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ORIGINAL ARTICLE

Termite eusociality and contrasting selective pressure on social and innate immunity

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Abstract

The evolution of termite eusociality has been influenced by their nesting and foraging ecology. This includes the evolution of a separate developmental line for specialized workers that forego direct reproduction (true workers), which coincides with the transition from inhabiting a dead-wood nest to foraging for food outside the nest. Foraging for extranidal food requires that termites move through an entomopathogen-rich rhizosphere. This suggests that improved defenses against these pathogens were required for the successful transition to foraging outside the nest. Soil is especially rich in insect pathogens such as *Metarhizium*, and termites use secreted salivary β-1,3-glucanases for protection from this fungus. These enzymes are likely to be dependent on hygiene behaviors, such as allogrooming of external surfaces after contact with fungal conidial spores or ingestion or burying of infected nestmates prior to sporulation of the cadaver. These social mechanisms of defense could compensate for internal innate mechanisms of defense and even relax selective pressure on these innate mechanisms. Here, we investigated whether the selective pressure was intensified or relaxed on secreted β-1,3-glucanases as well as internal innate immune proteins, especially those putatively involved in antifungal defense. An analysis of the molecular evolution of two termite β-1,3-glucanases (GNBP1,2) indicates that the intensity of selection on them significantly increased with the transition to foraging. The shift to foraging for extranidal food apparently required adaptive modification of secreted GNBPs to help cope with increased exposure to pathogenic conidia in the soil. This included modification of a conserved binding site in GNBP2. In contrast, there was either significant relaxation or no change of selection pressure on Toll and phenoloxidase pathway immune genes with the transition to foraging. Relaxation was also observed with the evolution of drywood termites, but this likely reflects a transition to a microhabitat with fewer pathogens. Selection intensified on a subset of immune genes that regulate intestinal microbes with the more recent radiation of the Termitidae and the diversification of their feeding strategies.

Significance statement

The evolution of termite worker eusociality coincides with a relaxation of selective pressure on innate components of the immune system, especially those involved in antifungal defense. In contrast, the selective pressure intensified on two β-1,3-glucanases that contribute to the elimination of fungal pathogens by social behaviors such as allogrooming, cannibalism, and undertaking. This change in selective pressure appears to reflect an increased reliance on social immunity by dedicated altruistic workers in response to novel or enhanced threat by fungal pathogens. This supports other studies in social insects that indicate that the evolution of social immunity not only compensates for an increased vulnerability to infectious disease associated with living in crowded conditions but relaxes selective pressure on components of the internal innate immune system. Our results also indicate that a shift to microbially depauperate habitats relaxed selective pressure on termite immune defenses whereas a more recent diversification of diet intensified selection on mechanisms for regulating intestinal microbes.

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Introduction

Social insects live in crowded conditions that are conducive to the spread of disease so selective pressure on their immune systems might have intensified relative to solitary insects (Cremer et al. 2007; Schmid-Hempel 1998). However, recent genomic and transcriptomic sequencing projects of honeybees, ants, and termites have revealed fewer immune genes than expected (Evans et al. 2006; Smith et al. 2011; Smith et al. 2011; He et al. 2021). The major immune pathways appear to be intact but represented by fewer genes than the non-social insects *Drosophila* and *Anopheles*. This reduction suggests that the evolution of allogrooming and other hygiene behaviors that depend on social interactions relaxed selective pressure maintaining innate immune gene diversity (Bulmer 2019; Evans et al. 2006). Many immune genes were then lost to genetic drift and the selective elimination of those that were costly to maintain. Recently, this behavioral hypothesis has been undermined, at least for bees, as the repertoire of bee immune genes appears to have contracted prior to the evolution of their sociality (Barribeau et al. 2015). Reduced immune gene number may therefore reflect other demographic and historical factors. However, a more direct phylogenetic approach to addressing this hypothesis is to specifically test for the relaxation of selective pressure on critical genes in innate immune pathways during transitions in sociality (Meusemann et al. 2020).

The sterility of soldiers, which are a terminal caste with no further developmental options, firmly establishes the ubiquity of eusociality in termites (Noirot and Pasteels 1987; Roux and Korb 2004). However, worker sterility was derived later in the evolution of termites, and its advent coincides with an important ecological transition from a one-piece nest life type, in which the nest itself (dead wood) provides food, to the use of extranidal cellulosic food (Shellman-Reeve 1997; Thorne 1997; Traniello and Leuthold 2000). Access to food outside the nest would have initially required construction of a gallery system through the soil connecting nests (multipe-piece nest life type). There have been at least three independent evolutionary transitions in termites from living in the confines of damp wood to foraging outside the nest (Legendre et al. 2008). All of these transitions were associated with the evolution of developmentally distinct workers, "true workers," and for the rhinotermitid lineage, the subsequent evolution of complex nest architecture and the diversification of feeding strategies in the Termitidae (Abe 1987; Noirot and Pasteels 1987).

Prior to the evolution of a separate developmental line for true workers, there was a single developmental line leading to winged alates (and soldiers). Older individuals in this developmental line constitute so called "false" workers (pseudergates) because they eventually develop into winged reproductives. They also have the potential to inherit the reproductive role of their parents (Thorne et al. 2003). The earliest termites likely resembled dampwood termites such as extant archotermopsids with false workers that occupy a microhabitat in the confines of decaying wood, rich in microbes and potential entomopathogens (Thorne 1997; Rosengaus et al. 2003; but see Thompson et al. 2000). False workers also occur in kalotermitids, a clade that transitioned to feeding on dry wood that is relatively free of microbes. The microbial load in nests and on the termite cuticle is reduced in drywood termites compared to dampwood termites (Rosengaus et al. 2003), and limited evidence suggests that drywood termites do not groom each other as frequently as dampwood termites, possibly because pathogenic pressure on hygienic defenses is reduced in a drier microhabitat (Korb et al. 2012; 2015).

