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Research article

Neoisoptera repeatedly colonised Madagascar after the Middle Miocene climatic optimum

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Madagascar is home to many endemic plant and animal species owing to its ancient isolation from other landmasses. This unique fauna includes several lineages of termites, a group of insects known for their key role in organic matter decomposition in many terrestrial ecosystems. How and when termites colonised Madagascar remains unknown. In this study, we used 601 mitochondrial genomes, 93 of which were generated from Malagasy samples, to infer the global historical biogeography of Neoisoptera, a lineage containing more than 80% of described termite species. Our results indicate that Neoisoptera colonised Madagascar between 7 and 10 times independently during the Miocene, between 8.4 and 16.6 Ma (95% HPD: 6.1–19.9 Ma). This timing matches that of the colonization of Australia by Neoisoptera. Furthermore, the taxonomic composition of the Neoisopteran fauna of Madagascar and Australia are strikingly similar, with Madagascar harbouring an additional two lineages absent from Australia. Therefore, akin to Australia, Neoisoptera colonised Madagascar during the global expansion of grasslands, possibly helped by the ecological opportunities arising from the spread of this new biome.

Keywords: Australia, endemism, historical biogeography, over-water dispersal, vicariance

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Introduction

Madagascar is the world's fourth largest island and is home to a great many endemic plant and animal species (Goodman and Benstead 2005, Buerki et al. 2013). One important reason for the peculiarity of its biota is its ancient isolation from other landmasses (Goodman and Benstead 2005). Madagascar, together with India, broke away from Africa ~ 160 million years ago (Ma) and has retained a distance of ~ 400 km from East Africa coast for the last 120 million years (Seward et al. 2004, Ali and Aitchison 2008). India subsequently broke away from Madagascar and started drifting northward, leaving Madagascar separated from other continental landmasses for the last ~ 88 Ma (Storey et al. 1995).

The fauna of Madagascar has been interpreted as the result of either vicariance or dispersal. Early biogeographers, unaware of the motion of continental landmasses, explained the origin of Madagascar's fauna by long-distance over-water dispersals (Matthew 1915, Simpson 1940). Subsequently, the validation of the continental drift hypothesis (Wegener 1912) in the 1960s initiated a paradigm shift, and vicariance became widely accepted as the dominant mechanism responsible for Madagascar's unique fauna (Bossuyt et al. 2006, Noonan and Chippindale 2006). However, the time-calibrated phylogenies produced during the last two decades have revealed that the majority of animal lineages found in Madagascar are younger than the split of Madagascar from continental Africa and India (Crottini et al. 2012). This timing implies that Madagascar predominantly acquired its fauna by means of long-distance over-water dispersals after its separation from other landmasses (Yoder and Nowak 2006). For instance, the distributions of the extinct elephant bird (Aepyornithidae) (Mitchell et al. 2014) and the iconic chameleons (Chamaeleonidae: Chamaeleoninae) (Raxworthy et al. 2002) are explained by such dispersals. Long-distance over-water dispersals also explain the distribution of several Malagasy insect lineages, such as the millipede assassin hemipteran bugs (Reduviidae: Ectrichodiinae) (Forthman and Weirauch 2016), the Scarabaeini dung beetle (Coleoptera: Scarabaeidae) (Sole et al. 2011), and the Oxyhaloinae cockroaches (Bourguignon et al. 2018). Only a few insect lineages, such as the Malagasy alderfly genus *Haplosialis* (Liu et al. 2015), the cascade beetles *Tritonus* (Hydrophilidae) (Toussaint et al. 2016), and the whirligig beetles (Gyrinidae) (Gustafson et al. 2017) are ancient enough to have their modern distribution potentially resulting from vicariance.

Termites are a group of social cockroaches feeding on ligno-cellulose at various stages of decomposition, from hardwood to the organic matter present in the soil (Donovan et al. 2000). They include ~ 3000 described species mainly distributed across the tropical and subtropical regions (Eggleton 2000, Krishna et al. 2013). The oldest known fossils of termites are ~ 130 million years old (Myo) and date from the Early Cretaceous (Thorne et al. 2000, Engel et al. 2016). Time-calibrated phylogenies provide slightly older age estimates and suggest that modern termites appeared 140–150 Ma (Engel et al.

2009, 2016, Bourguignon et al. 2015, Legendre et al. 2015, Bucek et al. 2019). The origin of termites therefore predates the breakup of Gondwana, indicating that vicariance may explain the current distribution of early-diverging termite lineages. However, the termite fauna of Madagascar is known to comprise derived genera of Kalotermitidae and Neoisoptera (Sjöstedt 1926, Cachan 1949, 1951, Emerson 1960, Paulian 1970, Eggleton and Davies 2003, Monaghan et al. 2009) and appears to lack early-diverging termite lineages, such as Stolotermitidae and Archotermopsidae, whose distribution may bear the signature of vicariance. We hypothesise that Madagascar acquired its modern termite fauna by means of long-distance over-water dispersals, presumably via rafting in floating wood pieces or vegetative rafts that contained parts of termite colonies (Thiel and Haye 2006).

The pathways and timing of the spread of termites across continents have been studied in detail in Neoisoptera (Bourguignon et al. 2016, 2017, Wang et al. 2019, Romero Arias et al. 2021), with the exception of Madagascar, which has been largely overlooked. The Neoisoptera contain more than 80% of described termite species classified into four families: the Stylotermitidae, the Serritermitidae, the Termitidae and the paraphyletic Rhinotermitidae (Engel et al. 2009, Krishna et al. 2013). In Madagascar, they are represented by a handful of endemic genera and a few genera also found in continental Africa and the Oriental region (Sjöstedt 1926, Cachan 1949, 1951, Emerson 1960). The only Malagasy termite lineage whose historical biogeography has been studied in detail is the fungus-growing termite genus *Microtermes*, which colonised Madagascar from continental Africa via a single long-distance over-water dispersal ~ 13 Ma (Aanen and Eggleton 2005, Nobre et al. 2010). This dispersal event was presumably facilitated by the acquisition of a vertical mode of transmission of *Termitomyces* fungal symbionts in *Microtermes* (Nobre et al. 2010). The number of dispersal events at the origin of the Neoisoptera fauna of Madagascar is still unknown. In this paper, we will reconstruct the historical biogeography of Malagasy Neoisoptera and test the hypothesis that they dispersed from Africa at a similar time as *Microtermes*.

