1 Genomic and transcriptomic analyses of the 2 subterranean termite *Reticulitermes speratus*: gene 3 duplication facilitates social evolution

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5 Shuji Shigenobu^{*+1,2}, Yoshinobu Hayashi⁺³, Dai Watanabe^{4,5}, Gaku Tokuda⁶,
6 Masaru Y Hojo^{6,19}, Kouhei Toga^{5,7}, Ryota Saiki⁵, Hajime Yaguchi^{5,8}, Yudai
7 Masuoka^{5,9}, Ryutaro Suzuki^{5,10}, Shogo Suzuki⁵, Moe Kimura¹¹, Masatoshi
8 Matsunami^{4,12}, Yasuhiro Sugime⁴, Kohei Oguchi^{4,10,13}, Teruyuki Niimi^{2,14}, Hiroki
9 Gotoh^{15,20}, Masaru K Hojo⁸, Satoshi Miyazaki¹⁶, Atsushi Toyoda¹⁷, Toru Miura^{*4,13},
10 Kiyoto Maekawa^{*18}

11

12 1) NIBB Research Core Facilities, National Institute for Basic Biology, Okazaki,
13 444-8585 Japan

14 2) Department of Basic Biology, School of Life Science, The Graduate University for
15 Advanced Studies, SOKENDAI, Nishigonaka 38, Myodaiji, Okazaki, Aichi 444-
16 8585, Japan

17 3) Department of Biology, Keio University, Hiyoshi, Yokohama, 223-8521, Japan

18 4) Faculty of Environmental Earth Science, Hokkaido University, Sapporo,
19 Hokkaido, 060-0810, Japan

20 5) Graduate School of Science and Engineering, University of Toyama, Toyama,
21 930-8555, Japan

22 6) Tropical Biosphere Research Center, COMB, University of the Ryukyus,
23 Nishihara, Okinawa 903-0213, Japan

24 7) Department of Biosciences, College of Humanities and Sciences, Nihon
25 University, Setagaya-ku, Tokyo, 156-8550, Japan

26 8) Department of Bioscience, School of Science and Technology, Kwansei Gakuin
27 University, Sanda, Hyogo, 669-1337, Japan

28 9) Institute of Agrobiological Sciences, NARO (National Agriculture and Food
29 Research Organization), Tsukuba, Japan

30 10) Bioproduction Research Institute, National Institute of Advanced Industrial
31 Science and Technology (AIST), Tsukuba, Japan

32 11) School of Science, University of Toyama, Toyama, 930-8555, Japan

33 12) Graduate School of Medicine, University of the Ryukyus, Nishihara, Okinawa
34 903-0125, Japan

35 13) Misaki Marine Biological Station, School of Science, The University of Tokyo,
36 Miura, Kanagawa, 238-0225, Japan

- 37 14) Division of Evolutionary Developmental Biology, National Institute for Basic
38 Biology, Okazaki, 444-8585 Japan
- 39 15) Ecological Genetics Laboratory, Department of Genomics and Evolutionary
40 Biology, National Institute of Genetics, Mishima, Shizuoka, 411-8540, Japan
- 41 16) Graduate School of Agriculture, Tamagawa University, Machida, Tokyo 194-
42 8610, Japan
- 43 17) Advanced Genomics Center, National Institute of Genetics, Mishima, Shizuoka,
44 411-8540 Japan
- 45 18) Faculty of Science, Academic Assembly, University of Toyama, Gofuku,
46 Toyama, 930-8555, Japan
- 47 19) Global Education Institute, University of the Ryukyus, Nishihara, Okinawa 903-
48 0213, Japan
- 49 20) Department of Biological Sciences, Faculty of Science, Shizuoka University,
50 Shizuoka, Shizuoka 422-8529, Japan
- 51
- 52
- 53 * corresponding authors. Email: shige@nibb.ac.jp, miu@mmbs.s.u-tokyo.ac.jp,
54 kmaekawa@sci.u-toyama.ac.jp
- 55
- 56 + contributed equally
- 57

58 Summary

59 Termites are model social organisms characterized by a polyphenic caste system.
60 Subterranean termites (Rhinotermitidae) are ecologically and economically
61 important species, including acting as destructive pests. Rhinotermitidae occupies
62 an important evolutionary position within the clade representing an intermediate
63 taxon between the higher (Termitidae) and lower (other families) termites. Here, we
64 report the genome, transcriptome and methylome of the Japanese subterranean
65 termite *Reticulitermes speratus*. The analyses highlight the significance of gene
66 duplication in social evolution in this termite. Gene duplication associated with
67 caste-biased gene expression is prevalent in the *R. speratus* genome. Such
68 duplicated genes encompass diverse categories related to social functions,
69 including lipocalins (chemical communication), cellulases (wood digestion and
70 social interaction), lysozymes (social immunity), geranylgeranyl diphosphate
71 synthase (social defense) and a novel class of termite lineage-specific genes with
72 unknown functions. Paralogous genes were often observed in tandem in the
73 genome, but the expression patterns were highly variable, exhibiting caste biases.
74 Some duplicated genes assayed were expressed in caste-specific organs, such as
75 the accessory glands of the queen ovary and frontal glands in soldier heads. We
76 propose that gene duplication facilitates social evolution through regulatory
77 diversification leading to caste-biased expression and subfunctionalization and/or
78 neofunctionalization that confers caste-specialized functions.
79

80 Significance Statement

81 Termites are model social organisms characterized by a sophisticated caste
82 system, where distinct castes arise from the same genome. Our genomics data of
83 Japanese subterranean termite provides insights into the evolution of the social
84 system, highlighting the significance of gene duplication. Gene duplication
85 associated with caste-biased gene expression is prevalent in the termite genome.
86 Many of the duplicated genes were related to social functions, such as chemical
87 communication, social immunity and defense, and they often expressed in caste-
88 specific organs. We propose that gene duplication facilitates social evolution

89 through regulatory diversification leading to caste-biased expression and functional
90 specialization. In addition, since subterranean termites are ecologically and
91 economically important species including destructive pests in the world, our
92 genomics data serves as a foundation for these studies.

93 Introduction

94 The evolution of eusociality, i.e., animal societies defined by the
 95 reproductive division of labor, cooperative brood care and multiple overlapping
 96 generations, represents one of the major transitions in evolution, having increased
 97 the level of biological complexity (1). Eusocial insects such as bees, wasps, ants
 98 and termites show sophisticated systems based on the division of labor among
 99 castes, which is one of the pinnacles of eusocial evolution (2). Recent advances in
 100 molecular biological technologies and omics studies have revealed many molecular
 101 mechanisms underlying eusociality and have led to the establishment of a new field
 102 of study known as “sociogenomics” (3). The genomes of major eusocial
 103 hymenopteran lineages, i.e., ants, bees and wasps, have been sequenced, and the
 104 differences in gene expression, DNA methylation (4–6)(7) (8–11) and histone
 105 modification (12) (13) among castes have been explored. These sociogenomics
 106 studies in hymenopterans revealed some genetic bases of social evolution,
 107 including the co-option of genetic toolkits of conserved genes, changes in protein-
 108 coding genes, cis-regulatory evolution leading to genetic network reconstruction,
 109 epigenetic modifications and taxonomically restricted genes (TRG) (14, 15).

