Social evolution in termites reduces natural selection efficacy

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Abstract

In eusocial species, reproduction is monopolized by a few reproductive individuals. From the perspective of population genetics, this implies that the effective population size (*Ne*) of these organisms is likely to be smaller compared to solitary species, as has been proposed in the literature for eusocial Hymenoptera. In this study, we investigate the genomic consequences of eusociality in termites (Isoptera) across two different timescales. First, by analyzing transcriptome data from 66 Blattodea species, we focus on the ratio of nonsynonymous to synonymous mutations (d_N/d_S) as a marker of natural selection efficiency and effective population size. Our results demonstrate an elevated d_N/d_S ratio in termites compared to other Blattodea members, which further generalizes the idea that convergent evolution toward eusociality strongly reduces effective population size and genome-wide efficiency of natural selection. Then, by comparing 68 termite transcriptomes, we show that this decrease in natural selection efficiency is even more pronounced in termites displaying high levels of social complexity. This study contributes to understanding the complex interplay between social structures and natural selection patterns, highlighting the genetic footprint of eusociality in shaping termite evolution.

Keywords: Eusociality; Genome Evolution; Natural Selection; Termites; Blattodea

Introduction

Eusociality represents the pinnacle of social organization within the animal kingdom, characterized by a clear division of reproductive labor among different castes, cooperative care for young and the presence of overlapping generations within a colony (Keller and Chapuisat, 2001). Various forms of social organizations have independently emerged in several taxa such as Coleoptera, Thysanoptera (Choe and Crespi, 1997), crustaceans (Duffy, 1996) or mammals (Jarvis et al., 1994), but the most complex forms of eusociality appeared only within Hymenoptera (e.g., bees, wasps, ants) and Isoptera (termites; Howard and Thorne, 2011). Both ants and termites display sharp contrasts in terms of levels of social complexity, ranging from small colonies of hundred individuals with low caste polymorphism, to large colonies of several millions of individuals and extreme caste differentiations.

For a given number of individuals making a population, the shift from solitary living to eusociality corresponds with a notable decrease in the effective population size *Ne*, mainly because reproduction becomes confined to a limited number of individuals within a colony (Romiguier, Lourenco, et al., 2014). Such reduction of *Ne* has important consequences on genome evolution because of the interplay between the efficiency of natural selection and random genetic drift (Kimura, 1983). The potency of selection in either promoting the fixation of advantageous mutations or removing deleterious ones hinges on the selection coefficient *vis-à-vis* the influence of genetic drift, whose intensity is increased for lower N_e values. When the influence of selection weakens in comparison to genetic drift ($|N_e.s| < 1$, s being the selective coefficient), alleles behave as if they are effectively neutral. Thus, genomes of species with a large effective size are characterized by a higher relative accumulation rate of slightly advantageous mutations compared to species with a small N_e. In the same direction, weakly deleterious mutations are not effectively eliminated from the genomes of low N_e species because genetic drift is stronger than purifying selection for them.

This phenomenon sets a theoretical threshold dubbed the "drift barrier" (Lynch and Walsh, 2007), delineating the boundary where random genetic drift limits the ability of selection to forestall the fixation of suboptimal alleles (Kimura et al., 1963; Ohta, 1973). The empirical demonstration of the drift barrier has established it as a key factor shaping the genetic architectures of living organisms. The central involvement of the N_e effect has since been empirically demonstrated to explain the evolution of different genomic components: mutation rate (Lynch, Ackerman, et al., 2016), intron emergence (Lynch, 2002), precision of intron splicing (Benitiere et al., 2022), optimization of codon usage (Galtier et al., 2018), complexity of protein-protein interactions (Lynch, 2012), protein optimization (Huber et al., 2017) and rate of adaptive evolution (Galtier, 2016).

The evolution of coding regions themselves is shaped by the mutation-selection-drift equilibrium, with life history traits significantly impacting the rates of nonsynonymous substitutions relative to synonymous substitutions (d_N/d_S; Botero-Castro et al., 2017; Figuet et al., 2016; Nikolaev et al., 2007; Popadin et al., 2007; Rolland et al., 2020; Romiguier, Ranwez, et al., 2013; Weyna and Romiguier, 2021). At synonymous positions, the majority of mutations are presumed to be neutral, although some exceptions exist (Galtier et al., 2018). This implies that the substitution rate d_S is a result of the number of mutations emerging per generation in the population ($2.N_e.\mu$, where μ represents the mutation rate per nucleotide per generation) multiplied by the probability of fixing a new neutral mutation ($\frac{1}{2.N_e}$). Hence, the evolution of d_S is solely driven by the mutation rate. Conversely, the fixation probability of new nonsynonymous mutations is directly reliant on the $N_e.s$ product: the smaller the N_e, the greater the accumulation of weakly deleterious nonsynonymous mutations in genomes. Consequently, any life-history trait impacting N_e is likely to increase the relative amount of deleterious nonsynonymous mutations within genomes by intensifying the role of genetic drift

Eusociality in ants, bees, wasps and termites appears to be such a life-history trait by restricting reproduction to few individuals. However, a recent study using an extensive dataset of 169 Hymenopteran species provide a more nuanced view of the link between eusociality and effective population size (Weyna and Romiguier, 2021). While social Hymenoptera featured an increase of d_N/d_S compared to solitary species, the results highlighted the notable exception of solitary bees displaying the same level of d_N/d_S than their social coun-

terparts. This results highlighted that strong decreases of effective population size is not necessarily a direct consequence of the emergence of eusociality, but might also predate it.