The Neoisoptera fauna of Madagascar shares similarities with the Australian fauna in terms of taxonomy and possibly timing of dispersal. For example, the Australian fauna of Termitidae is composed of several genera belonging to the Nasutitermitinae and *Termes* group and numerous species of *Microcerotermes* and the paraphyletic *Amitermes* that comprises *Drepanotermes* (Hill 1942, Miller 1991, Watson and Abbey 1993, Heimburger et al. 2022). These lineages colonized Australia via long-distance over-water dispersal 10–20 Ma (Bourguignon et al. 2016, Arab et al. 2017, Heimburger et al. 2022). With the exception of the lineage including *Amitermes* and *Drepanotermes*, all termitid lineages found in Australia have been recorded in Madagascar (Cachan 1949, 1951). We will test the hypothesis that their timing of dispersal in Madagascar and Australia matches.

In this study, we provide a comprehensive time-calibrated phylogeny of Neoisoptera, which we used to carry out a

worldwide ancestral range reconstruction of their historical biogeography with special emphasis on Madagascar. We sequenced mitochondrial genomes of 92 termite samples from Madagascar. We also sequenced 30 termite samples collected outside Madagascar and belonging to lineages poorly represented in previous datasets. Our specific aims were 1) to provide the first comprehensive phylogenetic tree of Malagasy Neoisoptera; and 2) to investigate the geographic origin and the timing of dispersal of neoisopteran lineages in Madagascar. We place these results in the context of global termite diversification, noting parallels with the Australian fauna.

Material and methods

Biological samples and mitochondrial genome sequencing

Neoisoptera include 2464 described species (Constantino 2020). Our sampling includes a total of 601 samples, representing over 425 neoisopteran species (~ 17% of extant diversity). The Malagasy termite samples sequenced in this study were collected in 2015 and 2019. Voucher samples are stored at the Univ. Libre de Bruxelles (ULB), the Czech Univ. of life sciences (CULS), the Okinawa Inst. of science and technology graduate Univ. (OIST), and the Univ. of Florida (UF; Prof. Rudolf Scheffrahn). Samples were tentatively identified based on available taxonomic works and similarity to publicly available cytochrome c oxidase subunit II (COII) sequences (Cachan 1949, 1951, Sands 1992, Inward et al. 2007, Monaghan et al. 2009, Krishna et al. 2013).

We sequenced the mitochondrial genomes of 92 termite samples from Madagascar, belonging to 33 species (Supporting information). We also sequenced an additional 30 mitochondrial genomes from termite samples collected outside Madagascar, including 13 samples from the Afrotropical realm, two samples from the Saharo-Arabian realm, nine Neotropical samples, one Oceanian sample, and five Nearctic samples (Supporting information). These 30 samples mostly belonged to termite lineages present in Madagascar and underrepresented in previous studies, such as *Amitermes*, *Psammotermes* and *Prorhinotermes*. We combined the 122 mitochondrial genomes sequenced in this study with the only previous mitochondrial genome of a Madagascan termite, that of *Prorhinotermes canalifrons* from Reunion Island (Holt et al. 2013, Bourguignon et al. 2015), and 477 previously published mitochondrial genomes representing most termite lineages (Cameron and Whiting 2007, Tokuda et al. 2011, Cameron et al. 2012, Bourguignon et al. 2015, 2016, 2017, Wang et al. 2019). We also obtained the mitochondrial genome of the cockroach *Cryptocercus relictus* (Cameron et al. 2012), a representative of Cryptocercidae, the sister group of termites.

We extracted DNA from pools of two or three individuals preserved in RNA-later® or 80% ethanol. Samples preserved in RNA-later were stored at −20 or −80°C until DNA extraction. Samples preserved in 80% ethanol were stored at room temperature for up to 20 years. We used one of the following three sequencing strategies: 1) long-range PCR followed by high-throughput DNA sequencing for samples stored in RNA-later; 2) whole-genome shotgun sequencing for samples stored in RNA-later; and 3) whole-genome shotgun sequencing for samples stored in 80% ethanol. In all three cases, DNA was extracted with the DNeasy Blood & Tissue extraction kit (Qiagen); and libraries were prepared using the NEBNext Ultra II FS DNA library preparation kit (New England Biolabs) and the unique dual indexing kit (New England Biolabs). Libraries were prepared with one-fifteenth of the reagent volumes recommended by the manufacturer.

For strategy 1) DNA was extracted using specimens from which the digestive tract was removed. The whole mitochondrial genomes were amplified in two long-range PCR reactions using the TaKaRa LA Taq polymerase and the primer sets and PCR conditions previously described in Bourguignon et al. (2016). We mixed both amplicons in equimolar concentration and prepared one library for each sample separately. Libraries were pooled in equimolar concentration and paired-end sequenced using the Illumina Miseq2000 platform. For strategy 2) whole genomic DNA was extracted from whole body of termite workers including guts. Libraries were pooled in equimolar concentration and paired-end sequenced using the Illumina Hiseq2500 or Hiseq4000 platforms. For strategy 3) whole genomic DNA was extracted from whole body of termite workers including gut. Libraries were prepared without enzymatic fragmentation step. Libraries were pooled in equimolar concentration and paired-end sequenced using the Illumina HiSeq X or Novaseq platforms.

