

# Caste- and development-associated gene expression in a lower termite

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Published: 26 September 2003

*Genome Biology* 2003, **4**:R62

The electronic version of this article is the complete one and can be found online at <http://genomebiology.com/2003/4/10/R62>

Received: 18 June 2003

Revised: 16 July 2003

Accepted: 26 August 2003

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## Abstract

**Background:** Social insects such as termites express dramatic polyphenism (the occurrence of multiple forms in a species on the basis of differential gene expression) both in association with caste differentiation and between castes after differentiation. We have used cDNA macroarrays to compare gene expression between polyphenic castes and intermediary developmental stages of the termite *Reticulitermes flavipes*.

**Results:** We identified differentially expressed genes from nine ontogenic categories. Quantitative PCR was used to quantify precise differences in gene expression between castes and between intermediary developmental stages. We found worker and nymph-biased expression of transcripts encoding termite and endosymbiont cellulases; presoldier-biased expression of transcripts encoding the storage/hormone-binding protein vitellogenin; and soldier-biased expression of gene transcripts encoding two transcription/translation factors, two signal transduction factors and four cytoskeletal/muscle proteins. The two transcription/translation factors showed significant homology to the *bicaudal* and *bric-a-brac* developmental genes of *Drosophila*.

**Conclusions:** Our results show differential expression of regulatory, structural and enzyme-coding genes in association with termite castes and their developmental precursor stages. They also provide the first glimpse into how insect endosymbiont cellulase gene expression can vary in association with the caste of a host. These findings shed light on molecular processes associated with termite biology, polyphenism, caste differentiation and development and highlight potentially interesting variations in developmental themes between termites, other insects, and higher animals.

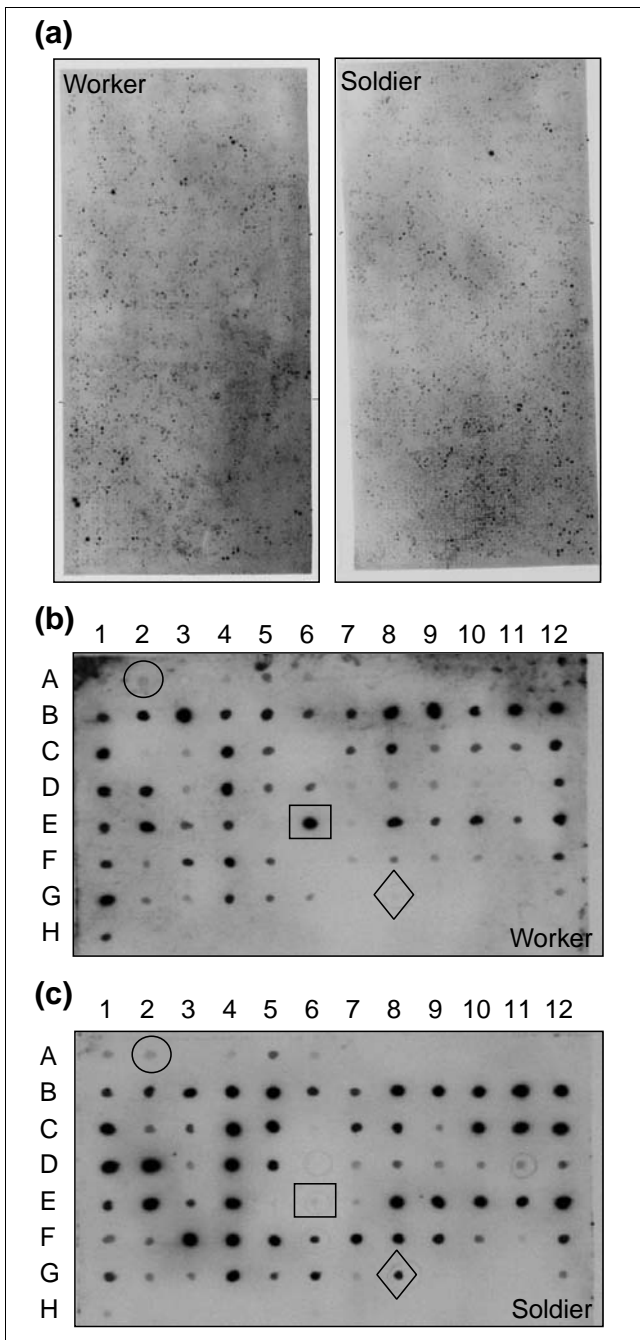
## Background

The lower termites possess one of the most ancient forms of insect eusociality, and a social organization that has arisen independently from that of the eusocial Hymenoptera [1,2]. Lower termites of the genus *Reticulitermes* have a worldwide distribution, are the most common termites in the United States and Europe, and are extremely important from both an

economic and an ecological perspective [3]. Lower termites, including *Reticulitermes*, undergo incomplete (hemimetabolous) metamorphosis and a caste-differentiation process that is unparalleled among the social insects (Figure 1) [4-6].