During the transition to foraging, movement through soil would have substantially increased exposure to ubiquitous fungal pathogens such as *Metarhizium* and *Beauveria*. Conidia of *Metarhizium* reach concentrations up to a million spores per gram of soil (Milner 1991). Its ubiquity in the rhizosphere may stem in part from its symbiotic association with plant roots, although this relationship in conjunction with its role as an entomopathogen is not well understood (Behie and Bidochka 2014). The intense and specific alarm response of subterranean termites to *Metarhizium* spores (conidia) suggests that this fungus represented one of the most serious pathogenic challenges associated with this transition (Myles 2002; Bulmer et al. 2019). *Metarhizium* conidia readily adhere to the insect cuticle and directly penetrate through the external cuticular surface to gain access to the host's hemocoel. Once inside, *Metarhizium* cells can both disrupt and hide from innate mechanisms of immune defense, which includes producing a collagenous coat that masks the cell wall from degradation by host effectors such as β-1,3-glucanases (Wang and Leger 2006; Hamilton et al. 2011).

The ability to move through the soil allowed termite colonies to occupy several nest and feeding sites and to expand their territorial range once a food source had been depleted. Selection for increased foraging efficiency may have been critical for the evolution of true workers (Rupf and Roisin 2008). Increased foraging efficiency in a pathogen rich environment could have also depended on an enhanced social immune system in the foragers, especially with respect to defense against fungi such as *Metarhizium*. β-1,3-glucanases secreted from the salivary glands appear to be an integral component of a social mechanism of defense because they

help inactivate conidia when termites mutually groom each other with their mouthparts (Hamilton et al. 2011; Hamilton and Bulmer 2012). This hygienic grooming rapidly increases 2–3 min after termites have been exposed to *Metarhizium* conidia (Bulmer et al. 2019; Liu et al. 2019). Behaviors such as allogrooming and cannibalism, which appear to be ubiquitous in termites, may have been adaptively modified with the transition to soil. The burial of infected individuals with masticated soil and wood (undertaking) may have evolved with this transition.

If the transition to foraging in termites enhanced social immune systems, this in turn could have relaxed selective pressure on components of the innate immune system. Here, we investigated whether selective pressure intensified on β-1,3-glucanases with the transition to foraging. This intensification potentially includes both purifying and diversifying selection in different regions of the proteins. We also tested whether selective pressure was relaxed on key components of the innate immune system with the transition. We focused on five Toll pathway (*cactus*, *myd88*, *pellino*, *toll*, *traf6*) and two phenoloxidase cascade genes (*PPO2*, *spn28*) that are likely to be critical for defense after a fungal pathogen has invaded the hemocoel (Hoffman 1995). Activation of the Toll pathway results in the production of antifungal peptides in the hemolymph. Activation of the phenoloxidase cascade results in melanization around sites where the fungus penetrates the cuticle as well as phagocytosis of fungal cells by hemocytes. This priming of internal mechanisms of defense is much slower to activate than rapid hygienic responses. For example, Toll pathway genes in locusts, which are critical for their antifungal defense, are upregulated 8 h after exposure to *Metarhizium* conidia (Zheng et al. 2020). The importance of the innate immune response for survival in termites was potentially diminished with the improvement of social responses that are quickly activated in minutes or that result in the elimination of infected individuals. In addition, we investigated changes in selective pressure on these social and innate immune genes with the transition from feeding on damp wood to feeding on dry wood, a transition to an environment with fewer pathogens that could have led to a relaxation in selective pressure on both social and innate immunity.

Another key evolutionary transition occurred more recently with a shift from multiple-piece nests to central-site nests in which termites forage outside distinct nests that harbor the young and specialized reproductives (Abe 1987; Noirot and Pasteels 1987). This transition coincides with the rapid radiation of termite species with diverse feeding strategies and a restructuring of the hindgut microbiome, including the loss of symbiotic protists. We tested for changes in selective pressure on the immune genes described above as well as two additional genes in the IMD pathway (*PGRP-SC2* and *relish*) that have been shown to contribute to the regulation of the insect microbiome (Guo et al. 2014; Vandehoef et al. 2020). We used published sequence from NCBI and new β-1,3-glucanase sequence from an additional one piece-life type termite, *Archotermopsis wroughtoni*, to test for changes in selective pressure during these transitions by comparing selective pressure in subsets of branches of a phylogenetic tree corresponding with dampwood, drywood, multiple-piece, and central-site nest types.

Methods

Termite β-1,3-glucanases

Termite sequences of two termite immune genes, *GNBP1* and *GNBP2*, were identified with BLAST searches using previously published sequence. These genes appear to have a unique role in immunity in termites and their closest cryptocercid woodroach relatives (Bulmer et al. 2012). They are distinct from β-1,3-glucan recognition proteins (βGRPs). Very early duplication of an ancestral β-1,3-glucanase appears to have produced βGRPs that lost their catalytic activity (Pauchet et al. 2009; Hughes 2012) whereas the GNBPs have maintained this activity. The respective identity of *GNBP1* and *GNBP2* was confirmed with alignments of translated sequence with Se-Al (Rambaut, 2002). *GNBP2* can be quickly distinguished from *GNBP1* with a deletion of 5 amino acids in the C-terminal region, which overlaps with a putative GPI anchor site (Bulmer et al. 2012). The alignments spanning 1089 nucleotides did not include the N-terminal signal sequence, which together with the GPI anchor site characterizes them as secreted proteins.