110
 111 Isoptera (termites) is another representative insect lineage exhibiting highly
 112 sophisticated eusociality and a wide range of social complexities (16). Termites are
 113 hemimetabolous and diploid insects that are phylogenetically distant from
 114 hymenopterans with holometaboly and haplodiploidy. Termite societies are
 115 characterized by reproductives of both sexes, workers and soldiers. In the termite
 116 lineage, eusociality is thought to have evolved once, although the levels of social
 117 complexity features, such as colony size, feeding habitat, symbiosis with
 118 microorganisms and caste developmental pathways, diverged among termite
 119 species. These characteristics are especially different between the two major
 120 termite sublineages, i.e., the early-branching families (called “lower” termites) and
 121 the most apical family Termitidae (“higher” termites) (Fig. 1a). To date, based on
 122 the whole-genome sequences of a few termite species, the commonality and
 123 diversity of genetic repertoires between Isoptera and Hymenoptera or between
 124 termite lineages and their solitary outgroup (i.e., cockroach) have been investigated
 125 (17, 18). Additionally, in *Zootermopsis nevadensis*, clear differences in gene

126 expression levels among castes (17) and in DNA methylation between alates and
127 workers (19) were detected.

128

129 Among the more than 2900 extant species of termites (Isoptera) (20),
130 subterranean termites (Rhinotermitidae), especially two genera, *Reticulitermes* and
131 *Coptotermes*, occupy an important evolutionary position (Fig 1a). Recent
132 phylogenetic studies showed that Rhinotermitidae is paraphyletic, and a clade
133 including *Reticulitermes*, *Coptotermes* and *Heterotermes* was shown to be sister to
134 Termitidae (21, 22). In particular, *Reticulitermes* exhibits intermediate social
135 complexity between those of higher (Termitidae) and lower (all the other families)
136 termites (23), for example, this genus displays primitive feeding ecology and gut
137 symbiont features, a relatively complex colony structure and a caste development
138 mode termed the bifurcated pathway (Fig. 1b). Moreover, *Reticulitermes* is the most
139 common termite group in palearctic (24) and nearctic (25) regions and a major pest
140 causing serious damage to human-made wooden structures (26). For these
141 reasons, members of this genus are probably among the most studied termites
142 (16). Nevertheless, despite their evolutionary, ecological and economic relevance,
143 subterranean termites remain an understudied group in terms of both genetics and
144 genomics.

145

146 In this study, we targeted the Japanese subterranean termite *Reticulitermes*
147 *speratus*. We conducted whole-genome sequencing, caste-specific RNA-seq
148 analysis and whole-genome bisulfite sequencing of *R. speratus* to understand the
149 genomic, transcriptomic and epigenetic bases of the social life of this termite
150 species. *R. speratus* nymphoids are almost exclusively produced
151 parthenogenetically by automixis with terminal fusion in primary queens, such that
152 the genome should be homozygous at most loci (27), which provides an advantage
153 in genome sequencing. We also compared the omics data of *R. speratus* with those
154 of sequenced higher and lower termites (Fig. 1a). Our integrative analyses of the
155 genome and transcriptome of *R. speratus* and other termites revealed that gene
156 duplications are often associated with caste-biased gene expression and caste-
157 specific functions, which highlights the significant role of gene duplication in
158 eusocial evolution in the termite lineage.

159

Results and Discussion

Genomic features of *Reticulitermes speratus*

Genome sequencing of *R. speratus* was performed with genomic DNA isolated from female secondary reproductives (nymphoids) [Fig. 1b]. *R. speratus* nymphoids are almost exclusively produced parthenogenetically by automixis with terminal fusion in primary queens, such that the genome should be homozygous at most loci (27) and thus ease de Bruijn-graph-based genome assembly. We generated a total of 86 Gb of Illumina HiSeq sequence data and assembled them *de novo* into 5817 scaffolds with an N50 of 1.97 Mb and total size of 881 Mb [Table 1], covering 88% of the genome based on the genome size (1.0 Gb) estimated by flow cytometry (28). The assembled *R. speratus* genome has high coverage of coding regions, capturing 99.2% (98.5% complete; 0.7% fragmented) of 1367 Insecta benchmarking universal single-copy orthologs (BUSCOs) (29) [Table 1]. The *R. speratus* genome is rich in repetitive elements, which make up 40.4% of the genome. A total of 15,591 protein-coding genes were predicted by combining the reference-guided assembly of RNA-seq reads (36 libraries derived from different castes, sexes and body parts; see below for details) and homology-based gene prediction followed by manual curation of gene families of interest [Fig. 1c]. Whole-genome bisulfite sequencing revealed extensive gene body methylation of the *R. speratus* genome, amounting to 8.8% of methylated cytosines in the CG context [Supplementary Fig. 1]. The genome-wide DNA methylation landscape was similar to that of a dampwood termite *Z. nevadensis* (12%) (19). These omics data and a genome browser are available at <http://www.termite.nibb.info/retsp/>.

We compared the *R. speratus* gene repertoire with those of 88 other arthropods, including the two termites *Z. nevadensis* and *Macrotermes natalensis* (17, 30). Ortholog analysis showed that 12,032 (82.9%) of the 15,591 genes in *R. speratus* were shared with other arthropods, and 1773 were taxonomically restricted (TRGs) to Isoptera, among which 430 were shared with the other two termites and 1343 were unique to *R. speratus* [Fig. 1c]. Whole-genome comparison with two sequenced termites, *M. natalensis* and *Z. nevadensis*, showed a high degree of synteny conservation [Fig. 1d]. We identified 2799 syntenic blocks (N50: 858.4 kb) shared with *M. natalensis* that covered 95.1% of the *R. speratus* genome

192 where 560.4 Mb of nucleotides was aligned, while 3650 syntenic blocks (N50: 591.1
193 kb) shared with *Z. nevadensis* covered 72.1% of the *R. speratus* genome where
194 116.7 Mb was aligned. Only a few cases of large genomic rearrangements were
195 found between termite genomes, at least, at the contiguity level of the current
196 assemblies, suggesting overall conservation of genome architecture in the lineage
197 of termites over 135 MY (Fig. 1a). Interestingly, despite such high conservation of
198 macrosynteny, interruptions or breaks in local synteny were observed and often
199 associated with tandem gene duplications. For example, when we examined
200 regions containing large tandem gene duplications (> 5-gene tandem duplications),
201 synteny between the *R. speratus* and *M. natalensis* genomes was interrupted in 10
202 of 21 regions (examples shown in Supplementary Fig 3).

204 Transcriptome differentiation among castes

205 Distinct castes arise from the same genome, a phenomenon called caste
206 polyphenism which is a distinctive hallmark in social insects (31, 32). To elucidate
207 caste-biased gene expression in order to understand the mechanism underlying the
208 caste-specific phenotypes, we compared the transcriptomes of three castes
209 (primary reproductives, workers and soldiers) in *R. speratus* [Fig. 1b;
210 Supplementary Table 2]. We sequenced 36 RNA-seq libraries, representing three
211 biological replicates of both sexes and two body parts ("head" and "thorax +
212 abdomen") for each of the three castes.

