *R. speratus*, is composed of two main lineages that may be subspecies (Kim et al., 2012; Park et al., 2006). However, our study also highlights the need for further research into the taxonomy and systematics of the genus *Reticulitermes*. It is particularly necessary for the eastern European and western Asian lineages (EE + WA), for which only three of ten taxa (i.e., *R. urbis*, *R. balkanensis*, and *R. clypeatus*) have been described at the species level. Furthermore, some of the names given to these undescribed taxa are misleading and should be avoided in future studies. For instance, the name “*R. lucifugus* Turkey”, which has been given to distinct taxa in the EE + WA lineage, should no longer be used since it refers to the “true” *R. lucifugus* species complex, which belongs to the WE lineage (Figs. 1–4). It is also worth noting that introgressive hybridization might occur among *Reticulitermes* taxa. In this study, introgression is indeed suggested by the sample collected in Iran (*R. sp.* 6, IRA), which exhibited a different position

in the phylogenies obtained using the mtDNA versus the ncDNA dataset (Figs. 1 and 2).

The present study also supports the idea that many previously described species are in fact synonymous. For example, out of the 138 *Reticulitermes* species described thus far (Krishna et al., 2013), 119 species are from China. This number is probably largely overestimated and may not reflect the true species diversity of this region. Many Chinese *Reticulitermes* specimens have been described exclusively using morphological characters. Future taxonomic studies should therefore combine morphological approaches with molecular tools.

4.3. Evolution of asexual queen succession (AQS) in *Reticulitermes*

Our findings showed that the three *Reticulitermes* species exhibiting AQS (i.e., *R. speratus*, *R. virginicus* and *R. lucifugus*) do not form a monophyletic group but, instead, belong to three distinct phylogenetic lineages (i.e., EA, NA and WE) (Figs. 3 and 5). These results also revealed that *Reticulitermes* species exhibiting AQS are closely related to species in which AQS was not found. The best hypothesis to explain these results is that AQS was originally absent in the ancestral lineage of *Reticulitermes* but evolved at least three times independently during the diversification of the genus. If we consider this hypothesis to be true, our analyses allow us to estimate the time periods during which AQS evolved. In the eastern Asia lineage, *R. speratus* expresses AQS, but six other species, namely *R. kanmonensis*, *R. miyatakei*, *R. amamianus*, *R. okinawanus*, *R. yaeyamanus* (Kawatsu and Matsuura, 2013) and *R. chinensis* (Huang et al., 2013) do not. Because *R. chinensis*, the closest relative of *R. speratus*, does not exhibit AQS, this trait may have evolved in EA less than 7.8–6.9 Ma (Fig. 4), when the EA.2 lin-

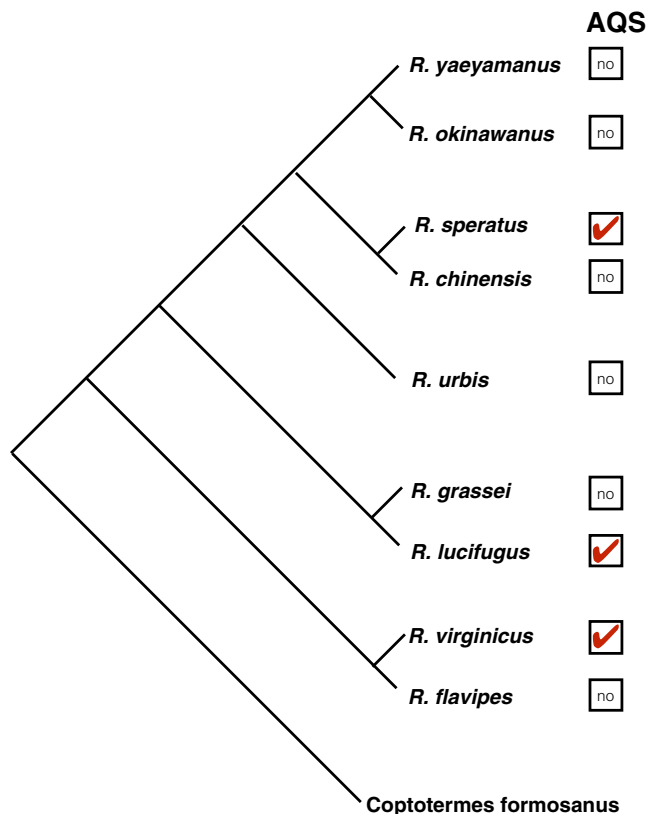


Fig. 5. Cladogram of *Reticulitermes* species that have been examined for the presence of asexual queen succession (AQS). AQS seems to have independently evolved three times in *Reticulitermes*. AQS has not been detected in the outgroup *Coptotermes formosanus*.

age diverged into *R. speratus* and *R. chinensis/R. leptomandibularis*. Both of the two *R. speratus* lineages, which diversified around 3.8 Ma, express AQS. Thus, AQS seems to have evolved before then in the eastern Asian lineage. In the western European lineage (WE), *R. lucifugus* appears to have diverged from *R. grassei* populations 10.2 Ma, and the divergence between the subspecies *R. l. lucifugus* and *R. l. corsicus* likely occurred about 4.7 Ma. Because both of the *R. lucifugus* subspecies exhibit AQS (Luchetti et al., 2013b) and *R. grassei* does not (F. Dedeine, unpublished data), AQS may have evolved in the WE lineage between 10.2 and 4.7 Ma. In the North American lineage (NA), *R. flavipes* has diverged from *R. virginicus* around 14.1 Ma. Because *R. virginicus* displays AQS but *R. flavipes* does not, AQS may have evolved in the NA lineage less than 14.1 Ma.

Although more empirical studies are required to test the hypothesis of an independent evolution of AQS in *Reticulitermes* termites, it is interesting to note that AQS was recently reported in the higher termite species *Cavitermes tuberosus* (Roisin et al., 2014) and *Embiratermes neotenicus* (Fougeyrollas et al., 2015), suggesting that AQS might be much more widespread in termites than previously thought, and that it evolved independently in at least two different termite families (Fougeyrollas et al., 2015).

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ympev.2015.10.020>.

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