Assembly and alignment

Raw reads were quality-checked with Fastp ver. 0.20.1 (Chen et al. 2018). Read adaptors were trimmed. Filtered reads were assembled using metaSPAdes ver. 3.13.0 (Nurk et al. 2017), and retrieved and annotated with MitoFinder ver. 1.4 (Allio et al. 2020). The Iterative Mapping and ReAssembling method (IMRA) was used as an attempt to elongate mitochondrial genomes that were not assembled in one contig (Kinjo et al. 2015). The control regions were omitted because they present repetitive patterns difficult to assemble with short reads.

All genes were aligned separately. The 22 transfer RNA genes and the two ribosomal RNA genes were aligned as DNA sequences with MAFFT ver. 7.305 (Katoh and Standley 2013). The 13 protein-coding genes were translated into amino acid sequences using EMBOSS ver. 6.6.0 (Rice et al. 2000) and aligned using MAFFT. Amino acid sequence alignments were back-translated into DNA sequences using Pal2Nal (Suyama et al. 2006). The 37 gene alignments were concatenated with FASconCAT-G_v1.04.pl (Kück and Longo 2014).

Phylogenetic analyses

The concatenated sequence alignment was partitioned into five subsets: one for the combined transfer RNA genes,

one for the combined ribosomal RNA genes, and one for each codon position of the protein-coding genes. The phylogenetic analyses were performed with and without third codon positions. Phylogenetic relationships were inferred using maximum likelihood and Bayesian inference methods. We used IQ-TREE ver. 1.6.12 (Nguyen et al. 2015) to reconstruct maximum likelihood phylogenetic trees. The best-fit nucleotide substitution model was determined with the Bayesian information criterion using ModelFinder (Kalyaanamoorthy et al. 2017) implemented in IQ-TREE. Branch supports were estimated using 1000 bootstrap replicates (Hoang et al. 2018). Bayesian analyses were implemented in MrBayes ver. 3.2.3 using a GTR+G model of nucleotide substitution (Ronquist et al. 2012) and the model selection step was skipped as suggested by Abadi et al. (2019). The tree and the posterior distribution of parameters were estimated from MCMC samplings. Each analysis was run with four chains, three hot and one cold. Each analysis was run in four replicates to ensure the convergence of the chains. For the analyses with third codon positions included, the chains were run for 40 million generations with a 25% burn-in fraction. For the analyses without third codon positions, the chains were run for 20 million generations with a burn-in fraction set to 10%. All the chains were sampled every 5000 generations. The burn-in fractions, mixing of the chains, and the behaviour of all parameters were examined and determined in Tracer ver. 1.7.1 (Rambaut et al. 2018). The analyses with third codon positions included were run for more generations than those without third codon positions as the chains converged at a slower rate. For all analyses, the topology was constrained to ensure a sister relationship between the subfamilies Sphaerotermitinae and Macrotermitinae, as supported by both transcriptome- and ultraconserved-elements-based phylogenies (Bucek et al. 2019, Hellemans et al. 2022).

Divergence time estimation

We analysed the concatenated sequence alignments with and without third codon positions and reconstructed time-calibrated phylogenetic trees using BEAST ver. 2.6.2 (Bouckaert et al. 2014). Each analysis was run twice to ensure the convergence of the chain. The rate variation across branches was modelled using an uncorrelated lognormal relaxed clock. We used the Yule model as a tree prior. A GTR+G model of nucleotide substitution was assigned to each partition, as suggested by Abadi et al. (2019). All partitions were unlinked. For the analyses without third codon positions, we sampled the tree and parameter values of the chain every 50 000 steps over a total of 350 million generations. The first 10% of generations were discarded as burn-in. For the analyses with third codon positions included, the chain was run over 600 million generations and the first 20% of sampled trees were discarded. The burn-in fractions, mixing of the chains, and the behaviour of all parameters were examined and determined with Tracer ver. 1.7.1 (Rambaut et al. 2018).

The molecular clock was calibrated using 14 fossils as minimum age constraints (Supporting information). We selected fossils using the five criteria described by Parham et al. (2012), which aim to ensure that the phylogenetic position and age of fossils are justified. For each fossil calibration, we used the youngest possible age reported in the Fossilworks database (Alroy 2016) last accessed on 31 January 2021 as minimum age constraints. We determined a soft maximum bound using the phylogenetic bracketing approach (Ho and Phillips 2009, Parham et al. 2012), as previously applied to this set of fossils by Bucek et al. (2019). Each calibration was implemented as exponential priors on node time to generate a maximum clade credibility consensus tree in TreeAnnotator (Bouckaert et al. 2014).

Biogeographic analyses

We reconstructed the historical biogeography of Neoisoptera using the R package BioGeoBEARS (www.r-project.org, Matzke 2013). We recognized nine biogeographic realms: the Afrotropical, Australian, Madagascan, Nearctic, Neotropical (including Panamanian), Oriental (including Sino-Japanese), Palaearctic, Saharo-Arabian, and Oceanian realms, as defined by Holt et al. (2013). The Madagascan realm includes Madagascar and neighbouring islands: Comoros, Mascarenes, and Seychelles. We used sampling locations to assign each tip to a biogeographic realm. A total of six phylogenetic reconstructions, estimated with IQ-TREE, MrBayes, and BEAST2 (with and without third codon positions), were subjected to ancestral range reconstructions with BioGeoBEARS. For each phylogenetic tree, we carried out ancestral range reconstructions with the DEC model (dispersal-extinction-cladogenesis), the DIVALIKE model (dispersal-vicariance analysis), and the BAYAREALIKE (Bayesian inference of historical biogeography for discrete areas) model. We used the likelihood version of DIVA and BAYAREA, which are referred to as DIVALIKE and BAYAREALIKE models, to make direct comparisons among different models. We run each model with and without the parameter '+ j' allowing jump dispersals, which correspond to speciation events following long-distance dispersals (Matzke 2014). The best-fit model was determined for each phylogenetic reconstruction using AICc (Akaike information criterion with sample size correction). We also compared the range probabilities of each node for all six phylogenetic trees, and only origins reconstructed with higher than 65% probabilities in all reconstructions were accepted for the final summary (Supporting information). In particular, we focused on the nodes representing dispersals to Australia or Madagascar.