213 The results clearly showed that termite castes were distinctively
214 differentiated at the gene expression level. The multidimensional scaling (MDS) plot
215 depicted the three castes as clearly distinct transcriptomic clusters for both the
216 head and thorax + abdomen transcriptomes [Fig. 2a]. However, little sexual
217 difference was detected within each caste, although reproductives showed
218 substantial transcriptomic differences in thorax + abdomen samples between
219 queens and kings, probably due to the difference in the reproductive organs [Fig.
220 2a]. Using a generalized linear model (GLM) with caste and sex as explanatory
221 variables, we identified 1579 and 2076 genes differentially expressed among castes
222 in head and thorax + abdomen samples, respectively, with the criteria of a false
223 discovery rate (FDR)-corrected $P < 0.01$, while we identified only 6 and 79 genes

that were differentially expressed between sexes in the head and thorax + abdomen samples, respectively, with the same criteria. We focused on the genes that were differentially expressed among castes (caste-DEGs) and further classified them into three categories of caste-biased genes (i.e., reproductive-, worker-, and soldier-biased genes), with a criterion of >2-fold higher expression relative to that in the other two castes. These caste-biased genes should account for the specialized functions of each caste. Soldier samples exhibited the highest number of caste-specific genes, suggesting the highly specialized functions of the soldier caste. This is consistent with the finding of a previous RNA-seq analysis of the Eastern subterranean termite *Reticulitermes flavipes*, reporting that a majority of DEGs were soldier-specific (33); 73 of the 93 DEGs identified were up- or downregulated specifically in the soldier caste. In addition to these soldier-specific *R. flavipes* genes (e.g., *troponin C* and fatty *acyl-CoA reductase*), the caste-biased genes identified in our transcriptome analysis of *R. speratus* included genes previously reported as caste-biased genes in other termites (34–37), e.g., *vitellogenin* (reproductives), *geranylgeranyl pyrophosphate synthase* (soldiers), and *beta-glucosidase* (probably associated with cellulase; workers). This consistency between transcriptome analyses of different termite species indicates that the RNA-seq analysis in this study is reliable and that the regulation and perhaps the functions of these caste-biased genes are conserved across the termite lineage.

The caste-DEGs were enriched for Gene Ontology terms related to a wide array of functions [Supplementary Table 4], such as hormone metabolism, chitin metabolism, hydrolase activity, oxidoreductase activity, lipid metabolism, signaling and lysozyme activity. Protein motifs enriched in the caste-DEGs were also identified, including cytochrome P450, lipocalin, lysozyme, glycosyl hydrolase family, TGF-beta and trypsin [Supplementary Table 5]. Among the 1773 taxonomically restricted genes (TRGs) that were restricted to Isoptera (see above), the termite-shared TRGs showed strong enrichment for caste-DEG (Fisher's exact test, $P < 1.0e-7$ for head samples, $P < 1.0e-10$ for thorax+abdomen samples), while the TRGs found only in *R. speratus* (orphan genes) did not ($P = 0.99$ and $P = 0.97$, respectively).

To investigate the relationship between caste-biased gene expression and DNA methylation, we analyzed differential methylation levels among three castes (reproductives, workers, and soldiers). The BS-seq data showed that the global CpG methylation patterns were very similar among the castes [Supplementary Fig. 2ab], in contrast to the methylation pattern of *Z. nevadensis*, in which DNA methylation differed strongly between castes (winged adults vs. final-instar larvae) and was strongly linked to caste-specific splicing (19). Instead, gene body DNA methylation of *R. speratus* seems to be important for the expression of housekeeping genes, as reported in the drywood termite *Cryptotermes secundus* (18). Housekeeping genes exhibited a high degree of gene body methylation in all castes of *R. speratus*, while caste-biased genes showed a significantly lower level of DNA methylation [Supplementary Fig. 2cd].

Gene duplication and caste-biased gene expression

Evolutionary novelties are often brought about by gene duplications (38) (reviewed in (39)), and the transition to eusociality in Hymenoptera has been associated with gene family expansion (18, 40, 41). Our ortholog analysis comparing the *R. speratus* gene repertoire with those of 88 other arthropods identified 1396 multigene families duplicated in the *R. speratus* genome. Interestingly, compared to the genome as a whole, the set of caste-DEGs identified above was significantly enriched for genes in multigene families (X-squared = 218.62, df = 1, p-value < 2.2e-16). We also calculated the tau score as a proxy of caste specificity of gene expression for all genes and found that duplicated genes were significantly more caste-specific than single-copy genes (p < 2.2e-16, Wilcoxon rank sum test) in both transcriptome data sets (head and thorax+abdomen) [Fig. 2b]. Additionally, gene set tests showed that sets of duplicated genes were differentially expressed in all pairwise comparisons between castes [Fig. 2c]. These data highlight the important roles of gene duplication in the caste evolution of termites.

285 Multigene families related to caste-specific traits in *R.* 286 *speratus*

287 Caste-biased multigene families were associated with diverse functional categories,
288 some of which were strongly related to caste-specific behaviors and tasks. Here,
289 we highlight five families, namely, lipocalins (protein transporters for social
290 communication and physiological signaling), cellulases (carbohydrate-active
291 enzymes for worker wood digestion), lysozymes (immune-related genes for social
292 immunity), geranylgeranyl diphosphate synthases (metabolic enzymes for the
293 production of soldier defensive chemicals), and a novel termite-specific gene family
294 with unknown functions, as examples of multigene families relevant to termite
295 sociality. Molecular evolution studies have shown that the redundancy caused by
296 gene duplication may allow one paralog to acquire a new function
297 (neofunctionalization) or divide the ancestral function among paralogs
298 (subfunctionalization) (38, 39). We are particularly interested in the evolutionary
299 impact of gene duplication on caste specialization through neo/subfunctionalization.

300 Lipocalins

301 Lipocalins belong to a family of proteins, with molecular recognition properties such
302 as the ability to bind a range of small hydrophobic molecules (e.g. pheromones)
303 and specific cell surface receptors, and to form complexes with soluble
304 macromolecules (42). A previous study identified a gene of the lipocalin family,
305 SOL1, that is exclusively expressed in the mandibular glands of mature soldiers of
306 the rotten-wood termite *Hodotermopsis sjostedti* (43). SOL1 is thought to function
307 as a signaling molecule for defensive social interactions among termite colony
308 members (31). Moreover, RNA-seq analysis showed that a lipocalin gene, *Neural*
309 *Lazarillo homolog 1* (*ZnNLaz1*), was specifically expressed in soldier-destined larvae
310 in an incipient colony of *Z. nevadensis* (44). Gene function and protein localization
311 analyses suggested that ZnNLaz1 was a crucial regulator of soldier differentiation
312 through the regulation of trophallactic interactions with a queen. Thus, it was of
313 interest that the lipocalin-related motif (Pfam PF00061; lipocalin/cytosolic fatty-acid
314 binding protein family) was significantly enriched in the list of caste-DEGs (FDR <
315 0.05; Supplementary Table 5).

316 We identified 18 lipocalin family genes in the *R. speratus* genome [Fig. 3a-c;
317 Supplementary Table 7]. The number of lipocalin genes was larger than those in
318 other insects [Fig. 3c]. Phylogenetic analysis of lipocalin family genes identified in
319 arthropods, including three termite species, revealed a highly dynamic evolutionary
320 history of this protein family [Fig. 3a]. A couple of subfamilies, namely clades A and
321 B, had experienced extensive expansion in the termite lineage. The most drastic
322 expansion was found in clade A, which includes *H. sjostedti* SOL1. In this clade, 7,
323 9 and 5 genes were identified in *R. speratus*, *M. natalensis* and *Z. nevadensis*,
324 respectively, and extensive and independent gene expansions occurred in each
325 species. Clade B was also composed of genes with a termite lineage specific
326 expansion. In many cases, these lipocalin genes were found in tandem arrays in
327 the *R. speratus* genome [Fig. 3b]. The inferred phylogenetic tree indicated that
328 duplications in each clade occurred after the divergence of termites from a common
329 ancestor.