Caste differentiation and development in *Reticulitermes* termites can follow imaginal (winged) and apterous (wingless)





**Figure 2**  
 Representative macroarray and dot blot results. **(a)** Duplicate macroarrays were probed separately with mRNAs that were labeled with digoxigenin (DIG) after isolation from worker or soldier *R. flavipes*. Shown are representative autoradiograms from arrays probed with worker (left) or soldier (right) mRNAs. **(b-c)** Plasmid DNA was obtained from array-positive cultures and spotted on duplicate membranes in equal quantities. The resulting dot blots were probed with aliquots of the same DIG-labeled worker or soldier mRNA pools that were used to probe the array membranes originally. The secondary dot blots verified elevated transcript abundance in approximately 50% of instances. Examples of worker- and soldier-associated cDNAs are in positions noted by squares and diamonds, respectively, while a control gene with similar expression (actin-related protein 66B) is circled.

differential expression between soldiers and workers were identified in the array screens. These clones were the basis of further analysis. Clones corresponding to each of the 153 positive array positions were sampled from frozen cultures in 384-well plates and grown as liquid cultures. Plasmid DNA was obtained from each culture and spotted on duplicate membranes in equal quantities. The resulting dot blots were probed with aliquots of the same DIG-labeled worker or soldier mRNA pools that were used to originally probe the array membranes. The secondary dot blots verified elevated transcript abundance in around 50% of instances (Figures 2b,c). In the dot blots shown in Figure 2, examples of worker- and soldier-specific cDNAs are in positions noted by squares and diamonds, respectively.

Dot blots were also used to determine the expression of two potential genes with similar expression levels between castes: these encoded NADH dehydrogenase and actin-related protein 66B (ARP 66B). These two transcripts were identified previously from expressed sequencing tag (EST)-sequencing studies [12] and were used as candidate control genes for expression. Similar expression levels of the two control genes can be seen in the upper left corners of the dot blots, with ARP 66B being circled (Figure 2b,c). In subsequent experiments it was determined that expression levels of these two genes were not robust enough to serve as controls in quantitative PCR. As a result,  $\beta$ -actin was used as the control in all subsequent experiments (see below).

**EST sequencing and bioinformatic summaries**

Following verification on dot blots, 68 clones were confirmed with clear differences in expression between workers and soldiers. High-quality ESTs were obtained for each of these clones (Additional data file 1). Because the cDNA library was created with 5'-3'-oriented cDNAs, EST sequencing was only performed in one pass from the 5' ends of clones. EST sequencing results are summarized in Table 1 in order of BLASTx E-value significance scores. Lengths of high-quality, vector-trimmed sequence ranged from 66 to 632 nucleotides. On the basis of BLASTx database similarity searches, eight cDNAs occurred more than once in the array screen (Additional data file 1). These multiply-occurring cDNAs, shown in order of their identity-significance scores, correspond to genes encoding: termite salivary cellulases (3 total), termite endoglucanase (10), endosymbiont endoglucanase (3), endosymbiont cellulase (2), mitochondrial cytochrome oxidase I (24), muscle-specific LIM protein (2), mitochondrial 18K protein (2), and unknown #24 (3). For simplicity, only single clones representing the longest sequence are reported in Table 1. Clones 1-16 possess significant E-values of less than  $1 \times 10^{-8}$  and therefore their identities are considered valid (Tables 1, 2). Because clones 17-25 have larger E-values, the identities of these clones, when available, are only considered putative.