Results

Topology of the phylogenetic trees

Our six phylogenetic trees were largely congruent with respect to interfamily and intergeneric relationships, with

the exception of a few nodes with low posterior probabilities and bootstrap supports (Fig. 1, Supporting information). Neoisoptera were represented by ten lineages of Rhinotermitidae and Termitidae in the Madagascan realm (Fig. 1, Supporting information). The Rhinotermitidae were represented by three species: *P. canalifrons*, *Coptotermes truncatus* and *Psammotermes voeltzkowi*. All three species belonged to genera also present in other biogeographic realms. The seven remaining lineages were part of the Termitidae and formed clades endemic to the Madagascan realm, including one clade of Macrotermitinae, two clades of Nasutitermitinae, and four clades of Termitinae. The only Madagascan clade of Macrotermitinae included several species of *Microtermes* that formed the sister group of a clade composed of African *Microtermes* and the Oriental *Ancistrotermes pakistanicus*. One of the two Madagascan clades of Nasutitermitinae contained *Malagasitermes milloti*, *Coarctotermes*, and several species assigned to the polyphyletic *Nasutitermes*. The sister group of this clade varied among analyses. The other Madagascan clade of Nasutitermitinae only included *Nasutitermes* sp. 1, recovered as sister to a group of Oriental species. The four Madagascan clades of Termitinae comprised *Microcerotermes*, *Amitermes* and two clades within the *Termes* group. The Madagascan *Microcerotermes* included at least 14 independently evolving lineages. Support values for *Amitermes* were sometimes low and internal topologies inconsistent between phylogenies; however, all phylogenies recovered the same branching order, including a sister-group relationship between American *Amitermes* and all other *Amitermes*. Within the latter, the two Madagascan *Amitermes* were consistently recovered as forming the sister group of the *Amitermes* from the remaining biogeographic regions. One of the two Madagascan clades of the *Termes* group included *Quasitermes*, *Capritermes* and a species resembling *Quasitermes*. This first clade was sister to a clade containing the Madagascan and Oriental species of *Termes* as well as the Australian members of the *Termes* group.

Divergence times

The time-calibrated phylogenetic trees reconstructed with and without third codon positions of protein-coding genes differed in their age estimates by up to five million years (Fig. 1, Supporting information). The differences were smaller than 2.2 million years for the nodes representing the splits between Madagascan clades and their sister groups (Fig. 1, Supporting information). Given the similar divergence age estimates obtained with both analyses, we will only discuss the results of the analysis with third codon positions excluded for the sake of simplicity.

All Madagascan clades of Neoisoptera diverged from their sister groups during the Miocene (Fig. 2). Within the Rhinotermitidae, the Madagascan *Prorhinotermes*, *Psammotermes* and *Coptotermes* diverged from their sister groups 9.0 Ma (95% height posterior density (HPD): 6.0–12.1 Ma), 9.0 Ma (95% HPD: 5.0–13.2 Ma), and 8.4 Ma (95% HPD: 6.1–10.8 Ma), respectively. Within the

Termitidae, the Madagascan macrotermitine *Microtermes* diverged from their sister group 16.6 Ma (95% HPD: 13.4–19.9 Ma). The Madagascan nasutitermitine clade containing *M. milloti* and *Coarctotermes* diverged from its sister group 14.4 Ma (95% HPD: 12.3–16.5 Ma). The other Madagascan nasutitermitine clade, composed of *Nasutitermes* sp. 1, diverged from its Oriental sister group 10.7 Ma (95% HPD: 8.2–13.2 Ma). Within the termitines, we dated the divergence between the Madagascan *Amitermes* and other *Amitermes* species at 13.1 Ma (95% HPD: 11.3–15.1 Ma). The most recent common ancestor of all Madagascan *Microcerotermes* and their sister group was estimated at 14.8 Ma (95% HPD: 12.5–17.2 Ma). The Madagascan *Quasitermes* + *Capritermes* clade diverged from its sister group 14.0 Ma (95% HPD: 11.6–16.5 Ma) and the Madagascan *Termes* sp. B diverged from its sister group 11.7 Ma (95% HPD: 9.5–13.9 Ma).

Biogeographic reconstruction

We reconstructed the ancestral range distribution of Neoisoptera on our six phylogenetic trees using six different models. The DIVALIKE + *j* model was the best-fit model for all trees, except for the BI tree with third positions for which the best-fit model was the DEC + *j* model (Table 1). The models with the parameter + *j* fit the data better than the models without this parameter, indicating that jump dispersals played a major role in the biogeographic history of Neoisoptera.