The two *GNBP* genes were also identified and sequenced from preserved *Archotermopsis wroughtoni* larvae and pseudergates. These termites were collected in 2017 from Himachal Pradesh, India (Jibhi), and stored in RNA later (Sigma-Aldrich) for a year, following the manufacturer's recommendations for storage. Complementary DNA was prepared for PCR and Sanger sequencing as described previously (Bulmer et al. 2012).

Termite sequences of the other immune genes were identified with BLAST searches, of Blattodea sequence in the NCBI nt/nr and TSA databases, initially using *Drosophila* immune gene sequence. These included five Toll pathway genes (*cactus*, *myd88*, *pellino*, *toll*, *traf6*), two phenoloxidase cascade genes (*PPO2*, *spn28*), and two IMD pathway genes (*relish*, *PGRP-SC2*). Translated sequences were manually aligned and trimmed with Se-Al (Rambaut, 2002). We also searched for three additional termite Toll pathway genes (βGRP, *spatzle* and *dorsal*) but did not include these because we did not identify a sufficient number of orthologous sequences that could be aligned for our comparative analysis. We did not include termicin, which is a secreted

antifungal protein that may act synergistically with the GNBPs (Bulmer et al. 2009) because of the difficulty in differentiating between paralogues and orthologues (Bulmer and Crozier 2004).

Tests of selection

Bucek et al. (2019) constructed a termite phylogenetic tree with mRNA sequence from over 4000 orthologous genes. The topology of this tree was used for the tests of selection. The ancestral states of nest type were inferred from their distribution in extant species and confirmed with Arbor, which uses a maximum likelihood approach for estimating ancestral character states (Lewis 2001).

Transitions in selective pressure between the termite groups were tested with RELAX (Wertheim et al. 2015). RELAX detects one of three possible transition categories, which include relaxation, intensification, or no detectable change in selective pressure. Both negative (purifying) and positive (diversifying) selections are considered in this test. An intensification of selective pressure could therefore result from selection constraining amino acid change ($dN/dS < 1$, purifying selection) or instigating amino acid change ($dN/dS > 1$, diversifying selection). Positive or relaxed selective pressure can increase the fixation rate of non-synonymous mutations that alter proteins. By modelling three parameters for dN/dS, RELAX reliably distinguishes between an increase in dN/dS due to relaxed selection and an increase due to positive selection (Wertheim et al. 2015). Intensification of selection is indicated when the RELAX test statistic K is significantly greater than one, relaxation of selection when K is significantly less than one, or no change in selective pressure when K is not significantly different from one. A lack of change in selective pressure with an evolutionary transition could be due to a continuation of relaxation or intensification of selective pressure rather than its absence. For example, K might not differ significantly between two groups if pervasive positive selection occurs in both groups.

Three evolutionary transitions were tested for each gene: (1) the shift by termites with a one-piece nest type (OP) that feed on moist, decaying wood (dampwood termites) to OP termites feeding on wood with low moisture content (drywood termites); (2) the shift from OP dampwood termites to a multiple-piece nest type (MP), which occurred two or three times in the phylogenies employed in this study; and (3) the shift from MP to a central-site nest type (CS). The transition from OP to MP that coincides with the evolution of harvester termites (Family Hodotermitidae) was not included in the analysis as sequence from this clade was unavailable (Legendre et al. 2008). The transition from OP to MP that coincides with the evolution of *Mastotermes* was included in the GNBP phylogenies but not in the innate immune gene phylogenies because its sequence was unavailable among these genes. The termite species used for the innate immune genes were not identical for each gene due to differences in sequence availability for each gene (see supplementary material).

Our focus was on ecological transitions in termites that could influence the evolution of their immune systems. Social immune defenses such as allogrooming and possibly cannibalism appear to have evolved prior to the evolution of the termites (Bulmer et al. 2012). Allogrooming and secreted β-1,3-glucanases are critical for defense against fungal pathogens in lightly sclerotized juveniles of *Cryptocercus* that resemble the woodroach-like ancestor of termites. Similar to the dampwood termites, these woodroaches occupy a microhabitat in the confines of decaying wood. However, we were unable to identify a sufficient number of phenotypically unique orthologues in the cryptocercids that could be aligned for an analysis of relaxation or intensification of selective pressure with the evolution of the termites from a woodroach-like ancestor.

The RELAX statistic K can also be influenced by demographic changes that affect the effective population size. Equivalent selection pressure in the two GNBPs, which included identical species, would be expected when K is affected by demographic changes that influence genetic drift. For example, increased genetic drift and relaxation of selection in particular termite lineages could occur as a result of decreases in effective population size. The effect of this type of demographic change would be expected to be the same in both genes for all transitions and also reflected by parallel transition types (relaxation, no change, intensification) in the other immune genes.