**Table 1****EST sequence summary of 25 positive *R. flavipes* clones identified from array screens**

Clone *	Size (bp)	Identity match by BLASTx (%)	E-value	Gene ontology†
1 (D04)	615	98% to <i>Reticulitermes speratus</i> salivary cellulase	1e-106	E
2 (C02)	423	89% to <i>Reticulitermes speratus</i> endoglucanase 2	1e-79	E
3 (E11)	505	57% to <i>Pseudotriconympha grassii</i> cellulase	7e-69	E
4 (B02)	518	87% to <i>Blattella germanica</i> cytochrome oxidase I	7e-54	ET
5 (A10)	449	Unknown (65% to <i>Anopheles gambiae</i> unknown)	2e-49	UNK
6 (C07)	505	>65% to <i>Drosophila melanogaster</i> $\beta$ -NAC homolog, 'bicaudal'	2e-45	TF
7 (B09)	585	69% to <i>Holomastigotoides mirabile</i> endoglucanase 2	1e-47	E
8 (C11)	509	69% to <i>Drosophila melanogaster</i> muscle-specific 'LIM' protein	2e-35	CSP
9 (B11)	569	80% to <i>Drosophila melanogaster</i> troponin I 'wings up'	1e-35	CSP
10 (H04)	242	98% to <i>Bombyx mori</i> $\beta$ -tubulin	6e-33	CSP
11 (D10)	499	39% to <i>Drosophila melanogaster</i> troponin I 'wings apart', 'heldup'	3e-29	CSP
12 (F10)	527	36% to <i>Athalia rosae</i> vitellogenin	4e-20	SP
13 (D03)	387	>64% to <i>Drosophila melanogaster</i> 'BAB-I' (BTB/POZ domain containing protein)	3e-18	TF
14 (G12)	296	81% to <i>Drosophila melanogaster</i> mitochondrial-ribosomal protein S2	1e-17	MOB
15 (G03)	340	49% to <i>Mus musculus</i> SH3-domain kinase binding protein	5e-10	ST
16 (D02)	645	43% to <i>Homo sapiens</i> Rab3 GTPase-activating protein	2e-8	ST
17 (H08)	297	39% to <i>Caenorhabditis elegans</i> RNA-dependent DNA polymerase (RTase)	0.001	DRF
18 (B10)	350	Unknown (40% to <i>Drosophila melanogaster</i> GC1077 gene product)	0.03	UNK
19 (A05)	630	38% to goldfish mitochondrion hypothetical 18K protein	0.12	MOB
20 (D07)	471	44% to <i>Oncorhynchus mykiss</i> cyclic GMP-gated channel	0.39	IC
21 (H06)	632	27% to <i>Musca domestica</i> nanos posterior determinant homolog	1.2	TF
22 (E12)	372	34% to <i>Moraxella bovis</i> endonuclease Mbo II	3.2	DRF
23 (G06)	370	Unknown (36% to <i>Anopheles gambiae</i> unknown)	>5	UNK
24 (A02)	510	Unknown	>5	UNK
25 (E03)	392	Unknown	>5	UNK

Clones are listed according to the significance of their identify-based BLASTx score E-values. \* Clone number, with sequence identifier position shown in parentheses. See Additional data file 1 for sequence details. †Gene ontology classification based on Adams *et al.* [13]: E (enzyme), CSP (cytoskeletal/structural protein), DRF (DNA replication factor), ET (electron transport), IC (ion channel), MOB (mitochondrial organization and biogenesis), SP (storage protein), ST (signal transduction), TF (transcription factor), UNK (unknown).

Because the six ESTs noted above corresponded to multiple positive array positions, the total number of distinct/unique sequences present in the array-positive EST pool was 25. These 25 cDNAs can be categorized into 10 gene ontology categories following the summary presented by Adams *et al.* [13]. These are: enzyme, cytoskeletal/structural, electron transport, transcription factor, mitochondrial organization and biogenesis, signal transduction, storage, DNA replication, ion channel, and five unknown (Table 1).

### Relative gene expression

Relative expression patterns for 24 discrete gene transcripts, determined by quantitative PCR, are shown in Table 2. Raw data from quantitative PCR analyses are provided in Additional data file 2. There was only one gene transcript that could not be amplified and evaluated by quantitative PCR. This sequence encodes a putative cyclic GMP-gated ion channel (clone 20) with highly repetitive nucleotide sequences and

high CG content [14]. Because of these properties, no PCR primer pairs could be designed that would provide the high-fidelity PCR necessary for quantitative PCR analysis.

Transcripts having highest worker-associated expression were those encoding cellulase and endoglucanase enzymes involved in cellulose digestion. Cellulases and endoglucanases can have origins in either termites or their microorganismal endosymbionts. Cellulases and endoglucanases of apparent endosymbiont origin had substantially higher expression relative to those of apparent termite origin. Other transcripts with elevated expression in workers were either related to mitochondrial organization and biogenesis (mRpS2 and 18K protein-like; also elevated in soldiers), or had unknown identity (clones 18 and 24).

More than half of the transcripts evaluated had exclusive soldier-biased expression (13 of 24). These included two