330 A comparison of the transcriptome among castes revealed that most
331 lipocalin genes (15 of 18) showed caste-biased gene expression [Fig. 2, Fig. 3ab].
332 The caste specificity, however, varied among genes, regardless of sequence
333 similarity and positional proximity on the genome. In particular, the expression
334 levels of genes in clades A and B drastically changed among castes. For example,
335 *RS008823* and *RS008824* displayed soldier-specific expression, the expression of
336 *RS013912* was biased toward workers, and *RS013913* was downregulated in
337 soldiers. *RS008881* and *RS008884* were exclusively expressed in queen bodies
338 (thorax + abdomen), while *RS008882*, a gene next to the aforementioned two
339 genes, showed quite different expression patterns and high expression levels in
340 heads, especially those of workers. These results indicate that termite lipocalin
341 genes underwent dynamic expansion in terms of gene repertoire, and regulatory
342 diversification of caste-biased expression. This gene expansion and regulatory
343 diversification of lipocalins may facilitate the evolution of the molecules involved in
344 signaling during caste development and among individuals through social
345 interactions.

346 To address the caste-specific function of the lipocalin paralogs, the
347 expression patterns of several selected caste-biased lipocalin genes were
348 examined by *in situ* hybridization [Fig. 3d-h, Supplementary Fig. 6]. *RS008881*, a
349 queen-biased lipocalin gene, was found to be expressed exclusively in the
350 accessory glands of the ovary [Fig. 3d]. The next gene on the same scaffold,

351 *RS008882*, was shown to be specifically expressed in worker antennae and
 352 maxillary/labial palps [Fig. 3f-h]. *RS008823*, a soldier-biased gene, was expressed
 353 exclusively in the frontal gland cells of the soldier heads [Fig. 3e]. Note that ovaries
 354 and frontal glands develop during postembryogenesis in a caste-specific manner
 355 (i.e., ovaries in queens and frontal glands in soldiers) in the pathway of caste
 356 differentiation in *R. speratus*. Antennae and maxillary/labial palps are not caste-
 357 specific but crucial sensory organs, especially for blind termite immatures, such as
 358 workers. Given that animal lipocalins generally work as carrier proteins (45), there
 359 is a possibility that focal termite lipocalins bind and convey some molecules to the
 360 targets from caste-specific organs (e.g., egg-recognition pheromone and soldier
 361 defensive and/or inhibiting substances; (46–48)), or participate in sensory
 362 reception, such as the role of odorant-binding proteins (49).

363 Cellulases

364 Lignocellulose degradation in termites is achieved by a diverse array of
 365 carbohydrate-active enzymes (CAZymes) produced by the host and their intestinal
 366 symbionts. The repertoire of CAZyme families in the genome of *R. speratus* did not
 367 show considerable differences from those of other nonxylophagous insects, such as
 368 a honeybee and a fruit fly (Supplementary Fig. 4). However, we found gene family
 369 expansion and expressional diversification for glycoside hydrolase family (GH) 1
 370 and GH9 members. The majority of GH1 and GH9 members are β -glucosidase
 371 (BGs; EC 3.2.1.21) and endo- β -1,4-glucanases (EGs; EC 3.2.1.4), respectively,
 372 which are essential for cellulose digestion in termites (50).

373 We identified 16 GH1 paralogs [Supplementary Table 8]. Such gene expansion
 374 of GH1 was also observed in the genome of other termites, but the reason for the
 375 gene expansion remains elusive (51). Although the phylogenetic tree divided these
 376 GH1 paralogs into four distinct groups (clades A to D in Fig. 4a), most of them were
 377 tandemly located in the genome of *R. speratus* (Fig. 4bc). The predominantly
 378 expressed BG gene was *RS004136*, while the expression of this gene was clearly
 379 biased toward the bodies (thorax + abdomen) of reproductives and workers (Fig.
 380 4b). This gene formed a rigid clade with *bona fide* BGs reported from the salivary
 381 glands or midgut of termites (clade A in Fig. 4a) (52), suggesting that this gene is
 382 involved in cellulose digestion in *R. speratus*. Indeed, *in situ* hybridization analysis
 383 showed that *RS004136* was specifically expressed in the salivary glands of workers

(Fig. 4de, Supplementary Fig. 7a). Other GH1 members showed a wide variety of expression patterns across castes and body parts [Fig. 4b]. Some of them might have diversified their functions, other than wood digestion, related to termite sociality, such as egg-recognition pheromones (53). A typical example displaying such diversification was *RS004624*, which was expressed specifically in the abdomens of queens [Fig. 4b]. The peptide sequence of this gene showed a monophyletic relationship with that of Neofem2 of *Cryptotermes secundus* (clade D in Fig. 4a), which is a queen recognition pheromone probably functioning in the suppression of reproductive emergence (54). *In situ* hybridization showed that *RS004624* was specifically expressed in the accessory glands of queen ovaries (Fig. 4fg, Supplementary Fig. 7b), suggesting that *RS004624* is involved in enzymatic activities in queen-specific glands. Together with the results for a queen-biased lipocalin (*RS008884*), this finding indicates that the queen accessory glands may produce some queen-specific pheromones. Like lipocalins, GH1 paralogs are also typical examples of multigene family members participating in caste-specific tasks, which may be acquired by gene duplication resulting in neo- or subfunctionalization.

We found four paralogs of GH9 in *R. speratus* [Supplementary Fig. 5, Supplementary Table 8]. Although several insect GH9 EGs have acquired the ability to hydrolyze hemicellulose (55), neo- or subfunctionalization of termite EGs has yet to be clarified. Intriguingly, we found that the GH9 member *RS006396* was weakly but uniformly expressed across all termite body parts and castes. This result suggests that some GH9 members also perform a function other than that of cellulase, as is the case for GH1.

Lysozymes

The immune system of termites is of particular interest, because the group living of termites with nonsclerotized and nonpigmented epidermis and microbe-rich habitat puts them at high risk for pathogenic infections (56). Thus, defense against pathogenic microbes is important for termites. In the *R. speratus* genome we identified 251 immune-related genes [Supplementary Information 1.9, Supplementary Table 26]. The repertoire and number of immune-related genes of *R. speratus* showed no large differences compared to those of other insect species, but a notable exception was found for lysozymes [Supplementary Fig. 8].

Lysozymes are involved in bacteriolysis through hydrolysis of β -1,4-linkages in the peptidoglycans present in bacterial cell walls, and three distinct types of lysozymes, chicken- or conventional-type (c-type), goose-type (g-type), and invertebrate-type (i-type) lysozymes, have been found in animals (57). We identified 13 and 3 genes encoding c-type and i-type lysozymes, respectively, and the number of lysozyme genes was larger than those in other insects [Fig. 5, Supplementary Table 9]. Phylogenetic analysis revealed that c-type lysozymes underwent extensive gene duplications in the sublineage leading to *R. speratus* [Fig. 5a]. Seven c-type lysozymes formed a tandem array on scaffold_859 [Fig. 5b], probably generated by repeated tandem gene duplication events. Interestingly, most of the c-type lysozyme genes showed caste-biased expression. Three genes (*RS014698*, *RS100022*, and *RS100023*) exhibited high expression levels compared to those of other lysozyme genes and were expressed in a soldier-specific manner, while *RS100026* was expressed in a worker-specific manner and *RS100024* and *RS100025* were highly expressed in both workers and soldiers [Fig. 5b]. The differential expression patterns of the lysozyme genes in *R. speratus* may represent division of labor among castes in terms of colony-level immunity.