We previously constructed a computational model of GNBP and identified six key amino acids that interact with a tetrasaccharide of β-1,3-D-glucan (Bulmer et al. 2009). These are represented by sites 39, 126, 129, 155,162, and 293 in the alignment used for this analysis (see GNBP2 alignment in supplementary materials). Two of these sites were polymorphic in GNBP1 with a minimum requirement that each variant was represented by at least two identical amino acids (sites 129 and 293), and two of these sites were polymorphic in GNBP2 (sites 129 and 293). Contrast-FEL was used to test for a significant difference in selective pressure at these sites between termite groups representing the three evolutionary transitions, OP dampwood to OP drywood, OP dampwood to MP, and MP to CS. Contrast-FEL (fixed-effect likelihood) uses a maximum-likelihood approach to calculate non-synonymous (dN) and synonymous (dS) substitution rates at each codon site of an alignment and was used to test for a difference in selective pressure on GNBP codons between the different termite groups using the Bucek et al. (2019) phylogeny (Kosakovsky Pond and Frost 2005).

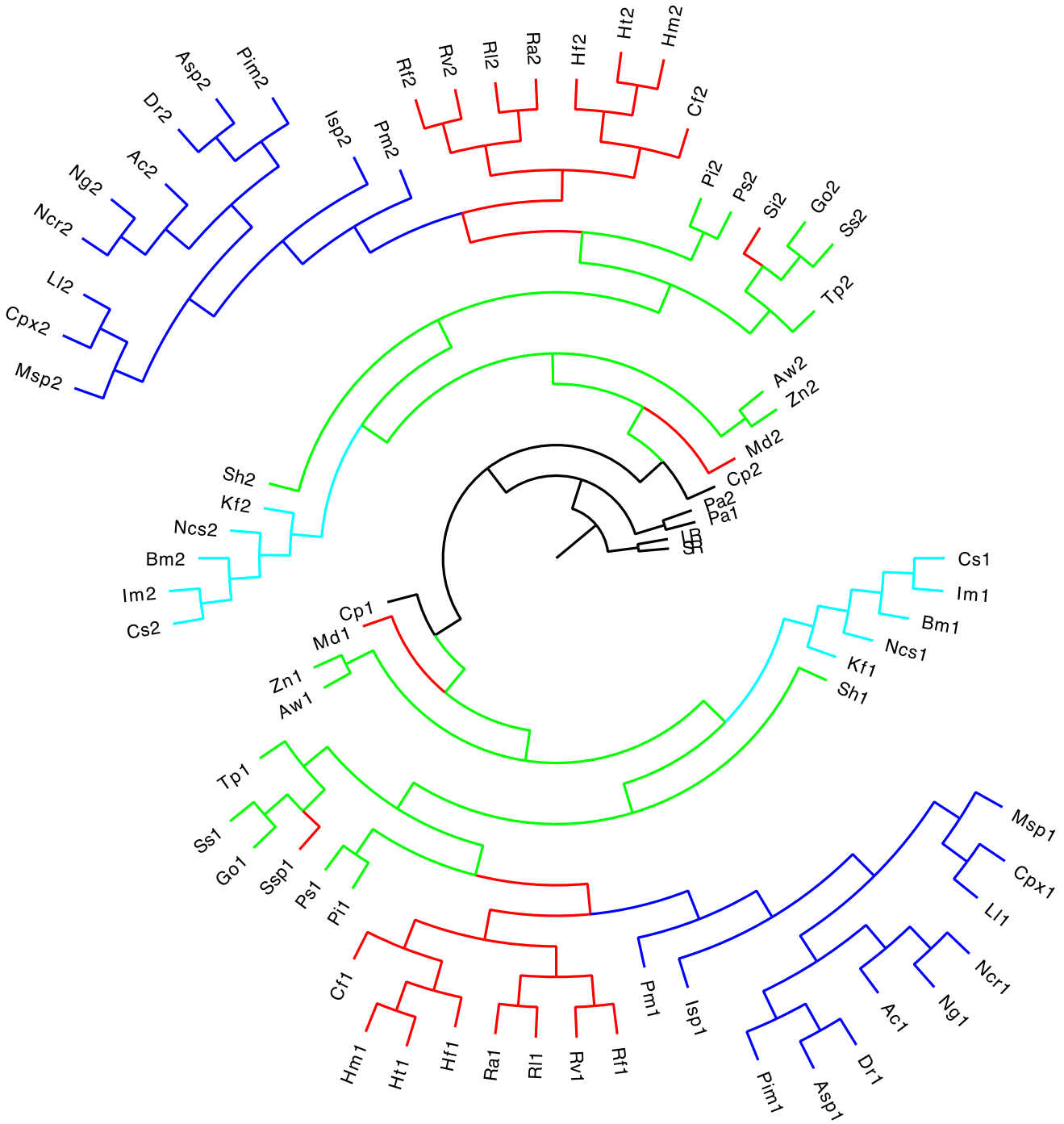

Fig. 1 Phylogenetic tree used for the selection analysis. The tree shows the duplication of GNBP1 and GNBP2 (Cp1 and Cp2) in a direct ancestor of *Cryptocercus* woodroaches (Bulmer et al. 2012). One-piece nest type lineages include dampwood termites depicted in green and drywood termites depicted in cyan; multiple-piece nest type lineages are depicted in red, and central-site nest type lineages are depicted in blue. Species designations and sequence accession numbers are shown in Table 3 and the alignment in supplementary materials. The tree topology is based on Bucek et al. (2019) and is rooted with sequence from *Periplaneta* (Pa1 and Pa2) and *Parcoblatta* cockroaches (SR and LR)