**Table 2****Relative expression levels of *R. flavipes* gene transcripts**

Gene ontology category and clone identity	Accession number <sup>‡</sup>	Caste		Developmental stage	
		Worker	Soldier	Presoldier	Nymph
<b>Standard/control</b>					
β-actin <sup>†</sup>	N/A (N/A)	1.1 ± 0.1	1.0 ± 0.4	1.0 ± 0.1	1.1 ± 0.1
<b>Transcription/translation factor</b>					
6. Rf β-NAC-1, 'bicaudal'	AY258589	5.2 ± 0.2	<b>13.8 ± 0.3</b>	1.4 ± 0.1	1.0 ± 0.1
21. Rf PDL, 'nanos'-like	BQ788190	3.0 ± 0.0	<b>5.0 ± 0.1</b>	1.0 ± 0.5	2.2 ± 0.1
13. Rf BTB/POZ-1, 'BAB'	AY258590	<b>2.2 ± 0.3</b>	1.7 ± 0.0	1.0 ± 0.1	<b>2.6 ± 0.2</b>
<b>Signal transduction</b>					
15. SH3-domain kinase-binding	CB518313	85.5 ± 0.1	<b>131.9 ± 0.2</b>	1.0 ± 0.2	3.6 ± 1.3
16. GTPase-activating protein	BQ788178	2.3 ± 0.3	<b>4.5 ± 0.1</b>	1.0 ± 0.4	1.3 ± 0.0
<b>Enzyme</b>					
1. Termite salivary cellulase	CB518295	<b>31.5 ± 0.1</b>	1.6 ± 0.0	1.0 ± 0.0	19.5 ± 0.0
2. Termite endoglucanase 2	CB518296	<b>32.3 ± 4.8</b>	2.5 ± 1.5	1.0 ± 0.1	<b>30.1 ± 1.1</b>
3. Symbiont cellulase	CB518297	<b>1939.7 ± 0.0</b>	1.7 ± 0.2	1.0 ± 0.4	208.9 ± 0.2
7. Symbiont endoglucanase 2	CB518298	<b>62.7 ± 0.9</b>	2.7 ± 1.1	1.0 ± 0.6	34.4 ± 0.1
<b>Cytoskeletal/structural protein</b>					
8. Muscle LIM protein	CB518301	1.6 ± 0.3	<b>5.0 ± 0.2</b>	1.8 ± 0.3	1.0 ± 1.1
9. Rf1-troponin I	CB518302	2.3 ± 0.1	<b>3.9 ± 0.4</b>	1.4 ± 0.2	1.0 ± 0.0
10. β-tubulin	CB518304	1.6 ± 0.3	<b>5.0 ± 0.2</b>	1.8 ± 0.3	1.0 ± 1.1
11. Rf2-troponin I	CB518303	1.1 ± 1.3	<b>11.5 ± 0.6</b>	1.0 ± 1.2	1.9 ± 3.0
<b>Storage/hormone binding</b>					
12. Vitellogenin	CB518311	1.2 ± 0.6	1.0 ± 0.1	<b>4.9 ± 0.2</b>	1.4 ± 1.7
<b>Electron transport</b>					
4. Cytochrome oxidase I	CB518306	3.1 ± 0.6	<b>6.6 ± 0.1</b>	2.5 ± 0.2	1.0 ± 0.0
<b>Mitochondrial organization/biogenesis</b>					
14. mRpS2	CB518308	<b>4.3 ± 0.0</b>	<b>4.0 ± 0.0</b>	1.0 ± 0.7	1.9 ± 0.1
19. I8K protein-like	CB518307	<b>2.9 ± 0.0</b>	<b>1.3 ± 0.9</b>	1.0 ± 0.1	<b>2.3 ± 1.5</b>
<b>DNA replication factor</b>					
17. Reverse transcriptase-like	CB518309	<b>1.4 ± 1.5</b>	<b>2.1 ± 0.7</b>	1.2 ± 0.1	1.0 ± 0.7
22. <i>MbolI</i> endonuclease-like	CB518310	1.1 ± 1.3	<b>5.3 ± 0.3</b>	2.8 ± 0.1	2.2 ± 0.4
<b>Unknown</b>					
5. Unknown	CB518314	2.2 ± 0.8	<b>6.1 ± 0.4</b>	1.0 ± 0.9	2.6 ± 0.1
18. Unknown	CB518315	<b>9.5 ± 0.2</b>	1.2 ± 0.5	1.1 ± 0.1	1.0 ± 0.3
23. Unknown	CB518318	1.0 ± 0.2	<b>5.9 ± 0.2</b>	<b>5.4 ± 2.5</b>	1.2 ± 2.0
24. Unknown	CB518317	<b>12.4 ± 0.1</b>	2.3 ± 0.1	1.0 ± 0.0	1.9 ± 0.1
25. Unknown	CB518319	1.1 ± 0.1	<b>2.1 ± 0.2</b>	1.5 ± 0.2	1.0 ± 0.5

Relative expression levels of *R. flavipes* gene transcripts (average ± standard error), identified using cDNA macroarrays and quantified by quantitative PCR. Data shown in bold-face type represent the highest relative expression for a given gene (LSD *t*-test;  $P \leq 0.05$ ). Relative expression calculated using the method described by Pfaffl et al. [44] ( $n = 3$ ). See Additional data file 2 for raw data. <sup>†</sup>All results are normalized to the control gene β-actin. Primers designed from mouse and rat sequences (Accession nos. X03672 and V01207), as described by Giulietti et al. [43]. <sup>‡</sup>Clone numbers correspond to the order shown in Table 1 (numbers 16 and lower have significant identity scores). See Additional data file 1 and 9, respectively, for sequence and primer details.