It is also possible that duplicated lysozymes may have functions other than immunity. A previous study indicated that the salivary glands of *R. speratus* secrete c-type lysozymes to digest bacteria ingested by termites through social feeding behavior (58). The same lysozyme genes are also expressed in the queen ovaries and eggs and play a role in egg recognition as proteinaceous pheromones in *R. speratus* (48, 53). We could not find identical sequences of these lysozyme genes in our gene models, but these sequences were most closely related to *RS002400* with 88% nucleotide identity, which occupied the basal position of the lineage-specific gene expansion (Fig. 5a).

GGPP synthase

Whole-genome comparison of *R. speratus* with *Z. nevadensis* and *M. natalensis* revealed a 270 kb *R. speratus*-specific fragment in scaffold_31, while the rest of this scaffold showed very high syntenic conservation among the three termites [Fig 6a]. We found that the *R. speratus*-specific region was encompassed by a tandemly duplicated gene cluster composed of 13 genes encoding geranylgeranyl diphosphate (GGPP) synthase [Fig. 6b, Supplementary Table 10]. GGPP synthase

450 catalyzes the consecutive condensation of an allylic diphosphate with three
451 molecules of isopentenyl diphosphate to produce GGPP, an essential precursor for
452 the biosynthesis of diterpenes, carotenoids and retinoids (59–61). The extensive
453 duplication of GGPP synthase paralogs observed in *R. speratus* is unusual
454 because the genomes of other insects surveyed have only a single copy of GGPP
455 synthase gene. The phylogenetic analysis of GGPP synthase homologs revealed
456 two clusters, a possibly ancestral group (including *RS007484*) and an apical group
457 (including other paralogs identified) [Fig. 6c]. The latter cluster also contained some
458 GGPP synthase paralogs obtained from the termitid *Nasutitermes takasagoensis*
459 (34)

460 Transcriptome data indicated that all of the GGPP synthase genes, except
461 *RS007484* which was a member of the ancestral group in the phylogenetic tree,
462 showed caste-biased expression, and caste specificity varied across the paralogs
463 [Fig 6b]. Specifically, *RS100010*, *RS007480*, *RS100012*, *RS100015*, *RS100016*,
464 *RS100017* and *RS007483* showed soldier-specific expression, while *RS007481*,
465 *RS007482* and *RS100013* showed reproductive-specific expression [Fig 6b].
466 Several GGPP synthase genes have been identified in some termite species and
467 are known to function in a caste-specific manner; for example, the soldiers of *N.*
468 *takasagoensis* synthesize defensive polycyclic diterpenes by high expression of the
469 GGPP synthase gene in the frontal gland to use chemical defense (62). It has been
470 reported that the soldiers of *Reticulitermes* have a frontal gland in which diterpenes
471 are synthesized, although the biological role is not fully understood (63–65).
472 Consequently, it is possible that the soldier-specific GGPP synthases identified to
473 date are involved in chemical defense. Indeed, *in situ* hybridization revealed that
474 the soldier-specific GGPP synthase *RS100016* was expressed exclusively in the
475 soldier frontal gland, as shown in a previous study (66) [Fig. 6d, Supplementary Fig.
476 7c]. It is also possible that reproductive-specific GGPP synthases are involved in
477 the metabolism of other diterpenes, such as pheromone synthesis, especially
478 *RS007481*, which shows strong queen-specific expression in the thorax and
479 abdomen and may play a role in the synthesis of queen substances.

480 Under the branch-site (BS) model of codon substitutions (67), significant
481 positive selection was detected on five branches of *R. speratus* GGPP synthase
482 family tree [Fig. 6e]: ancestral branches #1 and #2, and the branches leading to
483 *RS100017* (branch #3), *RS100012* (branch #4) and *RS007483* (branch #5). These
484 results suggest that all GGPP synthase paralogs of *R. speratus* except the

485 ancestral type *RS007484* have experienced positive selection and finally acquired
 486 novel roles for the production of defensive and/or pheromonal substances.
 487

488 The TY family, a novel gene family restricted to termites

489 Numerous studies have shown that novel genes (e.g., TRG) play important roles in
 490 the evolution of novel social phenotypes in hymenopteran social insects (8, 68, 69).
 491 We found that termite-shared TRGs showed strong enrichment for caste-DEGs
 492 (see above). A striking example of caste-biased TRGs is a tandem array of three
 493 novel genes [Fig. 7a; Supplementary Table 11], *RS001196*, *RS001197* and
 494 *RS001198*, that have no significant homologs in any organisms outside termite
 495 clades. These three genes were expressed at extremely high levels (up to 250,000
 496 RPKM), which constituted approximately 30% of the worker head transcriptome,
 497 and strongly biased across the three castes [Fig. 7a]. Each gene was composed of
 498 a single exon encoding a short peptide ~60 aa in length that contained a secretion
 499 signal peptide in the N-terminal region followed by a middle part rich in charged
 500 amino acid residues and C-terminal part rich in polar amino acids with unusually
 501 high number of tyrosine residues [Fig. 7b]. Here, we named this novel class of
 502 peptides the termite-specific tyrosine-rich peptide family (TY family). The three TY
 503 genes showed modest sequence similarity with each other, suggesting that they are
 504 paralogs derived by tandem duplication. TY family orthologs were also found in the
 505 genomes of *Z. nevadensis* and *M. natalensis* [Fig. 7b]. We estimated pairwise
 506 evolutionary rates (the ratio of nonsynonymous to synonymous substitutions, i.e.,
 507 dN/dS) between *R. speratus* and *Z. nevadensis* for these three peptides. The
 508 dN/dS for each gene ranged from 0.03 to 0.16 (Fig. 7c), indicating that they evolved
 509 under strong purifying selection and suggesting a conserved function in the termite
 510 lineage. Indeed, *Z. nevadensis* orthologs were also expressed at a high level in the
 511 soldier and worker castes in a pattern similar to that in *R. speratus*.

512 Facilitation of caste specification by gene duplication

513 Recent advances in sociogenomics in different social insects are promoting our
 514 understanding of the genetic bases of social evolution, which include the co-option

515 of genetic toolkits of conserved genes, changes in protein-coding genes, cis-
 516 regulatory evolution leading to genetic network reconstruction, epigenetic
 517 modifications and TRGs (15, 70). In addition to these components, our genomic
 518 and transcriptomic analyses in *R. speratus* highlighted the significance of gene
 519 duplication for caste specialization. Gene duplication is, in general, a key source of
 520 genetic innovation that plays a role in the evolution of phenotypic complexity; gene
 521 duplication allows for subsequent divergent evolution of the resultant gene copies,
 522 enabling evolutionary innovations in protein functions and/or expression patterns
 523 (71–73). Regarding eusocial evolution in insects, Gadagkar (74) first pointed out the
 524 importance of gene duplication; 'genetic release followed by diversifying evolution'
 525 made possible the appearance of multiple caste phenotypes in social insects. Many
 526 decades later, genomic analyses revealed gene family expansion, especially in
 527 relation to chemical communication, in both ants (odorant receptors; (75–77)) and
 528 termites (ionotropic receptors; (18)). Based on Godagkar's hypothesis, duplicated
 529 genes can be released from the constraints of original selection, leading to new
 530 directional evolution, i.e., for caste-specific functions (e.g., queen- or worker-trait
 531 genes). However, the detailed roles and significance of gene duplication in social
 532 evolution have been elusive.