Table 1 Change in selective pressure with evolutionary transitions

Gene	Contrast	K-value	LRT	*p*-value	Selection transition	Role
GNBP1	Dry vs damp	1.85	4.46	0.035	Intensification	External defense
GNBP1	MP vs damp	3.45	8.02	0.005	Intensification	
GNBP1	CS vs MP	0.47	14.76	< 0.001	Relaxation	
GNBP2	Dry vs damp	0.36	8.19	0.004	Relaxation	External defense
GNBP2	MP vs damp	1.49	5.50	0.019	Intensification	
GNBP2	CS vs MP	1.33	0.25	0.620	No change	
cactus	Dry vs damp	0.22	22.09	< 0.001	Relaxation	Toll pathway
cactus	MP vs damp	0.37	617.06	< 0.001	Relaxation	
cactus	CS vs MP	0.85	4.50	0.034	Relaxation	
myd88	Dry vs damp	0.03	2.93	0.87	No change	Toll pathway
myd88	MP vs damp	0.03	4.16	0.041	Relaxation	
myd88	CS vs MP	0.73	1.59	0.207	No change	
pellino	Dry vs damp	0.45	43.27	< 0.001	Relaxation	Toll pathway
pellino	MP vs damp	0.80	4.56	0.033	Relaxation	
pellino	CS vs MP	0.39	44.07	< 0.001	Relaxation	
toll	Dry vs damp	1.11	1.25	0.264	No change	Toll pathway
toll	MP vs damp	0.88	1.52	0.217	No change	
toll	CS vs MP	0.76	7.83	0.005	relaxation	
traf6	Dry vs damp	1.21	1.57	0.211	No change	Toll pathway
traf6	MP vs damp	0.01	3.39	0.066	No change	
traf6	CS vs MP	0.00	11.38	0.001	Relaxation	
PPO	Dry vs damp	0.88	2.87	0.090	No change	PPO cascade
PPO	MP vs damp	0.86	2.96	0.085	No change	
PPO	CS vs MP	3.57	21.28	< 0.001	Intensification	
spn28	Dry vs damp	0.55	10.23	0.001	Relaxation	PPO cascade
spn28	MP vs damp	0.07	11.39	0.001	Relaxation	
spn28	CS vs MP	2.46	2.82	0.093	No change	
PGRP-SC2	Dry vs damp	1.48	1.93	0.165	No change	IMD pathway
PGRP-SC2	MP vs damp	0.72	0.24	0.628	No change	
PGRP-SC2	CS vs MP	2.05	15.72	< 0.001	Intensification	
relish	Dry vs damp	0.47	9.69	0.002	Relaxation	IMD pathway
relish	MP vs damp	0.76	10.57	0.001	Relaxation	
relish	CS vs MP	2.52	18.34	< 0.001	Intensification	

Results

For the transition from dampwood to MP lineages, which occurred independently 3 times (Fig. 1), there was a significant intensification of selective pressure in both *GNBP1* and *GNBP2* (Table 1 and 2). In contrast, there was significant relaxation ($p < 0.05$) of selective pressure in three of the five toll pathway genes (*cactus*, *myd88*, *pellino*), one of the two PPO cascade genes (*spn28*), and one of the two IMD pathway genes (*relish*). There was no significant change in selective pressure on *PPO2*, *toll*, the toll pathway gene *traf6*, and the IMD pathway gene *PGRP-SC2* (phylogenetic trees, species identification, and alignments in supplementary figures and tables).

For the transition from dampwood termites to drywood termites, there was a significant relaxation of selective pressure on *GNBP2* but an intensification of selective pressure for *GNBP1*. There was significant relaxation of selective pressure in two of the five toll pathway genes (*cactus*, *pellino*), one of the PPO cascade genes (*spn28*) and one of the IMD pathway genes (*relish*). There was no significant change in selective pressure on three toll pathway genes (myd88, *toll*, *traf6*), *PPO2*, or *PGRP-SC2*.

For the transition from MP lineages to CS lineages, there was a significant relaxation of selective pressure on *GNBP1* but no significant change for *GNBP2*. The lack of change during the transition indicated that the intensification of selective pressure on GNBP2 in multiple-piece nest lineages was maintained in central-site lineages. There was significant relaxation of selective pressure in four of the five toll pathway genes (*cactus*, *pellino*, *toll*, *traf6*). There was no significant change in selective pressure on one toll pathway

Table 2 Summary of selective pressure changes

Contrast	Relaxation	No change	Intensification
dry vs damp	*cactus***	*myd88*	*GNBP1**
	*GNBP2***	*toll*	
	*pellino**	*traf6*	
	*relish***	*PPO2*	
	*spn28***	*PGRP-SC*	
MP vs damp	*cactus****	*PPO2*	*GNBP1***
	*myd88**	*toll*	*GNBP2**
	*pellino**	*traf6*	
	*relish***	*PGRP-SC*	
	*spn28****		
CS vs MP	*cactus**	*GNBP2*	*relish****
	*GNBP1****	*myd88*	*PPO2****
	*pellino****	*spn28*	*PGRP-SC****
	*toll***		
	*traf6***		

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$***

gene (myd88) and one PPO cascade gene (*spn28*). There was a significant intensification of selective pressure on *relish*, *PPO2*, and *PGRP-SC2*.