In this study, we aim to ascertain whether the negative impact of eusociality on the efficacy of natural selection applies to termites. Similarly to ants despite ≈ 383 my of divergence (Kumar et al., 2017), termites features various level of eusociality, the most complex forms being characterized by particularly large colonies (up to $> 10^6$ individuals; Porter and Hawkins, 2001) and high caste specialization. We thus hypothesize further that termite species with the highest levels of social organization might feature the lowest effective population size, in a similar way than in mammals or birds where species with large body size feature lower effective population size than small body size species (Botero-Castro et al., 2017; Romiguier, Ranwez, et al., 2013).

To explore this inquiry, we leverage two publicly accessible sequencing datasets delineating phylogenetic information at two different evolutionary levels:

1. across Blattodea, allowing for the comparison between 6 species of termites and 60 non-termites species (Evangelista et al., 2019).

2. across 68 species of termites, to study the effect of variation in levels of social complexity on d_N/d_S (Bucek et al., 2019).

Our findings reveal that termites (*i.e.* eusocial Blattodea) exhibit higher d_N/d_S ratios than solitary Blattodea species. Furthermore, among termites, species displaying the highest levels of social complexity tend to exhibit higher d_N/d_S ratio in comparison to those considered to exhibit simpler social organization.

Material and methods

The analysis presented here focuses on two phylogenetic scales: within the Blattodea in first, then a zoom on the Isoptera. For both scales, all the data including molecular alignments, phylogenetic trees and life history traits were taken from the literature. d_N/d_S by branch was then inferred in this study, based on the available aligned transcriptomes and the reconstructed phylogenetic trees.

Blattodea dataset

The molecular data (sequence alignments + phylogenetic tree) for the Blattodea come entirely from the study by Evangelista et al. (2019). This dataset corresponds to 3,244 coding genes aligned in 66 species, and is from the Genbank Umbrella Bioproject (ID PRJNA183205). The 66 Blattodea species had been chosen by the authors to represent the major lineages (except the Anaplectidae). Evangelista et al. (2019) sequenced the transcriptomes of 31 Blattodea species, and complemented this dataset by transcriptomic data coming from Misof et al. (2014) and Wipfler et al. (2019), consisting of 14 additional Blattodea and 21 outgroups. RNA extraction, library construction and paired-end sequencing (Illumina HiSeq 2000) are part of the 1KITE project (www.1kite.org). The used phylogenetic tree corresponds to the file Blattodea_FigTree_95_aa_run1_clean.treefile (Supplementary Material), was produced by Evangelista et al. (2019) using a maximum-likelihood approach implemented in IQ-TREE (Haeseler et al., 2014; Kalyaanamoorthy et al., 2017). The final dataset we used for the d_N/d_S analysis is composed over the 66 surveyed species by a median number of 2,102 coding genes per species, ranging from 1,243 in *Nyctibora* to 2,573 in *Zootermopsis nevadensis*.

Isoptera dataset

The Isoptera dataset comes from the study carried out by Bucek et al. (2019). They sequenced the transcriptomes of 53 termite species and two entire genomes. These data were used to complete publicly available genomes and transcriptomes for 13 termite species and 7 dictyopteran outgroups (*Blaberus atropos*, *Blattella germanica*, *Cryptocercus wrighti*, *Empusa pennata*, *Mantis religiosa*, *Metallyticus splendidus*, *Periplaneta americana*; Bourguignon, Šobotnıék, et al., 2016; Dedeine et al., 2015; Harrison et al., 2018; Hayashi et al., 2013;

Huang et al., 2012; Misof et al., 2014; Mitaka et al., 2016; Poulsen et al., 2014; Terrapon et al., 2014; Wu et al., 2016). Transcriptomes of workers were sequenced by Bucek et al. (2019) using the Illumina HiSeq 4000 platform. The authors then aligned the assembled orthologous using MAFFTv7.305 (Katoh and Standley, 2013). They inferred the phylogenetic tree using IQ-TREE 1.6.7 (Nguyen et al., 2015). The used phylogenetic tree corresponds to the file Isoptera_Bucek.treefile (Supplementary Material). The final dataset we used to estimate d_N/d_S corresponds to a median number of 3,604 coding genes per species, ranging from 2,391 (*Reticulitermes grassei*) to 3,927 (*Blattella germanica*).

Phylogenetic estimation of the d_N/d_S ratio

d_N/d_S values were computed using the mapnh program within the TestNH suite (J Dutheil and Boussau, 2008; JY Dutheil et al., 2012; Guéguen and Duret, 2018; Romiguier, Figuet, et al., 2012; https://github.com/BioPP/testnh) from the publicly available cleaned alignments, and from the tree topologies available for both the Blattodea and Isoptera datasets. Each loci which had sequences with over 90% non-informative sites were removed. mapnh estimates d_N and d_S for four substitution categories ($W \to S$, $S \to W$, $W \to W$, $S \to S$, referring to nucleotides affected by substitutions, AT = W for Weak and CG = S for Strong). These estimates were then associated with each terminal branch of the available phylogenies.

Social traits

The data from Evangelista et al. (2019) were utilized to investigate potential variations in d_N/d_S within Blattodea associated with eusociality. Each species was correlated with its social category following Michener's classification in 1969 Michener (1969) and average individual size. Sociality data were compiled from various bibliographic (Bohn, 1989; Cabrera and Scheffrahn, 2001; Legendre, 2007; Lihoreau, 2009; Picker, 2012; Roth, 1986, 2000) and online (Bugguide; Cockroachcare; Collections.museumvictoria; Jasa; Ozanimals; Padil; Roachcrossing; Virginiacheeseman; Wikipedia) sources. When species-level information was lacking, we utilized the average observation from within genera or subfamilies.