transcription factors, two signal transduction factors, four cytoskeletal/musculature proteins, one transcript associated with mitochondrial electron transport, one putative DNA replication factor, and three transcripts of unknown identity. Three additional transcripts had similar expression levels between workers and soldiers, yet had higher expression than the presoldier and/or nymphal intermediary developmental stages (mRpS2,18 K protein-like, and reverse transcriptase-like).

Two soldier-biased troponin I-encoding transcripts had both differential expression and sequence variation (Additional data file 3). The first of these (Rf1-troponin I) aligns with a portion of the 3' region of the *Drosophila* troponin I cDNA whereas the second (Rf2-troponin I) aligns with the 5' region, and includes the ATG start signal. The two troponin ESTs have a 60-nucleotide overlap, but share only 65% amino-acid identity in the overlap region. These termite troponin forms have significantly elevated expression levels in soldiers, which are 1.7- and 10.5-fold elevated for the Rf1 and Rf2 forms, respectively, relative to workers.

Transcripts were also observed with high expression levels in the intermediary presoldier and nymph developmental stages. In all cases, transcripts that were identified with elevated nymphal expression were also elevated in workers (as with cellulases/endoglucanases and Rf BTB/POZ-1) or both workers and soldiers (as with mitochondrial 18 K protein-like). Finally, a transcript encoding the storage/hormone-binding protein vitellogenin had highest expression in presoldiers, a stage incapable of feeding itself, and that is also hypothesized to serve as a sink for juvenile hormone [15].

#### Putative caste-regulatory genes

Two putative regulatory genes with significant homology to *Drosophila* and honey-bee (*Apis mellifera*) developmental genes were identified. Both these genes are transcriptional/translational regulators. One is *Rfβ-NAC-1*, which is related to the *bicaudal* locus of *Drosophila*, and the second is *Rf BTB/POZ-1*, which is related to the *bric-a-brac* (*BAB*) locus of *Drosophila*. Alignments of the respective translated amino-acid sequences are shown in Additional data files 4 and 5. A third related gene, *Rf-PDL*, has putative identity as a *nanos*-homologous posterior-determinant gene on the basis of weak homology to database sequences; however, hydropathy profiles for the translated amino-acid sequence bear striking similarities to *Drosophila* *nanos* protein and its homologs from other species (Additional data file 6).

*Rfβ-NAC-1* is a *Reticulitermes* homolog of the ribosomal translation factor  $\beta$ -NAC. A partial open reading frame (ORF) of 420 nucleotides (140 amino acids) was included in our cDNA clone. Database searches using the *Rfβ-NAC-1* translated amino-acid sequence revealed numerous proteins with significant homology, all of which are associated with ribosomal translational processes. Alignments of *Rfβ-NAC-1*

revealed 73 invariant amino-acid residues (Additional data file 4). Also observed was significant pairwise homology to  $\beta$ -NAC homologs from *Drosophila* (61.5%), *A. mellifera* (71.5%), and *Caenorhabditis elegans* (60.9%). These findings clearly establish identity of the *Reticulitermes* sequence as a  $\beta$ -NAC homolog.

Complete sequencing of the *Rf BTB/POZ-1* cDNA clone elucidated a complete ORF of 1,320 nucleotides (439 amino acids). Database searches with the translated amino-acid sequence of the entire ORF did not reveal homologs with high degrees of similarity across their entire lengths; however, significant similarity to BTB/POZ domains of many BTB/POZ proteins was identified (Additional data file 5). The presence of the highly conserved BTB/POZ domain indicates that the sequence encodes a zinc-finger protein involved in transcriptional regulation. Alignments of the amino-terminal regions of several BTB/POZ domain-containing proteins revealed an insertion of 60-65 amino acids in the amino-terminal region of *Rf BTB/POZ-1* that is not present in any of the homologous sequences, and the presence of three invariant sequence motifs in all BTB/POZ proteins shown (single-letter amino-acid code: LRWN, DVTL, LSACS). Overall identity between the BTB/POZ domains of termite *Rf BTB/POZ-1* and *Drosophila* BRcore, *Drosophila* fruitless, *A. mellifera* pipsqueak, and *Drosophila* pipsqueak are 44.5, 43.1, 41.8, and 35.8%, respectively.