There were no significant differences ($p < 0.05$) in selective pressure (dN/dS) on polymorphic GNBP1 β-1,3-D-glucan binding sites between termite groups representing the three evolutionary transitions. Selective pressure on site 162 was significantly different in polymorphic GNBP2 β-1,3-D-glucan binding sites for all three evolutionary transitions. Selective pressure was identified as significantly different at 16/363 sites for the transition from dampwood to drywood, 13/363 for the transition from dampwood to multiple-piece, and 25/363 for the transition from multiple-piece to central-site. Using these frequencies as probabilities for identifying a specific site by chance, the probability of identifying a significant difference in selective pressure at site 162 for all three transitions was < 0.001. Selective pressure on site 129 was significantly different in polymorphic GNBP2 β-1,3-D-glucan binding sites between the transition from multiple-piece to central-site nest type, and the probability of identifying a significant difference in selective pressure at this site was 0.069.

Discussion

The transition to extranidal foraging in termites coincides with a relaxation of selective pressure on key internal components of the Toll signaling pathway and phenoloxidase cascade. In contrast, there was an intensification of selective pressure during this transition on two β-1,3-glucanases that appear to be key components of social mechanisms of external antifungal defense. Conidial spores from pathogens such as *Metarhizium* attach to the cuticle, and after germination, fungal pathogen-associated molecular patterns (PAMPs) activate the phenoloxidase cascade and the production of antifungal peptides through the Toll signaling pathway (Hoffmann 1995). The increased selective pressure on foraging termites from pathogens such as *Metarhizium* could have enhanced social mechanisms of defense that afforded effective front-line external protection. These behavioral mechanisms may rely on β-1,3-glucanases as well as other antifungal compounds and include allogrooming, cannibalism, and burying of infected individuals (undertaking).

Undertaking possibly co-opted foraging traits associated with the construction of subterranean tunnels and more elaborate nest structures. β-1,3-glucanases are incorporated into material used to bury infected individuals where they and other antifungal compounds likely limit sporulation and the spread of infections (Bulmer et al. 2009; Hamilton et al. 2011). In addition to their role as effectors, the enzyme activity of GNBP2 may also release elicitors of the innate immune system after degrading the fungal cell wall (Bulmer et al. 2009; Hamilton et al. 2011). Recent evidence suggests that immune elicitors released by GNBP2 β-1,3-glucanase activity provide important cues for stimulating cannibalism of *Metarhizium*-infected subterranean termites (Esparza-Mora et al. 2020). It remains to be seen whether these elicitors influence other behaviors such as undertaking.

The transition from damp to dry wood also resulted in a relaxation of selective pressure on components of the Toll signaling pathway and phenoloxidase cascade. However, in this transition, the relaxation is likely associated with a shift to a microhabitat that is relatively free of microbes (Rosengaus et al. 2003, 2010). In support, selective pressure was also relaxed in one of the β-1,3-glucanases (GNBP2). The results from the contrast-FEL analysis indicate that modifications in GNBP2 are a direct response to fungal pathogens. The selective pressure on GNBP2 during all three transitions appears to have modified a PAMP binding site. This suggests that adaptive change in termite GNBP2 is a response to novel fungal pathogens with the evolutionary transitions or a counter response to the evolution of resistance in a co-evolving pathogen. In either case, this pathogenic threat was apparently reduced with the transition to a drier microhabitat. Pressure to modify binding sites for PAMPs is striking because PAMPs, in this case β-1,3-glucans, are usually highly conserved molecules that cannot be altered without disrupting their biochemical properties (Girardin et al. 2002; Janeway and Medzhitov 2002; Loker et al. 2004).

In contrast to GNBP2, there was an intensification of selective pressure on GNBP1 with the transition to dry wood. These two proteins duplicated in a woodroach-like ancestor of the termites and subsequent selection appear to have driven changes in the secretion system of GNBP2 (Bulmer et al. 2012). The original enzyme in earlier Blattodea ancestors is likely to have had a digestive function.