The data sourced from Bucek et al. (2019) were employed to identify potential variations in d_N/d_S within Isoptera linked to varying degrees of sociality. Each species was detailed with various characteristics, including category (Neoisoptera or Basal Isoptera), taxonomic family, queen(s) egg-laying count, approximate colony size, colony type (monogynous, polygynous, or both), polygyny type, worker type, dietary habits, geographical distribution, soldier and queen morphometrics, and invasive status. Notably, the Termitidae and Rhinotermitidae families, encompassing several paraphyletic clades in the dataset, were arbitrarily divided into Termitidae A and B and Rhinotermitidae A to E to study monophyletic groups. However, this division doesn't align with established subfamilies in taxonomy. Data on species characteristics were collected from various literature sources (Ahmad, 1963; Atkinson and ES Adams, 1997; Barbosa and Constantino, 2017; Bourguignon, Scheffrahn, et al., 2010; Chhotani, 1975; Constantino and De Souza, 1997; Constantino, 1990; De Saeger, 1954; Dejean and Fénéron, 1996; Dupont et al., 2009; Emerson and Banks, 1965; Evans, 2011; Florian et al., 2019; Fougeyrollas et al., 2015; F Gay, 1974; F Gay and Barrett, 1983; FJ Gay and JA Watson, 1982; Ghesini and Marini, 2013; Goodisman and Crozier, 2002; Hanus et al., 2006; Hellemans et al., 2017; Husseneder et al., 1998; Krishna, 1963; Krishna and CL Adams, 1982; Krishna and Araujo, 1968; Lewis, 2009; Liang et al., 2017; Maistrello and Sbrenna, 1996; Martius and Ribeiro, 1996; Matsuura, 2002; Miller, 1994; Miura et al., 2003; Noirot, 1959; Noirot, 1989; Parmentier and Roisin, 2003; Rasib and Saeed Akhtar, 2012; Scheffrahn and Krecek, 1999; Scheffrahn, Su, et al., 1999; Snyder, 1924; Soleymaninejadian et al., 2014; Thorne, 1984; Vargo, 2019; Vargo et al., 2006; J Watson, Brown, et al., 1989; J Watson, Metcalf, et al., 1977; Williams, 1966). Similar to the dataset in Evangelista et al. (2019), the search scope was extended to include closely related genera and subfamilies because of shared characteristics among closely related species.

Results and discussion

d_N/d_S evolution in Blattodea

We estimated the d_N/d_S ratio as a proxy of natural selection efficiency across a phylogenetic tree of 66 Blattodea species, including 6 termites (Isoptera). The dataset and phylogenetic tree employed in this investigation are derived from the study by Evangelista et al. (2019) encompassing 3,244 coding genes (Fig. 1).

Upon comparing the mean genomic d_N/d_S ratios calculated for the external branches leading to the 66 species under study, we observe a notably lower efficiency of natural selection within the clade that includes termites (mean=0.107; standard deviation=0.00687) as opposed to outside this group (mean=0.0675; standard deviation=0.00756). This disparity is statistically significant, as confirmed by a Wilcoxon signed-sum test ($V = 2211$; $P = 1.679 \times 10^{-12}$). The two distributions do not overlap, with the highest d_N/d_S for non-termites (0.092 in *Catara rugosicollis*) being lower than the lowest d_N/d_S for termites (0.097 in *Incisitermes marginipennis*).

In mammals, body size is known to strongly influence the d_N/d_S ratio because of its negative relationship with Ne. Thus, large and long-lived species display on average a higher d_N/d_S ratio than small and short-lived mammals (Nikolaev et al., 2007). Here we test whether a similar relationship is found in Blattodae (Fig. 2-A). To do this, we take advantage of the information available on adult body length in Blattodae and test its impact on the effectiveness of purifying selection. Contrary to mammals, where an equivalent relationship is strong, we found no relationship between body size and d_N/d_S in either termites (Spearman's $\rho = -0.6$, $P = 0.2417$) or non-termite Blattodae (Spearman's $\rho = 0.1645$, $P = 0.2439$). This result converges with what has been observed in another insect clade where eusociality has evolved, the Hymenoptera, whose d_N/d_S is not affected by body size (Benitiere et al., 2022; Weyna and Romiguier, 2021).

We then test the hypothesis that an increase in social organization within the Blattodae would reduce the effectiveness of natural selection by reducing the number of reproductive individuals. To do this, we assigned each of the 66 species studied to one of the social categories defined by Michener (1969).

- Solitary = no social behaviour, individuals live separately from each other.
- Gregarious = individuals live together but do not co-operate or care for young.
- Subsocial = individuals take care of their young for a certain period of time.
- Communal = individuals of the same generation cooperate in nest building but not in the care of young.
- Quasisocial = individuals from the same generation use the same nest and cooperate in the caring for the young.
- Semisocial = individuals from the same generation cooperate in caring for young and sharing reproductive tasks.
- Eusocial = individuals from several generations cooperate in caring for the young and where reproductive tasks are shared.