A third EST termed *Rf-PDL* (posterior determinant-like) shares modest similarity with *nanos*-homologous posterior determinant genes of *Musca domestica* (AAA87461) and *Xenopus laevis* (X72340) (part a in Additional data file 6). Striking similarities in hydropathy profiles exist, however, for sequenced portions of the termite cDNA versus *nanos* homologs from *Drosophila*, *Musca*, and *Xenopus* (part b in Additional data file 6). The *nanos* gene in *Drosophila* is a transcription factor that controls anterior-posterior polarity and is under direct control of  $\beta$ -NAC. Quantitative PCR results for *Rf-PDL* follow a similar caste and development-associated expression pattern to *Rf β-NAC-1*, implying a link between these two genes.

Two gene transcripts encoding signal transduction factors with established links to well-characterized developmental processes were also identified. The first of these genes, SH3-domain kinase, has significant homology to mammalian kinases occurring at the *Ruk* locus of mouse and rat (Additional data file 7), but interestingly, not to any sequences from insects. The second signal transduction factor, GAP, also has the highest degree of similarity to vertebrate sequences, although modest similarity also exists to a *Drosophila* GTPase-coding sequence (Additional data file 8). Relative to presoldiers, both of these transcripts showed substantial and significantly elevated expression in soldiers (132- and 4.5-fold, respectively), although expression was also significantly elevated in workers (86- and 2.3-fold, respectively).



with mirror-image duplications of abdominal segments. Markesich and co-workers [26] mapped the *bicaudal* locus to a region encoding  $\beta$ -NAC, a component of the ribosomal translational machinery and the nascent polypeptide-associated complex. *bicaudal* and a related mutation, *enhancer of bicaudal*, are caused by insertions into the  $\beta$ -NAC transcribed region. Both *bicaudal* mutations have been rescued by wild type copies of  $\beta$ -NAC, which regulates expression of the posterior determinant gene *nanos* (that is, *bicaudal* mutations lead to ectopic expression of *nanos* [26]). The observed high expression of Rf  $\beta$ -NAC-1 in soldiers, and the unique shape of the termite soldier head in relation to the rest of the body, suggest that Rf  $\beta$ -NAC-1 may influence the generalized soldier body plan and possibly direct ectopic expression of the posterior determinant *nanos* (see below).

In insects, *nanos* is an evolutionarily conserved organizer of anterior-posterior polarity, which acts as a translational repressor by directing expression of the *hunchback* gene [30,32]. The mechanism by which *nanos* acts is conserved in insects, and *nanos* sequence divergence among dipterans correlates with evolutionary relationships and natural history. As a result, *nanos* should be more highly diverged in *R. flavipes*. In a study by Curtis *et al.* [30], *nanos* homologs with only 28-63% amino-acid identity from evolutionarily distant dipteran species successfully rescued *nanos* activity in null mutants of *Drosophila*. Although the function encoded by the Rf-PDL transcript is unknown, its similar expression patterns to Rf  $\beta$ -NAC-1, and its similar hydropathy profiles to known *nanos* homologs, provide support that it may potentially encode *nanos*-homologous functions. Regardless of its true identity, the Rf-PDL transcript possesses caste-associated expression that suggests a developmental role.

BTB/POZ domains are conserved amino-acid motifs in the amino terminus of zinc-finger proteins, some of which function as transcriptional regulators. A total of 40 BTB/POZ domain-containing proteins are encoded in the *Drosophila* genome, and therefore these proteins appear to be responsible for a vast number of functions [27,28]. Several lines of evidence establish clear developmental roles for BTB/POZ-domain-containing proteins, however. Godt and co-workers [27] reported that the BTB/POZ protein *bric-a-brac* (*Bab-I*), which is most similar to our termite sequence, is involved in pattern formation along the proximal-distal axis of the leg and antenna of *Drosophila*, and that mutants possess legs and antennae with segmentation defects. *bric-a-brac* is also known to be involved in *Drosophila* ovary morphogenesis on the basis of the disrupted ovariole formation and female sterility in *bric-a-brac* mutants [33]. BTB/POZ protein domains can dimerize with themselves [34], possibly leading to self-inactivation. This suggests that the expression of termite Rf BTB/POZ-1 may repress gene expression in workers and nymphs, which have higher BTB/POZ expression than presoldiers and soldiers. Alternatively, elevated BTB/POZ

expression in workers and nymphs may relate to their gonadotropic/reproductive potential.

#### Signal transduction factors

We also identified two gene transcripts with significant homology to the signal transduction factors SH3-domain kinase and GAP. SH3-domain proteins are adaptor proteins involved in the regulation of signal transduction [35]. The SH3 domains of these proteins can interact with other kinases involved in apoptotic cell death and membrane trafficking. Expression of mouse SH3-Ruk proteins in cells appears to be involved in the regulation of their survival and other intracellular processes [35]. Extremely high levels of expression occurred for the SH3-domain protein in termite soldiers (139-fold relative to presoldiers), but expression was also high in workers (85.5-fold relative to presoldiers), suggesting that dose-dependent factors may be implicated in SH3-domain cascade-dependent processes.