Our analyses indicated that the Madagascan realm was colonised by 7–10 long-distance over-water dispersals (Fig. 2, Supporting information). Four Neoisopteran lineages unambiguously colonised the Madagascan realm once: *C. truncatus* colonised the Madagascan realm from the Oriental realm; *Microtermes* from the Afrotropical realm; and *Microcerotermes* and *Amitermes* from an unidentified realm. The colonization of the Madagascan realm by *Prorhinotermes* + *Psammotermes*, the Nasutitermitinae, and the *Termes* group involved one or two long-distance over-water dispersals. Following the most likely scenario, *Prorhinotermes* and *Psammotermes* independently colonised the Madagascan realm through long-distance over-water dispersals from undetermined biogeographic realms. The alternative scenario of an early arrival of the common ancestor of *Prorhinotermes* and *Psammotermes*, followed by subsequent long-distance over-water dispersals to other biogeographic realms, was less likely but could not be excluded. Similarly, the two Madagascan clades of Nasutitermitinae probably originated from two independent dispersals from Africa to the Madagascan realm. A less likely alternative featured one long-distance over-water dispersal from Africa to the Madagascan realm followed by two dispersal events from the Madagascan realm to the Afrotropical and Oriental realms. Lastly, the Madagascan realm was either colonised once by the *Termes* group followed by one or several dispersal events out of the Madagascan realm, or it was independently colonised twice, once by each Madagascan lineage of the *Termes* group.
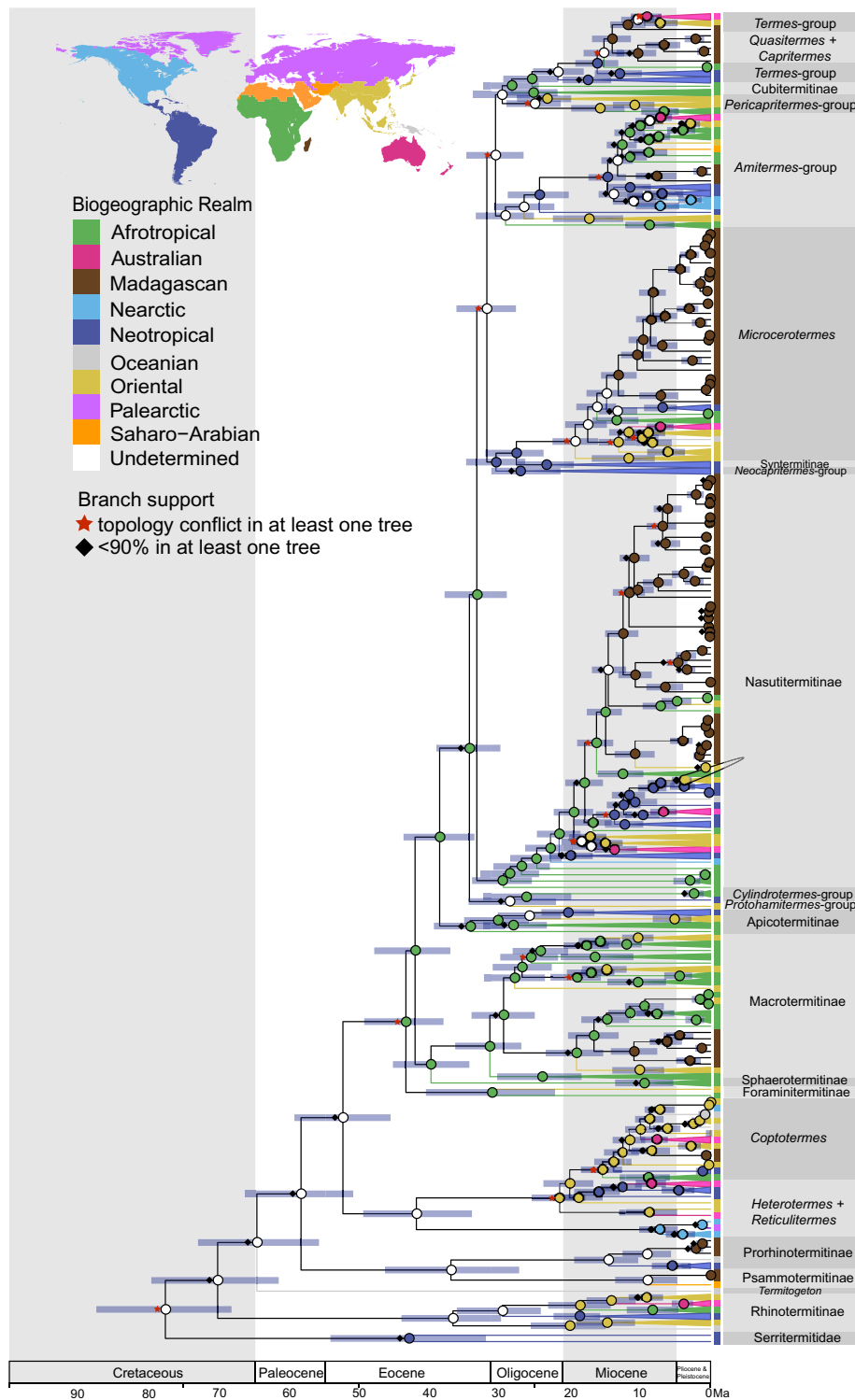
Figure 1. Time-calibrated Bayesian phylogeny inferred from 601 mitochondrial genomes, with the third codon position excluded. Node labels provide a summary of node supports across the six phylogenetic analyses: red stars indicate topology conflicts for at least one analysis; and black diamond indicate support value < 90% for at least one analysis. Nodes without labels have support values > 90% for all analyses. Node bars indicate the 95% height posterior density (HPD) intervals estimated with BEAST 2. Tips and node circles are color-coded to indicate biogeographic realms. The colours of node circles indicate ancestral ranges reconstructed with probabilities higher than 65% for the six phylogenetic trees inferred with IQ-TREE, MrBayes, and BEAST 2, with and without third codon positions. White circles indicate undetermined ancestral distribution. Clades containing species collected in the same biogeographic realm are collapsed, except for species collected in the Madagascan realm. The map shows the biogeographic realms recognised in this study (modified from Holt et al. 2013). The full reconstruction is available as the Supporting information.
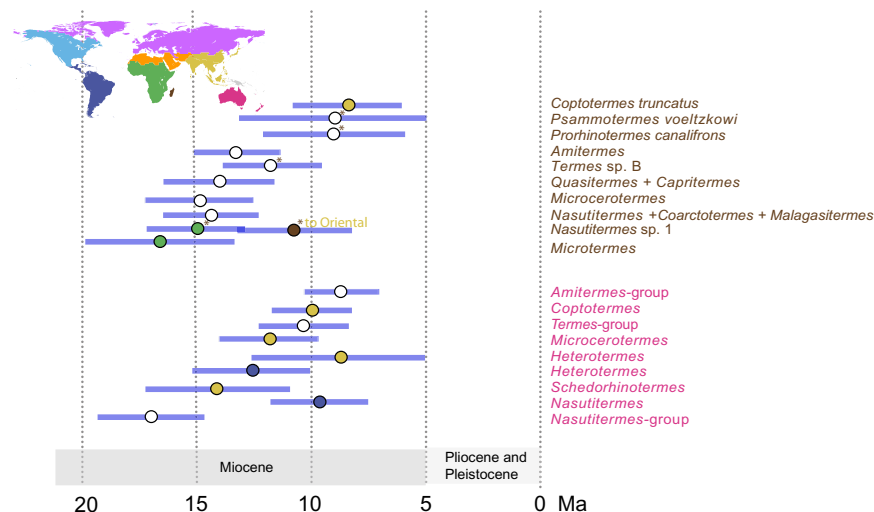
Figure 2. Summary of Madagascar and Australia colonization events. The scenario with 10 dispersal events is displayed. Node bars indicate the 95% HPD intervals estimated with BEAST 2. The colours of node circles indicate ancestral ranges reconstructed with probabilities higher than 65% for the six phylogenetic trees inferred with IQ-TREE, MrBayes, and BEAST 2, with and without third codon positions. White circles indicate undetermined ancestral distribution. The map shows the biogeographic realms recognised in this study (modified from Holt et al. 2013). Asterisks indicate ambiguous dispersal events.