Throughout the explored dataset, we found 24 solitary species, 31 gregarious, 3 subsocial and 6 eusocial. Social organization was not assigned for species of the genus *Tanzaniophasma* and for *Tryonicus parvus*. Before carrying out an ANOVA analysis to test an effect of social organization on d_N/d_S, we checked whether this ratio followed a normal distribution in each social group using a Shapiro-Wilk test (P-values ranging from 0.077 in the gregarious group to 0.838 in the eusocial group). Then we checked that variances of the d_N/d_S ratios are homogeneous between the different social systems using a Levene's test ($P = 0.3$). Once the normality of the d_N/d_S and the homogeneity of the variances between groups had been confirmed, we performed an ANOVA test highlighting a strong effect of the social system ($P < 2.66 \times 10^{-16}$). To identify specific differences

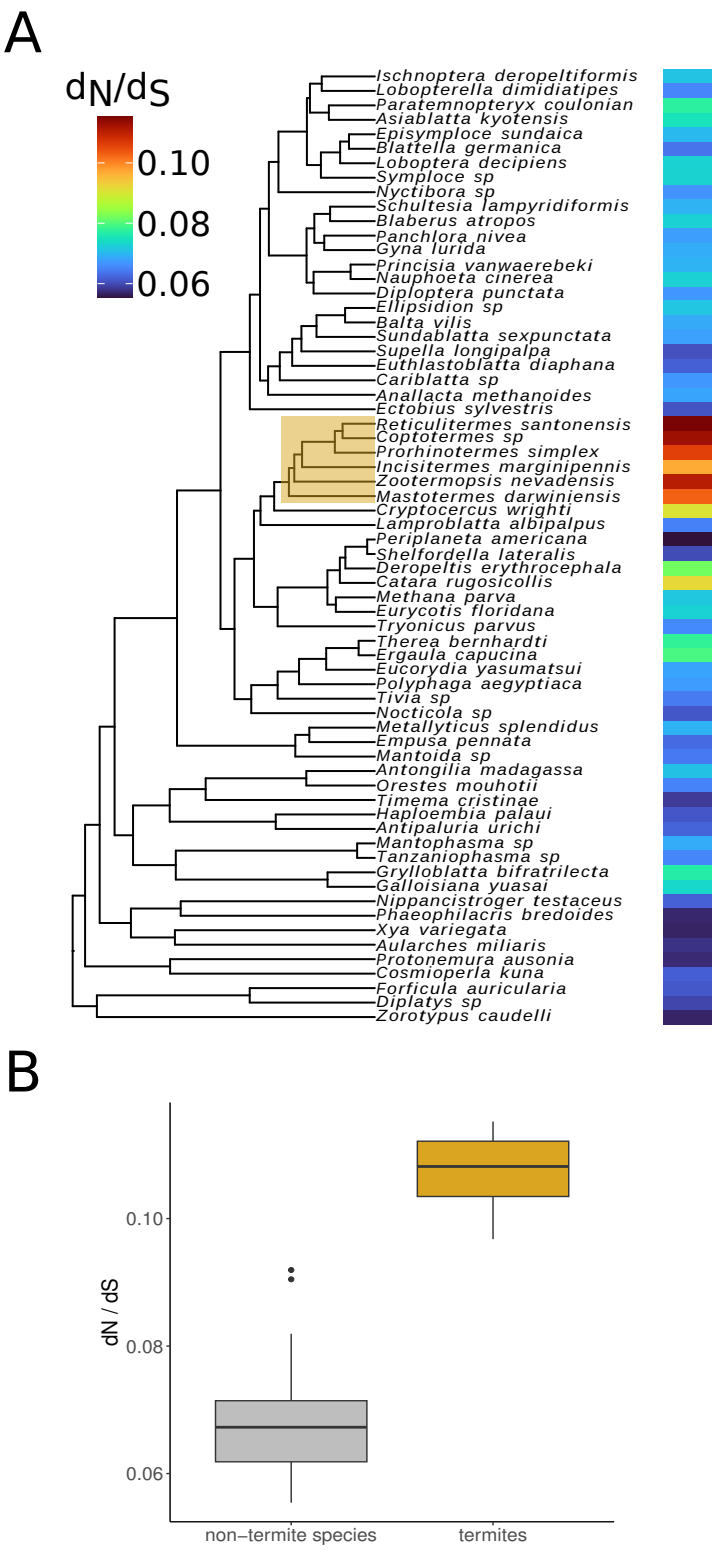

Figure 1. Distribution of the d_N/d_S ratio in Blattodea.
A. The phylogenetic tree employed in this study was produced by Evangelista et al. (2019), constructed from sequenced transcriptomes. Utilizing the identical coding genes, we estimated the d_N/d_S ratios along each phylogenetic branch by mapnh. In this study, we exclusively report the d_N/d_S values corresponding to the terminal branches, represented here by a gradient of colours between 0.055 (dark blue) and 0.115 (dark red). The clade encompassing termites is highlighted by a yellow rectangle. **B.** Boxplot representing the distribution of d_N/d_S ratios in the terminal branches of Blattodae. In grey: estimated values for non-termite species. In yellow: estimated values for termites. Horizontal lines indicate median values