GAPs are involved in regulating the transfer of phosphate groups by GTPases, and are particularly relevant to signal transduction processes. One related protein in *Drosophila*, named Ras1, mediates the modulation of homeotic gene function in cell and segment identity [36] and eye development [37]. Ras1-mediated cell signaling modifies nuclear activity of transcription factors, most notably those encoded by the homeobox genes *proboscopedial* and *ultrabithorax*. Ras1 is active when coupled with GTP but inactive in the presence of GDP, thus implicating GTPases as regulators of Ras1 (and homeobox) action. Ras1 impacts on homeobox gene expression are dose-dependent [36], which may explain high and intermediate (not absent) GAP expression in soldiers and workers, respectively. Together, our signal transduction-related findings suggest interesting possibilities for signal transduction-mediated transcriptional regulation, cascade-dependent processes, and possibly regulation of cellular death in termite caste-differentiation processes.

#### Conclusions

Here we have presented the first summary of a transcriptome-based survey for caste- and differentiation-associated genes in a termite. We have focused on the lower termite *R. flavipes* because it is the commonest termite in the United States, and because of its ecological and economic importance. Vast differences occurred in sequence composition of this directed array screen versus previous random EST sequencing [12], emphasizing the validity of our approach. Several biologically significant trends were apparent from our findings. First, expression of both termite- and endosymbiont-homologous cellulase genes is correlated with cellulose-feeding adaptations in workers and nymphs. Interestingly, endosymbiotic genes were represented in our arrays, suggesting additional utility of our approach in investigating host-endosymbiont relationships. Second, genes related to transcriptional regulation and signal transduction (some linked

to key developmental processes in *Drosophila* and honey-bees) appear to be important in the development and differentiation of the soldier caste. Third, genes associated with musculature and cytoskeletal architecture (including two tropomyosin I forms) were identified in association with the apterous and highly muscular soldier caste. Fourth, expression of a gene encoding the storage/hormone-binding protein vitellogenin is associated with a stage incapable of feeding and thought to serve as a juvenile hormone sink (the presoldier stage). Finally, several genes with unknown and/or putative identity, but with significant caste and development-associated expression were also identified as having differential expression between castes.

Because the two stages compared in our cDNA arrays represent a developmental end-point (soldiers) and a non-differentiated caste (workers), many important developmental genes were surely not identified. Future sociogenomic-based studies on intermediary developmental stages (nymphs and presoldiers) is likely to yield exciting new information. We observed strong similarities between termite genes and a number of well-characterized *Drosophila* mutants and genes, as well as honey-bee developmental genes. It is unclear if the differences we identified in developmental gene expression between *Drosophila*, honey-bees and termites relates to the holometabolous and hemimetabolous nature (respectively) of these insect groups. Nonetheless, the information on novel termite genes reported here sheds new light on completely uncharacterized aspects of basic termite molecular biology and physiology.

## Materials and methods

The authors will provide specific protocols on request.

### Termites, cDNA library, array preparation and array screening

Termites of multiple castes and developmental stages were obtained, and a polyphenic cDNA library synthesized as described previously [12]. Total RNA was isolated from groups of whole termites using the RNeasy total RNA isolation kit (Ambion, Austin, TX), and its integrity and quantity verified by formaldehyde-agarose electrophoresis [38]. Poly(A) RNA was purified from the total RNA (Ambion), cDNA was synthesized using an oligo-dT<sub>12</sub> adaptor primer, and the library constructed (see [12]). From the cDNA library, the Purdue University Genomics Core Facility prepared multiple identical 'macro' arrays, which are gridded bacterial colony filters. Each 22 × 22 cm filter was printed from 18,432 glycerol stocks contained in forty-eight 384-well plates. Duplicate filters were printed by using a Biorobotics robot (Total Array System, Woburn, MA) to spot samples from plates onto charged nylon membranes overlaid with LB agar containing 100 µg/ml ampicillin. The filters were grown overnight at 37°C, peeled away from the agar, placed on chromatography paper saturated with 5% SDS and 2x SSC for 2 min,

heated in a microwave for 2.5 min, then UV-cross linked in a Stratalink (Stratagene, La Jolla, CA).

Primary gene-expression comparisons between the soldier and worker castes were made as follows, using soldiers and workers isolated from laboratory colonies. mRNA was obtained separately from whole, pooled worker or soldier termites (Figure 1) using a commercially available protocol (Ambion), then digoxigenin (DIG)-labeled using a separate commercially available protocol (DIG Chem-link Labeling and Detection; Roche, Indianapolis, IN). Individual array membranes were probed with DIG-labeled mRNAs from either workers or soldiers (10 ml of 100 ng/ml). Autoradiograms of the membranes, showing the positions of specific clones, were produced using a commercially available protocol (Roche). Expression levels were compared between soldier and worker array autoradiograms by manual alignment.