Table 3 GNBP species identification

Species ID	Species	Accession no	NCBI database
Ac1	*Agnathotermes crassinasus*	GHYW01163249.1	TSA
Ac2	*Agnathotermes crassinasus*	GHYW01172791.1	TSA
Asp1	*Amitermes sp.*	GHXC01038740.1	TSA
Asp2	*Amitermes sp.*	GHXC01016139.1	TSA
Aw1	*Archotermopsis wroughtoni*	MT127784.1	nr/nt
Aw2	*Archotermopsis wroughtoni*	MT127785.1	nr/nt
Bm1	*Bifiditermes mutubae*	GHZC01054502.1	TSA
Bm2	*Bifiditermes mutubae*	GHZC01019942.1	TSA
Cf2	*Coptotermes formosansus*	JX876645.1	nr/nt
Csp1	*Coptotermes sp.*	GDUG01027636.1	TSA
Cpx1	*Cornitermes pugnax*	GHZG01103844.1	TSA
Cpx2	*Cornitermes pugnax*	GHZG01115046.1	TSA
Cp1	*Cryptocercus punctulatus*	JQ435785.1	nr/nt
Cp2	*Cryptocercus punctulatus*	JQ435786.1	nr/nt
Cs1	*Cryptotermes secundus*	XM_023855946.1	nr/nt
Cs2	*Cryptotermes secundus*	XM_023855989.1	nr/nt
Cusp1	*Cubitermes sp.*	GHZH01134505.1	TSA
Cusp2	*Cubitermes sp.*	GHZH01088236.1	TSA
Dr1	*Drepanotermes rubriceps*	DQ058921.1	nr/nt
Dr2	*Drepanotermes rubriceps*	DQ058934.1	nr/nt
Go1	*Glossotermes oculatus*	GHZQ01099032.1	nr/nt
Go2	*Glossotermes oculatus*	GHZQ01026633.1	nr/nt
Hf1	*Heterotermes ferox*	GHZZ01320974.1	TSA
Hf2	*Heterotermes ferox*	GHZZ01349968.1	TSA
Hm1	*Heterotermes malabaricus*	GHZS01012246.1	TSA
Hm2	*Heterotermes malabaricus*	GHZS01096386.1	TSA
Ht1	*Heterotermes tenuior*	GHZT01084533.1	TSA
Ht2	*Heterotermes tenuior*	GHZT01078798.1	TSA
Im1	*Incisitermes marginipennis*	GDBO01054019.1	TSA
Im2	*Incisitermes marginipennis*	GDBO01066582.1	TSA
Isp1	*Indotermes sp.*	GHZW01113074.1	TSA
Isp2	*Indotermes sp.*	GHZW01159705.1	TSA
Kf1	*Kalotermes flavicollis*	GHWY01163971.1	TSA
Kf2	*Kalotermes flavicollis*	GHWY01002111.1	TSA
Ll1	*Labiotermes labralis*	GIAA01170288.1	TSA
Ll2	*Labiotermes labralis*	GIAA01062626.1	TSA
Md1	*Mastotermes darwiniensis*	DQ058922.1	nr/nt
Md2	*Mastotermes darwiniensis*	DQ058935.1	nr/nt
Msp1	*Microcerotermes sp.*	GHYV01052830.1	TSA
Msp2	*Microcerotermes sp.*	GHYV01123677.1	TSA
Ncr1	*Nasutitermes corniger*	JF683377.1	nr/nt
Ncr2	*Nasutitermes corniger*	JF683378.1	nr/nt
Ng1	*Nasutitermes graveolus*	DQ058914.1	nr/nt
Ng2	*Nasutitermes graveolus*	DQ058927.1	nr/nt
Ncs1	*Neotermes castaneus*	GIAC01169962.1	TSA
Ncs2	*Neotermes castaneus*	GIAC01151916.1	TSA
Pim1	*Palmitermes impostor*	GIAD01194034.1	TSA
Pim2	*Palmitermes impostor*	GIAD01190650.1	TSA
PL	*Parcoblatta* (L)	JQ435783.1	nr/nt
PS	*Parcoblatta* (S)	JQ435784.1	nr/nt
Pi1	*Prorhinotermes inopinatus*	GHYS01001132.1	TSA

Table 3 (continued)

Species ID	Species	Accession no	NCBI database
Pi2	*Prorhinotermes inopinatus*	GHYS01029060.1	TSA
Ps1	*Prorhinotermes simplex*	GASE02024448.1	TSA
Ps2	*Prorhinotermes simplex*	GASE02021019.1	TSA
Pa1	*Periplaneta americana*	JQ435788.1	nr/nt
Pa2	*Periplaneta americana*	JQ435787.1	nr/nt
Pm1	*Pseudacanthotermes militaris*	GHYT01075496.1	TSA
Pm2	*Pseudacanthotermes militaris*	GHYT01034712.1	TSA
Ra1	*Reticulitermes aculabialis*	GHMS01050437.1	TSA
Ra2	*Reticulitermes aculabialis*	GHMS01074716.1	TSA
Rf1	*Reticulitermes flavipes*	JF683373.1	nr/nt
Rf2	*Reticulitermes flavipes*	JF683375.1	nr/nt
Rl1	*Reticulitermes labralis*	GHNP01002285.1	TSA
Rl2	*Reticulitermes labralis*	GHNP01023105.1	TSA
Rv1	*Reticulitermes virginicus*	JF683374.1	nr/nt
Rv2	*Reticulitermes virginicus*	GU906847.1	nr/nt
Si2	*Schedorhinotermes intermedius*	GIAM01532853.1	TSA
Ssp1	*Schedorhinotermes sp.*	GIAN01069171.1	TSA
Sh1	*Stylotermes halumicus*	GIAK01086774.1	TSA
Sh2	*Stylotermes halumicus*	GIAK01085695.1	TSA
Ss1	*Serritermes serrifer*	GIAH01075678.1	TSA
Ss2	*Serritermes serrifer*	GIAH01083085.1	TSA
Tp1	*Termitogeton planus*	GHXB01038592.1	TSA
Tp2	*Termitogeton planus*	GHXB01039633.1	TSA
Zn1	*Zootermopsis nevadensis*	XM_022060860.1	nr/nt
Zn2	*Zootermopsis nevadensis*	XM_022060861.1	nr/nt

With duplication, one of the enzymes (GNBP1) may have retained its original role, and the duplicate (GNBP2) evolved into a novel immune protein (Bulmer et al. 2009). However, this functional division into digestion or immunity is potentially misleading as immune function often overlaps with a digestive function (Broderick 2015; Van Niekerk and Engelbrecht 2015). Although we found no evidence of modification of the GNBP1 β-1,3-glucan binding site in any of the transitions, modifications in GNBP1 appear to reflect adaptations to newly encountered fungi. In the dry wood microhabitat, these may be represented by newly or more frequently encountered fungal pathogens that tolerate a dry microhabitat or possibly newly or more frequently encountered wood-rot fungi that are potential competitors in wood consumption (Martin and Bulmer 2018).