533 This study revealed that gene duplication associated with caste-biased gene
 534 expression is prevalent in the *R. speratus* genome. The list of duplicated genes
 535 encompasses a wide array of functional categories related to the social behaviors in
 536 termites as exemplified by transporters such as lipocalins (communication and
 537 physiological signaling; cf. (31, 44)), digestive enzymes such as carbohydrate-
 538 active enzymes, immune-related genes such as lysozymes (social immunity), and
 539 metabolic enzymes such as GGPP synthase (social defense). This study
 540 demonstrated that caste-specific expression patterns differed among in-paralogs.
 541 Although such paralogous genes were often observed in tandem in the genome,
 542 the expression patterns were often independent from one another, showing
 543 differential caste biases in many cases. Additionally, discordant caste biases in
 544 transcriptional expression were observed among closely related paralogs with
 545 similar coding sequences, as represented by little correlation between phylogenetic
 546 position and caste specificity (Fig. 3a). Although the regulatory and evolutionary
 547 mechanisms underlying caste-biased expression patterns are elusive, these
 548 examples strongly suggest that gene duplications have facilitated caste
 549 specialization, leading to social evolution in termites.

550 After the gain of caste-biased gene regulation, subfunctionalization and/or
 551 neofunctionalization seems to have occurred, leading to caste-specific expression
 552 and caste-specialized functions. For example, in the case of lipocalin family,
 553 lipocalin paralogs were generated by lineage-specific functional expansion in caste-
 554 specific organs or tissues: a queen-specific lipocalin (*RS008881*) was expressed
 555 specifically in the ovarian accessory glands, while a soldier-biased lipocalin
 556 (*RS008823*), was expressed exclusively in the frontal glands in soldier heads [Fig.
 557 3de]. Taken together, we hypothesize that, in termites, caste specification through
 558 gene duplication proceeds by the following three steps: 1) gene family expansion by
 559 tandem gene duplication, 2) regulatory diversification leading to an expression
 560 pattern restricted to a certain caste, and 3) subfunctionalization and/or
 561 neofunctionalization of the gene products conferring caste-specific functions. As an
 562 exaptation of these steps, the case in which one (or some) of the multiple functions
 563 of pleiotropic genes are allocated and specialized to a duplicated gene copy might
 564 have led to caste-specific subfunctionalization (38, 39).

565 Recently, it was suggested that the evolution of phenotypic differences
 566 among castes in the honey bee was associated with the gene duplication, by
 567 showing that duplicated genes had higher levels of caste-biased expression
 568 compared to singleton genes (78). It was also shown that the level of gene
 569 duplication was correlated with social complexity in bees (superfamily Apoidea)
 570 (78). Given the independent origin of eusociality in termites and honeybees, gene
 571 duplications might be a shared mechanism facilitating the evolution of caste
 572 systems in social insects.

573

574

575 Materials and Methods

576 Insects

577 All mature colonies of *Reticulitermes speratus* used for genome, RNA, and Bisulfite
 578 sequencing (BS-seq), were collected in Furudo, Toyama Prefecture, Japan
 579 [Supplementary Table 1]. Detailed sample information is described in *SI Appendix*,
 580 *Supplementary Methodology*.

581 Sample collection, genome sequencing and assembly

582 All colonies of *Reticulitermes speratus* used for genome, RNA, and bisulfite
583 sequencing were collected at Furudo, Toyama Prefecture, Japan. Detailed sample
584 information is described in Supplementary Table 1 and *SI Appendix*,
585 *Supplementary Methodology*.

586

587 We used female secondary reproductives (nymphoids I and II) for genome
588 sequencing. We excluded the gut and ovaries of nymphoids to avoid contamination
589 by DNAs from the king or other microorganisms. Genomic DNA was isolated from
590 each individual using a Genomic-tip 20/G (Qiagen). We used 5 microsatellite loci
591 (Rf6-1, Rf21-1, Rf24-2, Rs02, and Rs03) to confirm whether they were homozygous
592 at these loci and shared the same genotype. The purified genomic DNA purified
593 was fragmented with a Covaris S2 sonicator (Covaris), size-selected with
594 BluePippin (Sage Science), and then used to create two pair-end libraries using a
595 TruSeq DNA Sample Preparation Kit (Illumina) with insert sizes of ~250 and ~800
596 bp [Supplementary Table 3]. Four Mate-pair libraries with peaks at ~3 kb, ~5 kb, ~8
597 kb and ~10 kb, respectively, were also created using a Nextera Mate Pair Sample
598 Preparation Kit (Illumina) [Supplementary Table 3]. These libraries were sequenced
599 using an Illumina HiSeq system with 2 × 151 bp paired-end sequencing protocol.
600 Reads of the pair-end and mate-pair libraries were assembled using ALLPATHS-
601 LG (build# 47878), with default parameters. BUSCO
602 v4.0.6(29)(<https://busco.ezlab.org/>) was used in quantitative measuring for the
603 assessment of genome assembly using insecta_odb10 as the lineage input. A
604 genome browser was built using JBrowse (<https://jbrowse.org/>)

605 Gene prediction

606 A protein-coding gene reference set was generated with two main sources of
607 evidence, aligned *R. speratus* transcripts and aligned homologous proteins of other
608 insects, and a set of *ab initio* gene predictions. RNA-seq reads were assembled *de*
609 *novo* using Trinity, and then mapped to the genome using Exonerate. We
610 processed homology evidence at the protein level using the reference proteomes of
611 7 sequenced insects including *Z. nevadensis* and Blattodea protein sequences
612 predicted from RNA-seq of *Periplaneta americana* and *Nasutitermes*
613 *takasagoensis*. These proteins were split-mapped to the *R. speratus* genome with

Exonerate. These models were merged using the EvidenceModeler (EVM), which yielded 15584 gene models. Seventy-four genes were manually inspected and corrected. In particular, tandemly duplicated genes were liable to be incorrect gene prediction with erroneous exon–exon connections across homologs. The final set of 15591 genes was designated as Rspe OGS1.0 [Supplementary Data 2 (DOI:10.6084/m9.figshare.14267381)]. The quality of the OGS1.0 was evaluated by assessing two types of evidence, homology and expression. Among 15591 genes, 12996 (83.3%) showed any hits in the NCBI nr database, 10440 (70.0%) included known protein motifs defined in the Pfam database, and 14302 (91.7%) showed evidence of expression with a threshold of RPKM = 1.0 in any sample of caste-specific RNA-seq data. In sum, 15577 (99.9%) had evidence for the presence of homologs and/or expression.