For secondary verification, dot blots were performed using plasmid DNA affixed to nitrocellulose membranes (Millipore, Marlboro, MA). Liquid cultures were produced from array-positive cell lines, from which plasmid DNA was obtained using a commercially available protocol (Qiagen, Valencia, CA). Pure plasmid DNA (100-200 ng) was manually spotted onto duplicate membranes [38]. Membranes were probed with labeled aliquots of the same worker- and soldier-derived mRNAs noted above, using an identical procedure, then were manually superimposed to compare relative expression levels between castes.

### Sequencing and bioinformatics analyses

Array and dot blot-positive clones were sequenced from their 5' ends (T3 promoter) using a high-throughput EST sequencing procedure. For the putative regulatory genes *Rfβ-NAC-1* and *Rf BTB/POZ-1*, longer sequences shown in alignments (Additional data files 4 and 5) were obtained by sequencing from both the 5' and 3' ends, and by designing internal sequencing primers in the case of BTB/POZ (forward = GCAACGGATGACTACATGGA, reverse = TGGTTGAAGCCTGATTCACA). All sequencing-related procedures were performed by the Purdue University Genomics Core Facility. After contaminating vector sequences were trimmed, the ESTs were used as query sequences to search the GenBank nucleotide and translated databases by BLASTx [39]. Database searches were performed under default settings, with E-values for significance of identity being  $1 \times 10^{-10}$  and smaller [40]. Sequence alignments were performed using clustal analysis in DNASTar-Megalign software (Madison, WI).

### Quantitative real-time PCR

Quantitative PCR was performed to precisely quantify relative caste-associated gene expression. cDNA templates for quantitative PCR were synthesized separately from DNase-treated total RNA of whole worker, presoldier, soldier and

nymph termites. Total RNA isolation and DNase treatment were performed using commercially available protocols (Qiagen and Ambion, respectively). The quantity and quality of RNA was assessed before and after DNase treatment by spectrophotometry and formaldehyde-agarose electrophoresis [38]. cDNA was synthesized from 5 µg total RNA using an oligo-dT<sub>15</sub> primer and 50 U Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) at 42°C for 50 min.

Primers for use in quantitative PCR were designed from EST sequence data by the MIT Primer 3 program [41]. Primer sequences are provided in Additional data file 9. Quantitative PCR primers were designed to provide products of 100-200 base-pairs (bp) within putative ORFs; and had 45-55% GC content, T<sub>m</sub> = 57-62°C, non-complementarity between forward and reverse primers, and pyrimidine nucleotides on their 5' and 3' ends [42].

Quantitative PCR was performed using an iCycler iQ real-time PCR detection system (Bio-Rad, Hercules, CA) with SYBR Green I product tagging. To verify the amplification efficiency for both target and control genes, quantitative PCR conditions were optimized for cDNA template dilution (1:5, 1:10, 1:25, 1:50, and so on) and annealing temperature (T<sub>m</sub> ± 5°C). Before experiments, the production of single product bands and absence of 'primer-dimers' was confirmed by viewing products following agarose electrophoresis and ethidium-bromide staining. Simultaneous determination of relative expression levels for specific genes in workers, soldiers, presoldiers and nymphs was determined from quantitative PCR results in relation to the standard gene β-actin [43], using REST software [44] (see Additional data file 2).

#### Data deposition

Sequences have been deposited in GenBank under accession numbers AY258589, AY258590, BQ788178, BQ788190 and CB518295 through CB518319.

#### Additional data files

The following additional data are included with the online version of this article: Figures showing a summary of clones and clone sequences (Additional data file 1), the quantitative real-time PCR raw data (Additional data file 2), the troponin alignments (Additional data file 3), the β-NAC alignments (Additional data file 4), the BTB/POZ alignments (Additional data file 5), the *nanos* alignments and hydropathy profiles (Additional data file 6), the SH3-domain kinase alignments (Additional data file 7), the GAP alignments (Additional data file 8), and the real-time PCR primer sequences (Additional data file 9).

#### Acknowledgements

We thank Phillip San Miguel and Paul Parker of the Purdue University Genomics Core Facility for their expert assistance, Catina Ratliff, Jody Aleong and Jesse Hoteling for technical assistance with termites, and Bill

Pak and anonymous reviewers for helpful comments on manuscript drafts. This is journal article No. 17100 of the Agricultural Research Program of Purdue University, West Lafayette, Indiana.

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