Discussion

Long-distance over-water dispersals of Neoisoptera to and from the Madagascan realm: taxonomic identity, timing, and origin of the dispersers

We reconstructed the most comprehensive phylogenetic tree of Neoisoptera (17%) to date. The relationships among the main lineages of Neoisoptera were largely congruent with earlier molecular studies based on mitochondrial genome and transcriptome data (Bourguignon et al. 2015, 2017, Bucek et al. 2019). Our time estimates were generally younger than those found by these studies, but remained congruent, with overlapping HPD intervals. These differences may pertain to the use of different fossil calibrations and to changes in the estimated ages of the fossils since 2019. For example, Bourguignon et al. (2015, 2017) calibrated Kalotermitidae + Neoisoptera with the ~110 Myo *Cratokalotermes santanensis* (Grimaldi et al. 2008), while we follow Bucek et al. (2019), using the ~95 Myo *Archeorhinotermes rossi* (Krishna and Grimaldi 2003) to calibrate the same node.

Three genera of Rhinotermitidae, *Coptotermes*, *Prorhinotermes* and *Psammotermes*, as well as four groups of Termitidae, *Microtermes*, *Microcerotermes*, Nasutitermitinae, and the *Termes* group, were previously known from the Madagascan realm (Sjöstedt 1926, Cachan 1949, 1951, Emerson 1960, Eggleton and Davies 2003, Krishna et al. 2013). Our phylogeny recovered at least 40 independent evolving lineages, implying the existence of more than the 33 species currently described from Madagascar (Eggleton and Davies 2003). Most notable were two independent evolving lineages of *Amitermes*, a genus previously unknown from the island. In addition, Madagascan Nasutitermitinae and *Termes*

group samples did not form monophyletic groups. These results illustrate that the Madagascan termite fauna is more phylogenetically diverse than previously envisioned.

Our ancestral state reconstructions indicated that Neoisoptera colonised the Madagascan realm seven to ten times independently and possibly dispersed out of the Madagascan realm up to four times. The dispersal events to and from the Madagascan realm took place 8.4–16.6 Ma (95% HPD: 6.1–19.9 Ma), between the mid-Miocene climatic optimum (Zachos et al. 2001) and the end of the Miocene. Based on its geographic position at that time, the Madagascan realm must have acquired its Neoisoptera through long-distance over-water dispersal events.

Our ancestral range reconstructions also revealed one long-distance over-water dispersal event within the Madagascan realm, that of *P. canalifrons* between Madagascar and the Reunion Island 2.1 Ma (95% HPD: 1.3–3.0 Ma; Supporting information). This species is also known from Mauritius, Comoros and Seychelles (Krishna et al. 2013), potentially indicating additional over-water dispersals among islands of the Madagascan realm for this genus with high dispersal abilities and tolerance to salinity (Emerson 1955, Chiu et al. 2021). Two other species, *C. truncatus* and *Microcerotermes subtilis*, as well as the nasutitermitine genus *Kaudernitermes*, are also known from Madagascar and several neighbouring islands (Krishna et al. 2013), indicating further dispersals between islands. Whether these dispersals were mediated by human activities or were long-distance over-water dispersals, as was the case for *P. canalifrons* in Madagascar and the Reunion islands, is unclear. Additional sequence data from the termite fauna of the Reunion Island, Mauritius, Comoros and Seychelles are needed to identify the processes of colonization of these islands.

Table 1. Statistical comparisons of DEC, DEC+J, DIVALIKE, DIVALIKE+J, BAYAREALIKE and BAYAREALIKE+J with phylogenies generated based on both Maximum Likelihood and Bayesian analyses. Likelihood ratio test (LRT) were performed to determine the effect of jumping speciation events in pairs of models. Abbreviations: d – dispersal rate between biogeographic realms; e – local extinction rate. Models were statistically compared using corrected Aikake information criterion (AIC) weights.