The general productivity, size, and prevalence of termite colonies will have changed with the evolutionary transitions to extranidal foraging or feeding on dry wood. Genetic drift results in an increased fixation rate of mutations when the effective population size is small. When contrasting selective pressure between termite groups, selection constraining change could appear to have been relaxed in one termite group compared to the other because of the effect of demographic change. However, the effect of demographic change on the signatures of selection would be the same for equivalent branches in GNBP1 and GNBP2 and the different innate immune genes. The observed differences in selective pressure do not therefore appear to reflect demographic effects.

Adaptive changes required for increased foraging efficiency promoted the evolution of a separate developmental line leading to true workers (Rupf and Roisin 2008). The potential for direct reproductive opportunities would have been increasingly limited with this transition and compensated by conditions that promoted indirect reproductive benefits gained from altruistic behaviors (Hamilton 1964). Conditions that are likely to have promoted this altruism include an increase in colony size and longevity facilitated by foragers gaining access to new food sources. This would have allowed the expansion of colonies and the production of greater numbers of winged reproductive that initiate new colonies and therefore the potential for indirect benefit gains by individuals foregoing their own reproductive opportunities. These conditions would have promoted altruistic behavior even with their direct fitness costs including the cost associated with cleaning or disposing of infected individuals.

The transition from multiple-pieces nests to central-site nests resulted in a further relaxation of selective pressure on components of the Toll signaling pathway. This likely

reflects the evolution of novel social mechanisms of defense as well as the refinement of these systems. Again, secreted antifungal compounds were likely to contribute to these mechanisms of defense (He et al. 2021; Bulmer and Crozier 2004). There was a diversification of these secondary metabolites such as defensive terpenoids with the diversification of the Termitidae. For example, *Nasutitermes* termites may use terpenoids associated with the frontal gland of their numerous soldiers, and *Reticulitermes* may use mellein derived from hindgut symbionts for defense against microbial pathogens by incorporating them into the building material of their nests and tunnels (Mitaka et al. 2017, 2019).

The transition from multiple-pieces nests to central-site nests resulted in a relaxation of selective pressure in GNBP1 but no change on selective pressure on GNBP2. The lack of change in GNBP2 likely reflects continued selective pressure on this critical antifungal immune protein. The relaxation in GNBP1 suggests that its fungal targets became depauperate with the transition or other mechanisms of immunity or digestion led to its redundancy. This transition coincides with substantial changes in the microbial composition of the symbiotic hindgut community, which contributes to both digestion and immunity (Rosengaus et al. 2014; Xu et al. 2020). A diversification in diet from cellulose acquired from wood (all subterranean termites with multiple-piece nest types) to cellulose from grass and soil as well as arboreal nesting away from soil may have relaxed selective pressure imposed by wood-rot fungi that compete with termites for food. Wood-rots are more likely to be consumed by multiple-piece subterranean termites that consume wood in a greater state of decay, and the β-1,3-glucanase activity of GNBP1 may be important for their digestion and elimination (Martin and Bulmer 2018).

Although the focus in this study was on innate immune proteins that have been shown to be involved in fungal defense, our analysis also included Relish and PGRP-SC2. Relish is a transcription factor in the IMD pathway involved in defense against bacterial pathogens (Dushay et al. 1996). There was a relaxation in selective pressure on this protein with transition to foraging and the transition to dry wood. Social immune mechanisms in true workers may have relaxed selective pressure in the foragers, and a reduced microbial load may have relaxed selective pressure in the drywood termites in parallel with relaxation that occurred in the Toll and phenoloxidase pathways. In contrast, there was an intensification in selective pressure on Relish and PGRP-SC2 with evolution of the Termitidae, which may reflect the substantial changes that occurred in the composition of the termite hindgut microbiome. There was also an intensification of selective pressure on PPO2 with the evolution of the Termitidae. Recent research indicates that all three of these proteins contribute to the regulation of insect gut microbiomes (Guo et al. 2014; Vandehoef et al. 2020; Luo et al. 2020). Selective pressure on Relish also intensified in a *Nasutitermes* (Termitidae) lineage in which there appears to have been substantial change in gut morphology and diet that likely reflects a change in the gut microbiome (Bulmer and Crozier 2006).

Evidence of reduced gene diversity and reduced transcriptional activity in termite immune genes as well as reduced selective pressure on honeybee Toll pathway genes indicates that social mechanisms of defense can relax selective pressure on the innate immune system (Evans et al. 2006; Harpur and Zayed 2013; He et al. 2021). However, a termite genomic study did not detect any evidence of relaxation or intensification of selective pressure across multiple single-copy immune genes (Meusemann et al. 2020). Our analysis apparently captured signatures of selective pressure because it used a larger sample of sequences for each immune gene and focused on key evolutionary transitions to different microbial settings with a robust phylogeny. The coincidence of selective pressure intensification on GNBPs and relaxation on innate components of the antifungal immune system supports the behavioral hypothesis for relaxation on innate immune genes. Our results also indicate that termite transitions into different microbial settings impacted immune gene evolution in other ways. A shift to microbially depauperate habitats appears to have relaxed selective pressure on innate immune genes whereas shifts in diet intensified selection on a subset of immune genes that regulate intestinal microbes. Their regulatory role may have been instrumental in the diversification of termite foraging strategies.

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Data availability The data analyzed during the current study are available in GenBank. Hyperlinks to all data are included in the tables, including supplementary tables.

Declarations

Ethics approval Benefits from this research accrue from the sharing of our sequence data and results on public databases. Termites (*A. wroughtoni*) were not collected from forests with restrictions on removing biological samples nor transported across international borders.

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