Orthology inference and gene duplication analysis

Orthology determination among three termites: Orthologous genes among the proteomes of three termite species, *R. speratus*, *Z. nevadensis*, and *M. natalensis* (gene models RspeOGS1.0, ZnevOGSv2.229, and MnatOGS3, respectively), were determined by pairwise comparisons with InParanoid v4.1 followed by three-species comparison with MultiParanoid. *M. natalensis* gene set, MnatOGS3, was built in this study using a similar pipeline as used for *R. speratus*. Ortholog analysis with arthropod proteomes: Orthology relationships of *R. speratus* genes (OGS1.0) with other arthropod genes were analyzed by referring to the OrthoDB gene orthology database ver.8 (87 arthropod species) (<https://www.orthodb.org/>). We grouped *R. speratus* genes with the OrthoDB ortholog group using a two-step clustering procedure. For each *R. speratus* protein, BLASTP was used to find similar proteins among the arthropod proteins, and the ortholog group of the top hit was provisionally assigned to the query *R. speratus* gene. Then, the ortholog grouping was evaluated by comparing the similarity level (BLAST bit score) among members within the focal ortholog group. We keep the grouping if the BLAST bit score between the query *R. speratus* gene and top arthropod gene was higher than the minimal score within the original cluster members. Among 15591 *R. speratus* OGS1.0 genes, 12434 were clustered into 9033 OrthoDB Arthropod ortholog groups. Gene duplication was assessed based on this clustering. If two or more members of one species were included in a single ortholog group, they were regarded as a multigene family.

648 RNA-seq

649 W4–5 workers (old workers) and soldiers were collected from each colony. To
 650 collect primary reproductives, dealated adults were chosen randomly from each
 651 colony in accordance with the method of the previous literature (79), and female–
 652 male pairs were mated (Supplementary Table 1). Kings and queens were sampled
 653 after 4 months. Each individual was divided into head and body parts (thorax +
 654 abdomen). We prepared RNA-seq libraries for 12 categories based on castes
 655 (reproductives, workers and soldiers), sexes (males and females) and body parts
 656 (head, and thorax + abdomen). Three biological replications of the 12 categories
 657 were made with three different field colonies totaling 36 RNA-seq libraries
 658 [Supplementary Table 2]. All Illumina libraries prepared using a TruSeq Stranded
 659 mRNA Library Prep kit were subjected to a single-end sequencing of 101 bp
 660 fragments on HiSeq 2500. The cleaned reads were mapped onto the genome with
 661 TopHat v2.1.0 guided by the OGS1.0 gene models. Transcript abundances were
 662 estimated using featureCounts and normalized with the trimmed mean of M-values
 663 (TMM) algorithm in edgeR. Differentially expressed genes among castes and
 664 between sexes were detected in each body part (head / thorax and abdomen) using
 665 a generalized linear model with two factors, namely, caste and sex using edgeR
 666 with the conditions set as false discovery rate (FDR) < 0.01 and the log2 fold
 667 change of the expression level > 1.

668 Data Availability

669 Data from whole-genome sequencing, transcriptome sequencing, and methylome
 670 sequencing have been deposited in the DDBJ database under BioProject
 671 accessions PRJDB2984, PRJDB5589 and PRJDB11323, respectively. The
 672 analyzed data including genome assembly, gene prediction, annotation, and gene
 673 expression are available through FigShare
 674 (<https://doi.org/10.6084/m9.figshare.c.5483235>). The *R. speratus* genome browser
 675 is available at <http://www.termite.nibb.info/retsp/>.

676 Code availability

677 Custom R and Ruby scripts were deposited into Github
 678 (https://github.com/termiteg/retsp_genome_paper).

679

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690

691

692 **Author contributions**

693 S.S., Y.H., T.M., and K.M. designed and managed the project. D.W., K.T., R.S.,
694 H.Y., Y.M., R.S., and K.M. collected samples. D.W., R.S., Y.M., and R.S. performed
695 the DNA extraction. S.S., and A.T. performed the library construction and genome
696 sequencing. Y.H., D.W., K.T., R.S., H.Y., and Y.M. generated the RNA-Seq data.
697 S.S., Y.H., and R.S. generated the BS-Seq data. S.S., Y.H., D.W., G.T., M.Y.H.,
698 K.T., M.M., Y.S., K.O., T.N., H.G., M.K.H., and S.M. contributed to the genome
699 assembly and annotation. S.Su, and M.K. performed histological analyses. S.S.,
700 Y.H., G.T., T.M., and K.M. drafted the manuscript. All authors contributed to the
701 final version of the manuscript.

702

703 Tables

704 **Table 1. Summary of *Reticulitermes speratus* genome assembly, annotation**
705 **and methylome**

Genome	No. scaffolds	5,817
	No. contigs	63,310
	Total length	881 Mb
	Scaffold N50	1,967 kb
	Contig N50	37.5 kb
	Longest scaffold	14.3 Mb
	GC%	39.70%
	No. Ns	63 Mb
	Completeness (BUSCO insecta_odb10)	C:98.5% [S:98.1%, D:0.4%]
Annotation	No. genes (coding)	15,591
	Repeat content	40.4%
Methylome	%methylated CpG	8.79%

706

707 Figure legends

708 **Figure 1: Phylogenetic position of *Reticulitermes speratus* in Blattodea, its**
 709 **developmental pathway, and evolution of the gene repertoire and genome**
 710 **structure.**

711 **(a)** Phylogenetic tree of termites and cockroaches. Estimated divergence dates
 712 (mya: million years ago) are based on Bucek et al. (80). *R. speratus* is marked in
 713 red, and two termites mainly compared in this study are marked with bold
 714 characters. **(b)** Developmental pathway of *R. speratus*. There are 2 larval stages
 715 before the molt into a nymph (with wing buds) or worker (no wing buds). There are
 716 6 imaginal stages, and the 6th-stage nymphs molt into alates, which are primary
 717 reproductives (queen and king). Secondary reproductives (neotenics) differentiate
 718 from the 3rd- to 6th-stage nymphs. In the apterous line, there are at least 5 stages
 719 of workers. Some workers in the colony molt into presoldiers and soldiers. Female
 720 neotenics used for genome sequencing and 3 castes used for RNA-seq are marked
 721 with asterisks. **(c)** Gene repertoire of *R. speratus* categorized by orthology. *R.*
 722 *speratus* genes were compared to those of 88 arthropods and grouped into three
 723 classes: orthologs shared with other arthropods (labeled 'arthropod'), orthologs
 724 shared with other termites (*Z. nevadensis* and/or *M. natalensis*) but with no
 725 orthologs in other arthropods (labeled 'termite'), and orphan genes unique to *R.*
 726 *speratus* (labeled 'no hit'). **(d)** High conservation of synteny between termite
 727 genomes revealed by dot plots generated by comparing *R. speratus* with *Z.*
 728 *nevadensis* and *M. natalensis*. Scaffolds longer than 2.0 Mb in the *R. speratus*
 729 assembly are used for plotting. Forward alignments are plotted in red and reverse
 730 alignments are plotted in blue.

731
 732 **Figure 2: Caste-specific transcriptome analysis and the enrichment of**
 733 **duplicate genes for caste-biased genes.**

734 **(a)** Multidimensional scaling (MDS) plot of RNA-seq data showing relatedness
 735 between the expression profiles of different castes (reproductive, soldier and
 736 worker) and sexes (male and female). The left panel plots RNA-seq data from head
 737 samples, and the right panel plots data from thorax + abdomen samples. Three
 738 biological replicates were analyzed for each condition and plotted individually. **(b)**
 739 Numbers of caste-biased genes with >2-fold higher expression levels than the other

740 two castes. Colours in each bar indicate the differences of RNA-seq data obtained.
 741 **(c)** Violin plots showing the distribution of tau indexes of duplicate genes and single
 742 genes. Tau values range between 0 and 1, with low values indicating invariable and
 743 constitutive expression between castes and higher values supporting caste
 744 specificity. In both body part samples, the tau values of duplicate genes were
 745 significantly greater than those of single genes ($p < 2.2e-16$, Wilcoxon rank sum
 746 test). **(d)** Enrichment of caste-DEGs (differentially expressed genes among castes)
 747 for duplicate genes. In each comparison between castes (soldier vs worker,
 748 reproductive vs worker, and reproductive vs soldier), all genes are ranked and
 749 ordered by log-fold-change value along the horizontal axis. Black bars mark the
 750 positions of genes. Genes of sociality-related functions highlighted in the text are
 751 selected and plotted in the lower panels. Curved lines in the upper panel show
 752 relative enrichment of the duplicate genes (blue line) or single genes (green line)
 753 relative to uniform ordering.