	Model	LnL	No. of parameters	d	e	j	AICc	AICc_wt	p-value (LRT)
IQ-TREE (with the third codon position excluded)	DEC	−516.1	2	0.2	0.23	0	1036	< 0.001	< 0.001
	DEC+J	−396.6	3	< 0.001	0.092	0.0078	799.3	0.043	
	DIVALIKE	−490.6	2	0.23	< 0.001	0	985.3	< 0.001	< 0.001
	DIVALIKE+J	−393.7	3	< 0.001	< 0.001	0.0085	793.4	**0.86**	
	BAYAREALIKE	−677.2	2	0.23	2.84	0	1358	< 0.001	< 0.001
	BAYAREALIKE+J	−395.8	3	< 0.001	< 0.001	0.0085	797.7	0.098	
IQ-TREE (with the third codon position)	DEC	−510.6	2	0.17	0.16	0	1025	< 0.001	< 0.001
	DEC+J	−392.2	3	< 0.001	0.015	0.0092	790.5	0.42	
	DIVALIKE	−484.7	2	0.19	< 0.001	0	973.4	< 0.001	< 0.001
	DIVALIKE+J	−392	3	< 0.001	< 0.001	0.0085	790.1	**0.52**	
	BAYAREALIKE	−677.3	2	0.19	2.39	0	1359	< 0.001	< 0.001
	BAYAREALIKE+J	−394.2	3	< 0.001	< 0.001	0.0085	794.4	0.059	
MrBayes tree (with the third codon position excluded)	DEC	−510.9	2	0.25	0.2	0	1026	< 0.001	< 0.001
	DEC+J	−405.2	3	< 0.001	0.32	0.0087	816.4	< 0.001	
	DIVALIKE	−485.5	2	0.29	< 0.001	0	975.1	< 0.001	< 0.001
	DIVALIKE+J	−395.7	3	< 0.001	< 0.001	0.0086	797.5	**0.88**	
	BAYAREALIKE	−670.6	2	0.3	3.61	0	1345	< 0.001	< 0.001
	BAYAREALIKE+J	−397.7	3	< 0.001	< 0.001	0.0086	801.4	0.12	
MrBayes tree (with the third codon position)	DEC	−511.9	2	0.15	0.13	0	1028	< 0.001	< 0.001
	DEC+J	−394.5	3	< 0.001	< 0.001	0.0085	795	**0.65**	
	DIVALIKE	−487.8	2	0.17	< 0.001	0	979.7	< 0.001	< 0.001
	DIVALIKE+J	−395.3	3	< 0.001	< 0.001	0.0086	796.6	0.31	
	BAYAREALIKE	−675	2	0.16	2.02	0	1354	< 0.001	< 0.001
	BAYAREALIKE+J	−397.2	3	< 0.001	< 0.001	0.0086	800.5	0.043	
BEAST tree (with the third codon position excluded)	DEC	−506.8	2	0.0014	0.0013	0	1018	< 0.001	< 0.001
	DEC+J	−394.9	3	< 0.001	< 0.001	0.0085	795.8	0.44	
	DIVALIKE	−479.4	2	0.0016	< 0.001	0	962.8	< 0.001	< 0.001
	DIVALIKE+J	−394.7	3	< 0.001	< 0.001	0.0085	795.4	**0.53**	
	BAYAREALIKE	−873.6	2	0.01	0.01	0	1751	< 0.001	< 0.001
	BAYAREALIKE+J	−397.8	3	< 0.001	< 0.001	0.0086	801.6	0.024	
BEAST tree (with the third codon position)	DEC	−765.3	2	0.01	0.01	0	1535	< 0.001	< 0.001
	DEC+J	−393.8	3	< 0.001	< 0.001	0.0085	793.7	**0.65**	
	DIVALIKE	−484.2	2	0.0015	< 0.001	0	972.5	< 0.001	< 0.001
	DIVALIKE+J	−394.6	3	< 0.001	< 0.001	0.0086	795.2	0.31	
	BAYAREALIKE	−907.6	2	0.01	0.01	0	1819	< 0.001	< 0.001
	BAYAREALIKE+J	−396.5	3	< 0.001	< 0.001	0.0086	799.1	0.043	

We could identify the sources of three dispersal events to the Madagascan realm: *C. truncatus* from the Oriental realm, *Microtermes* from Africa (Aanen and Eggleton 2005), and at least one lineage of nasutitermitines from Africa. This picture is similar to many other taxa of the Madagascan realm (Yoder and Nowak 2006, Warren et al. 2010). However, the origin of most Madagascan Neoisoptera lineages remains unresolved, and ancestral range reconstructions do suggest the possibility of multiple dispersal events from the Madagascan realm. These questions can only be resolved with a more detailed data set, including additional sequences of potential sister clades from other biogeographical realms.

The colonization of the Madagascan and Australian realms by Neoisoptera coincides with the global expansion of grasslands

The Madagascan and Australian realms were colonised by related lineages of Neoisoptera (Lee et al. 2015, Bourguignon et al. 2016, 2017, Arab et al. 2017, Wang et al. 2019) (Fig. 2). The colonization of both realms began around the Miocene climatic optimum, 15–17 Ma, and continued over the next 5–10 million years while the world climate gradually cooled (Zachos et al. 2001) and grasslands expanded worldwide (Edwards et al. 2010). We tentatively attribute this concurrent timing to shared historical climatic and ecological changes in Australia and Madagascar.