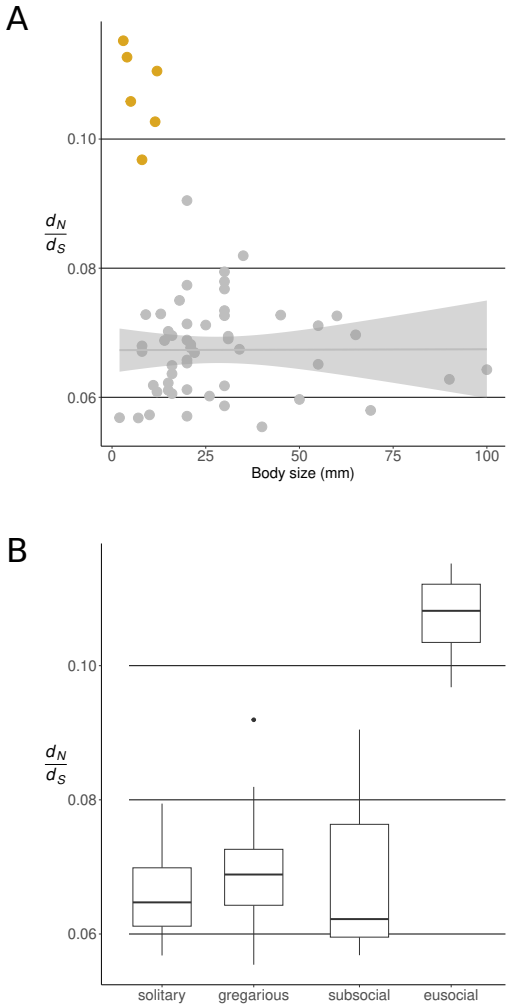

Figure 2. Comparative analysis of body size and social system influences on d_N/d_S ratios
Panel A: relationship between organism body size reported for adults (in mm) and d_N/d_S.
Panel B: distribution of d_N/d_S ratios within different social systems according to Michener (1969).

Table 1. Tukey multiple comparisons of means

comparison adjusted P	difference	lower	upper
Subsocial - Gregarious 0.9948	0.0011	-0.0110	0.0133
Solitary - Gregarious 0.5327	-0.0028	-0.0083	0.0027
Eusocial - Gregarious **<0.0001**	0.0386	0.0296	0.0475
Eusocial - Subsocial **<0.0001**	0.0374	0.0232	0.0517
Eusocial - Solitary **<0.0001**	0.0414	0.0322	0.0506
Solitary - Subsocial 0.8338	-0.0039	-0.0163	0.0084

between groups, we used a Tukey-HSD (Honest Significant Difference) test, which tests the differences in means between each pair of groups (Table 1).

The results of the Tukey-HSD test suggest that the eusocial social system is associated with a higher d_N/d_S than the other groups (gregarious, subsocial, and solitary). The other comparisons (subsocial *versus* gregarious, solitary *versus* gregarious, and solitary *versus* subsocial) did not show statistically significant differences, indicating that d_N/d_S is only increased for eusocial species. At this phylogenetic scale, our analysis is potentially influenced by a pronounced phylogenetic bias, given the fact that only termites are classified as eusocial in Blattodea. We therefore note:

1. d_N/d_S is a rate along a branch and is estimated in a phylogenetic framework, which means that technically two neighbouring branches are independent and should not have similar values due to phylogenetic inertia in the same way than two neighbouring nodes for other statistics (such as $\%GC$ or body mass).

2. When adding this result with those obtained in a very phylogenetically distant group, Hymenoptera (Weyna and Romiguier, 2021), this is the fourth observation of strongly increased d_N/d_S associated to the emergence of eusociality.

Our results reinforce the notion that eusociality is associated with a genome-wide reduction in natural selection efficiency, an effect not exclusive to eusocial Hymenoptera.

Interestingly, we note that the closest relative of termites, *Cryptocercus wrighti*, displays an intermediate d_N/d_S ratio between other non-social species and eusocial ones ($d_N/d_S = 0.0905$ compared to an average of 0.0675 in non-social Blattodea and 0.107 in eusocial ones). The *Cryptocercus* genus is characterized by biparental parental care of the brood (Evangelista et al., 2019), which likely precede the evolution of eusociality where parental care is extended to sibling workers. This is reminiscent of the situation in *Anthophila* bees, where solitary bees features high parental care (nest building and pollen collection for few larvae) and display elevated d_N/d_S, suggesting low effective population size preceding multiple evolution of eusociality in this group (Weyna and Romiguier, 2021). Our results reinforce the idea that high parental care and low effective population size might facilitate evolution towards eusociality. As eusociality is a reproductive strategy characterized by parental care shared by many sibling individuals, high parental care is likely to be a first pre-requisite to its evolution. High parental care has also been identified as the best ecological predictor of low genetic diversity (Romiguier, Lourenco, et al., 2014), which theoretically increases inbreeding and the relative advantages of kin selection (Tabadkani et al., 2012). Together with the example of bees, the evolution of eusociality in termites might have been facilitated by the combination of high parental care and reduced

effective population size. Whether these are universal pre-requisites for eusociality emergence is still open to question, as the same pattern is not observed for ants and social wasps (Weyna and Romiguier, 2021). This can either mean that eusociality can emerge without initial drop of effective population size, or just that we did not detect it because of a lack of genomic data for closely related non-social species for ants and social wasps.

To further understand the influence of sociality on the effectiveness of natural selection in termites, we analyzed an additional dataset derived from transcriptome sequencing (Bucek et al., 2019). This dataset encompasses 68 termite species as well as 7 dictyopteran outgroups, allowing us to test whether the diversity in levels of eusociality among termites correlates with d_N/d_S variations.