754

755 **Figure 3: Lipocalin genes in *R. speratus*.**

756 **(a)** Maximum likelihood (ML) tree of lipocalin homologs based on the amino acid
 757 sequences obtained with a log gamma (LG) model. Branches leading to clade A
 758 and clade B, which show gene family expansion specific to termite sublineages, are
 759 marked in yellow and green, respectively. Color gradients in the outer tracks show
 760 the expression levels as averaged $\log(\text{RPKM}+1)$ values in three castes
 761 (reproductive, soldier, and worker). Expression levels of head samples and thorax +
 762 abdomen samples are shown in purple and green, respectively. Caste-DEGs
 763 (differentially expressed genes among castes) are marked as R, S, or W beside the
 764 color gradients, indicating biases toward the reproductive, soldier, or worker caste,
 765 respectively. **(b)** Lipocalin multigene clusters in the *R. speratus* genome and their
 766 relative expression levels among castes. The heatmap shows the Z-scores of the
 767 $\log(\text{RPKM}+1)$ values in the caste-specific transcriptome. **(c)** Comparison of the
 768 number of lipocalin subclasses among representative arthropods. Note clades A
 769 and B are specific to termites. **(d)** Vertical cryosection of the queen abdomen
 770 subjected to *in situ* hybridization with an antisense DIG-labeled *RS008881* mRNA
 771 probe. The accessory gland cell layer is stained dark (arrowhead), in contrast to the
 772 other ovarian tissues, including the spermatika (asterisk). Bar = 0.2 mm. **(e)**
 773 Photographs of *in situ* hybridization for *RS008823* mRNA in the soldier head. The
 774 front of the head is on the left side. The gland cell layer surrounding the frontal

775 gland reservoir (R) is stained dark (arrowhead). The asterisk indicates the brain.
 776 Bar = 0.1 mm. **(f, g, h)** Vertical cryosection of the worker antenna (f) and horizontal
 777 cryosections of the worker labial palp (g, right palp) and maxillary palp (h, the last
 778 segment of the left (upper) and right (lower) palp) subjected to *in situ* hybridization
 779 for *RS008882* mRNA. Tissues around some sensilla are stained dark (arrowhead).
 780 Bar = 0.1 mm. Photographs of cryosections hybridized with sense probes (negative
 781 controls) are shown in Supplementary Fig. 6a-c.

782

783 **Figure 4: Glycoside hydrolase family (GH) 1 in the *R. speratus* genome.**

784 **(a)** ML tree of GH1 genes based on the amino acid sequences obtained with a
 785 LG+G+I model. Fourteen of 16 GH1 genes in *R. speratus* were used; two genes
 786 (*RS004146* and *RS100005*) were removed from the analysis due to incomplete
 787 retrieval of the coding sequences from gapped scaffolds. GH1 subclasses are
 788 colored and labeled A, B, C, and D. **(b)** GH1 multigene clusters in the *R. speratus*
 789 genome and their expression levels. Letters A-D on the gene structures represent
 790 GH1 subclasses categorized in the phylogenetic tree in (a). The heatmap shows
 791 the Z-scores of the log(RPKM+1) values in the caste-specific transcriptome. **(c)**
 792 Synteny comparison around the GH1 multigene cluster region (orange rectangle)
 793 between *R. speratus* and *M. natalensis* genomes. **(d)** Vertical cryosection of the
 794 worker thorax subjected to *in situ* hybridization with an antisense DIG-labeled
 795 *RS004136* mRNA probe. The head part is on the right side. Bar = 0.2 mm. **(e)**
 796 Magnified view of the worker thorax. The salivary gland cells are specifically stained
 797 dark (arrowhead). Bar = 0.1 mm. **(f)** Vertical cryosection of the queen abdomen
 798 subjected to *in situ* hybridization for *RS004624* mRNA. Bar = 0.2 mm. **(g)** Magnified
 799 view of the queen ovary. The accessory gland cell layer is stained dark
 800 (arrowhead), in contrast to the other ovarian tissues, including ovarioles with two
 801 oocytes (asterisks). Bar = 0.1 mm. See Supplementary Fig. 7a-b for negative
 802 controls of the *in situ* hybridization experiments (d-g).

803

804 **Figure 5: Lysozyme family in the *R. speratus* genome.**

805 **(a)** ML tree of lysozyme genes with a GTR+G model. The red curve indicates a
 806 lineage-specific gene expansion observed in the *R. speratus* genome for a c-type
 807 lysozyme. **(b)** Lysozyme multigene clusters in the *R. speratus* genome and their
 808 relative expression levels among castes. The heatmap shows the Z-scores of the
 809 log(RPKM+1) values in the caste-specific transcriptome.

810

811 **Figure 6: Geranylgeranyl diphosphate (GGPP) synthase homologs in the *R.***
812 ***speratus* genome.**

813 **(a)** Synteny comparison around GGPP synthase loci among three termites, *R.*
814 *speratus*, *M. natalensis* and *Z. nevadensis*. *R. speratus*-specific insertions were
815 found, where GGPP synthase paralogs were tandemly duplicated in the *R. speratus*
816 genome. **(b)** Genomic location and gene expression of *R. speratus* GGPPS
817 homologs. The heatmap shows the expression level calculated by mean-centered
818 $\log(\text{RPKM}+1)$. Yellow indicates high expression, while blue denotes low expression.
819 Black represents the mean level of expression among the castes. Note that the
820 heatmap of *RS007484* is almost entirely black for all samples, which indicates that
821 expression was invariable among castes, while most of the rest of the paralogs
822 showed caste-biased expression. **(c)** ML tree of GGPP synthase homologs with a
823 LG+G model. *R. speratus* genes are marked with blue circles. **(d)** Vertical
824 cryosection of the soldier head subjected to *in situ* hybridization for *RS100016*
825 mRNA. The front of the head is on the left side. The gland cell layer surrounding the
826 frontal gland reservoir (R) is stained dark (arrowhead). The asterisk indicates the
827 brain. The frontal pore (P) discharging frontal gland secretion is also observed. Bar
828 = 0.1 mm. See Supplementary Fig. 7c for the negative control experiment. **(e)**
829 Molecular evolutionary analysis of *R. speratus* GGPP synthase homologs by the
830 PAML branch-site test. Detected positive selection is marked with a single asterisk *
831 ($p < 0.05$) or double asterisks ** ($p < 0.01$) next to the corresponding branches.

832

833 **Figure 7: The TY family, a novel secretion gene family identified from termite**
834 **taxonomically restricted genes.**

835 **(a)** Genomic locations and caste-biased expression patterns of TY family genes. **(b)**
836 Multiple alignment of TY homologs of *R. speratus* and *Z. nevadensis*. Protein motifs
837 and structural characteristics are represented. **(c)** Orthology of TY homologs in
838 three termites and the results of the Ka/Ks analysis.

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