The climate of Australia became drier during the Middle Miocene ~14 Ma, and new biomes composed of flora and fauna adapted to arid conditions expanded (Martin 2006, Crisp and Cook 2013). The expansion of the arid-adapted biomes in Australia was accompanied by the opening of new ecological opportunities for local Australian taxa and for colonisers arriving from other continents (Crisp and Cook 2013), which included a dozen of lineages of Neoisoptera (Lee et al. 2015, Bourguignon et al. 2016, 2017, Arab et al. 2017, Wang et al. 2019) (Fig. 2). Unlike in Australia, the origin of grasslands in Madagascar is still

debated. Human activities have undoubtedly contributed to the expansion of modern Madagascar's grasslands, and some authors have argued that, prior to human arrival, the areas presently covered by grasslands were forested and only contained patches of grasslands (Joseph and Seymour 2020, Crowley et al. 2021). The alternative view is that Madagascar's grassland first appeared during the Miocene and gradually expanded, an expansion that was accelerated by human arrival (Bond et al. 2008, Vorontsova et al. 2015, Solofondranohatra et al. 2020). Whichever scenario turns out to be correct, the arrival of Neoisoptera in the Madagascan realm was concurrent with the diversification of grasses in Madagascar, whose number of species exponentially increased since around 20 Ma (Hackel et al. 2018). The divergence between the two grass-feeding species *Coarctotermes pauliani* and *Coarctotermes baharaensis* 6.5 Ma (95% HPD: 4.2–8.8 Ma) indicates an early adaptation of some termite species to grassland in Madagascar. However, the bulk of the termite diversity in Madagascar is associated with forested areas (Eggleton and Davies 2003). The colonization of Madagascar and Australia by Neoisoptera therefore coincides with the global spread of grasses. Other taxa also colonised Madagascar during the last 20 million years, possibly under the influence of climatic cooling and the expansion of grasslands. For instance, the social spider genus *Anelosimus*, which dispersed from South America to Madagascar, greatly diversified in both regions during the last eight million years, possibly as a consequence of climatic changes (Agnarsson et al. 2016, Luo et al. 2020).

In addition to the timing of colonization, another parallel that can be made between the Neoisopteran fauna of the Madagascan and Australian realms is the similarity of their taxonomic composition. The Madagascan realm was colonised by two genera not found in Australia, the rhinotermitid *Psammotermes* and the termitid *Microtermes* (Cachan 1949), while the Australian realm was colonised by three genera absent from Madagascar, the rhinotermitids *Schedorhinotermes*, *Parrhinotermes* and *Heterotermes* (Watson and Abbey 1993). Note that *Heterotermes philippinensis* was introduced in Madagascar and in Mauritius (Cachan 1950, Evans et al. 2013). In contrast, both realms were colonised by the rhinotermitid genera *Coptotermes* and *Prorhinotermes* and by the termitid genera *Microcerotermes*, *Amitermes*, *Termes* and *Nasutitermes* (Cachan 1949, Watson and Abbey 1993). Of note, the latter three genera are paraphyletic and include a number of genera endemic to the Madagascan and Australian realms nested within them. These two realms host taxonomically similar communities of Neoisoptera, acquired within the same geological time interval (Lee et al. 2015, Bourguignon et al. 2016, 2017, Arab et al. 2017, Wang et al. 2019, Heimburger et al. 2022). These observations suggest the existence of ecological pre-adaptations in the Neoisopteran lineages that colonised Madagascar and Australia, two distant landmasses presently dominated by grasslands and savannah biomes. Further studies are needed to determine whether the pattern we describe holds for other taxa.

Conclusion

Our phylogenetic analyses show that Madagascan Neoisoptera are more diverse than previously reported and provide an opportunity to refine the picture of the global spread of Neoisoptera. The higher termites, which make up over 80% of species of Neoisoptera (Krishna et al. 2013), originated from Africa and dispersed worldwide in two phases (Bourguignon et al. 2017). During the first phase, which spanned the Oligocene and the Early Miocene, ~34–20 Ma, higher termites colonised the Neotropical and Oriental realms via a dozen of over-water dispersal events (Bourguignon et al. 2017). Our results show that Neoisoptera dispersed to the Madagascan realm via long-distance transoceanic dispersals during a second phase, which took place during the Miocene, ~20–8 Ma. This timing coincides with the colonization of Australia, and the global expansion of grasslands. Pre-adaptations to grasslands in certain isopteran lineages presumably facilitated their colonization of both Madagascar and Australia.

Author contributions

Menglin Wang: Formal analysis (lead); Investigation (lead); Writing – original draft (equal). **Simon Hellemans**: Formal analysis-Supporting, Investigation-Supporting, Writing – original draft (equal); Writing – review and editing (equal). **Aleš Buček**: Formal analysis-Supporting, Investigation-Supporting, Writing – review and editing-Supporting. **Taisuke Kanao**: Investigation-Supporting. **Jigyasa Arora**: Investigation-Supporting. **Crystal Clitheroe**: Investigation-Supporting. **Jean-Jacques Rafanomezantsoa**: Resources-Supporting. **Brian L. Fisher**: Resources-Supporting. **Rudolf Scheffrahn**: Investigation-Supporting. **David Sillam-Dusses**: Investigation-Supporting. **Yves Roisin**: Investigation-Supporting, Writing – review and editing-Supporting. **Jan Sobotník**: Funding acquisition-Supporting, Investigation-Supporting, Writing – review and editing-Supporting. **Thomas Bourguignon**: Conceptualization (lead); Investigation-Supporting, Resources (lead); Supervision (lead); Writing – original draft (equal); Writing – review and editing (lead).

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Data availability statement

The mitochondrial genome sequences in this paper are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.6hdr7sr55 (Wang et al. 2023) or from GenBank (accession numbers are listed in the Supporting information).

Supporting information

The Supporting information associated with this article is available with the online version.

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