Contrasting d_N/d_S ratios among eusocial Blattodea (Isoptera)

In the second part of our study, we investigated the evolution of the d_N/d_S ratio within Isoptera using transcriptome data from Bucek et al. Similarly to the approach adopted for Blattodea, we computed the median genomic d_N/d_S for all branches of the Isopteran phylogenetic tree. The reported d_N/d_S per species corresponds to their respective terminal branch value (Fig. 3). The transcriptomes were available for a total of 75 species. Of these, 68 belong to the Isoptera, exhibiting a d_N/d_S variation range from 0.132 (*Glossotermes oculatus*) to 0.256 (*Occasitermes*). The remaining seven species constitute the outgroup, showing a d_N/d_S variation range from 0.0925 (*Periplaneta americana*) to 0.125 (*Cryptocercus wrighti*). This second dataset thus corroborates the overall trend we previously identified using the first dataset (Evangelista et al., 2019), also indicating an increased d_N/d_S in Isoptera, with values that do not overlap with the range found in non-eusocial Blattodea.

We then tried to explain the large variations of d_N/d_S across termites. More particularly, we hypothesized that the effect of eusociality on d_N/d_S could be modulated by the level of social complexity. As in ants (Romiguier, Borowiec, et al., 2022), termite societies can be more or less complex, with some species featuring extreme forms of eusociality with highly specialized division of labour, large and long-lived colonies. To test whether social complexity increase the effect of eusociality on effective population size (*Ne*), we tested the effect on d_N/d_S of two social traits recognized as two good proxies for social complexity in the literature: nesting strategies and developmental pathways (Mizumoto and Bourguignon, 2021) . Nesting strategies are typically divided in three categories: OP (One Piece), MP (Multiple Pieces), and SP (Separated Pieces). In the One Piece strategy, termites live in a single piece of wood used as both nest and food source, which means that colonies are typically small and short-lived. Multiple Piece nests are typically larger, but social complexity is at its highest in Separated Piece strategies, with large nests distinct from food source, meaning that they do not reduce in size over time and can growth in size over extended period of time. We conducted a Mann-Whitney U test between each pair of nesting strategies, and always found significant differences except for the MP *versus* SP comparison (W=211; adjusted P=1; Table S2). Although termites, collectively, exhibit a higher d_N/d_S ratio compared to non-social Blattodae species, the variations within termites are strongly associated with the complexity of their eusocial structures. Larger colonies, characterized by multiple pieces (MP) and separated pieces (SP) strategies, display higher d_N/d_S values than the smaller, single-piece (OP) colonies (Fig. 4-A). Within the same termite family, different species tend to exhibit identical nesting strategies, complicating the attribution of a direct correlation between nesting strategy and d_N/d_S. Nonetheless, a minimum level of variation in nesting strategies is at least observed within two families: Archotermopsidae, with two OP species and one MP species, and Rhinotermitidae B, comprising two OP species and three MP species. Despite the limited number of data points, we observed that within these families, the d_N/d_S ratio is consistently higher in MP species compared to OP (Fig. S1). This preponderance of species with less complex sociality in the Archotermopsidae could explain why it is the termite family with the lowest d_N/d_S (median=0.149) over the 11 surveyed families (Table S1).

This intricate social structure likely leads to higher d_N/d_S values due to the formation of larger colonies, which in turn reduces the number of breeding individuals, significantly affecting *Ne* and thus natural selection

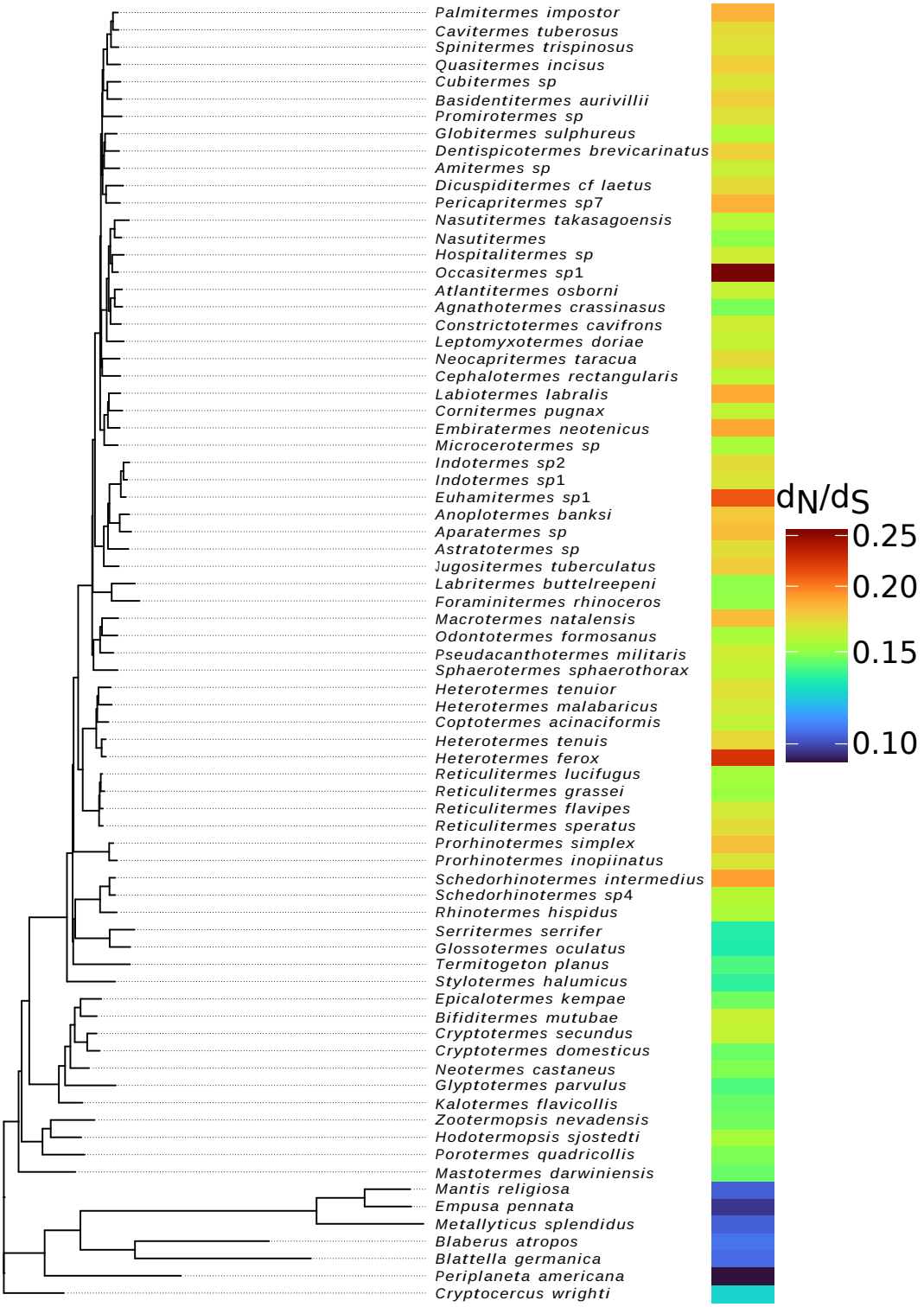

Figure 3. Distribution of the d_N/d_S ratio in Isoptera (and outgroup species).
The phylogenetic tree employed in this study was produced by Bucek et al. (2019), constructed from sequenced transcriptomes. Utilizing the identical coding genes, we estimated the d_N/d_S ratios along each phylogenetic branch by mapnh. In this study, we exclusively report the d_N/d_S values corresponding to the terminal branches, represented here by a gradient of colours between 0.0925 (dark blue for the outgroup *Periplaneta americana*) and 0.256 (dark red for *Occasitermes*).

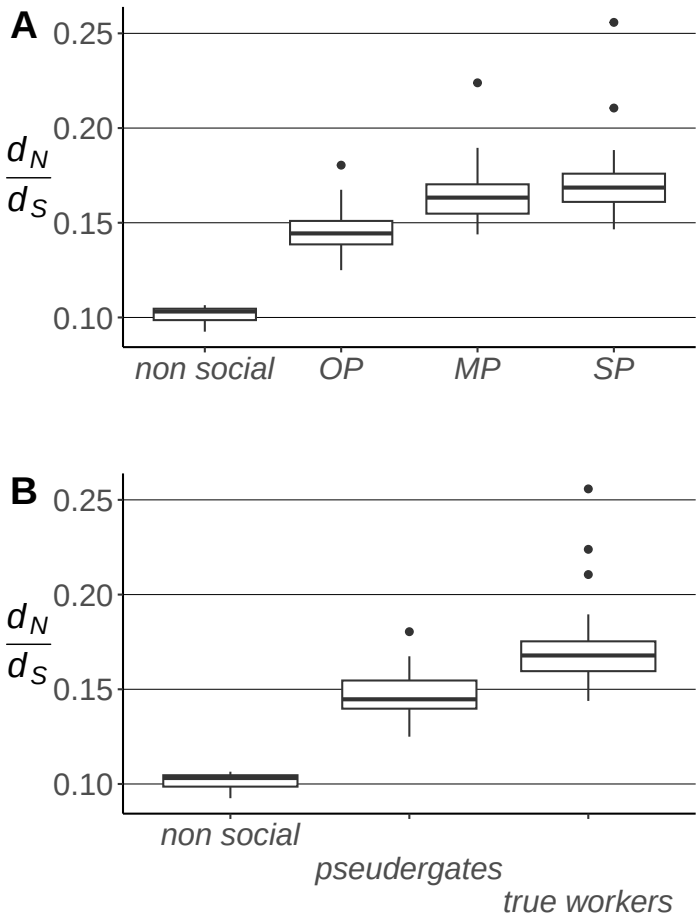

Figure 4. Relationship between social complexity in termites and d_N/d_S ratio.
A. Social complexity, as approximated by nesting strategy, following Mizumoto and Bourguignon (2021). Non-social species are represented solely by the outgroup. OP denotes One-Piece nesters, MP signifies Multiple-Pieces nesters, and SP refers to Separate-Pieces nesters. **B.** Social complexity, as approximated by the working castes, following Mizumoto and Bourguignon (2021). Pseudergates are workers with the plasticity to transition between castes, including reproductive roles, whereas true workers lack this ability to change castes.

efficiency. In contrast, colonies that inhabit a single piece of wood, referred to as OP (One Piece strategy; see Fig. 4-A), are typically smaller. Consequently, these are anticipated to exhibit lower d_N/d_S values, stemming from decreased reproductive variance among females. Here, we classify the nesting strategies of termites as OP (One Piece), MP (Multiple Pieces), and SP (Separated Pieces) following the categorization in the termite literature (Mizumoto and Bourguignon, 2021).

Mizumoto and Bourguignon (2021) suggests an alternative method for gauging the complexity of eusociality in termites by classifying species based on the developmental pathways of their worker caste, which are either pseudergates or true workers. Pseudergates are developmentally immature reproductive individuals, and can perform the colony's labor tasks but also differentiate later into either soldiers or reproductives. In contrast, true workers lack such developmental plasticity and are highly specialized to the colony's tasks. This specialization of true workers, as opposed to pseudergates, results in a higher division of labor, thereby indicating increased social complexity. Both species possessing pseudergates or true workers exhibit higher d_N/d_S ratios compared to non-social species (Mann-Whitney U test: W=0 in both comparisons; Adjusted P-value of 1.22×10^{-3} and 2.15×10^{-4} respectively). Interestingly, species with true workers display significantly higher d_N/d_S ratios than those with pseudergates (W=117; Adjusted P-value=1.87×10^{-5}). This reinforces the idea that effective population size is not only affected by eusociality, but also at a more finer scale by the

different levels of social complexity.

Our results suggest with two different proxies of eusociality level that there is molecular cost to social complexity. Such a finding have been suggested in ants, where a correlation between d_N/d_S ratios and another proxy of social complexity (queen/worker dimorphism) have been reported (Romiguier, Lourenco, et al., 2014). This finding was therefore based on only 7 species. Here, we confirmed this idea on a larger dataset of the other major eusocial taxa. Taken independently, both results are therefore sensitive to correction for phylogenetic inertia, as species with high social complexity tend to be clustered in few related clades within both ants and termites. Taken collectively, both results reinforce the conclusion of the other, as it seems unlikely that the association between high d_N/d_S and high social complexity is due to chance in the two taxa. Supplementary analyses on more species, including other eusocial clades such as bees or wasps will be useful to further confirm our conclusion.

Overall, this study confirms that eusociality is associated to significant decreases in terms of effective population size, which validates population genetic theory and highlights that eusocial insects undergo molecular evolution that is closer to large vertabrates than other insects. This support the view that social insects should be considered as superorganisms (Boomsma and Gawne, 2018), and that their population size vary depending on their social complexity in the same way than population size vary depending on life history traits for non-social species (Romiguier, Gayral, et al., 2014).

Conflict of interest disclosure

The authors declare that they comply with the PCI rule of having no financial conflicts of interest in relation to the content of the article.

Data, script, code, and supplementary information availability

Data and scripts are available online from a Zenodo depository: https://doi.org/10.5281/zenodo.11057544

Table S1. d_N/d_S values for termite families

Family	mean d_N/d_S	min d_N/d_S	max d_N/d_S
Apicotermitinae	0.179	0.168	0.211
Archotermopsidae	0.149	0.145	0.155
Kalotermitidae	0.149	0.140	0.163
Macrotermitinae	0.167	0.156	0.181
Nasutitermitinae	0.170	0.147	0.256
Rhinotermitidae_B	0.154	0.132	0.190
Rhinotermitidae_D	0.161	0.152	0.171
Rhinotermitidae_E	0.178	0.161	0.224
Syntermitinae	0.173	0.156	0.188
Termitidae_B	0.173	0.168	0.185
Termitinae	0.170	0.158	0.185

Table S2. Pairwise comparisons of differences in dNdS between alternative nesting strategies

Pair	*W*	Adjusted P-value
Non-social *vs.* OP	0	2.77×10^{-3}
Non-social *vs.* MP	0	3.72×10^{-3}
Non-social *vs.* SP	0	6.02×10^{-4}
OP *vs.* MP	40	1.77×10^{-2}
OP *vs.* SP	74	6.46×10^{-5}
MP *vs.* SP	211	1.00

Adjusted *p*-values from Mann-Whitney U tests comparing the d_N/d_S values between different nesting strategies of termite species. Adjustments were made using the Bonferroni method to correct for multiple comparisons.

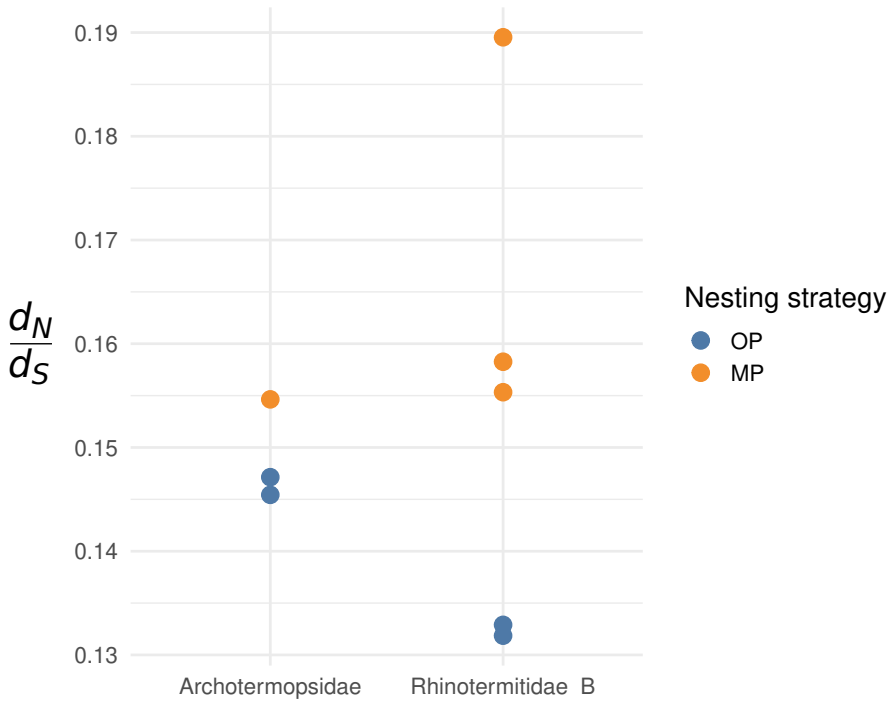

Figure S1. Association between social complexity and d_N/d_S ratios in Archotermopsidae and Rhinotermitidae B using the nesting strategy as proxy.

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