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SHORT COMMUNICATION

# Conservation of *fruitless*' role as master regulator of male courtship behaviour from cockroaches to flies

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**Abstract** In *Drosophila melanogaster*, male courtship behaviour is regulated by the *fruitless* gene. In *D. melanogaster*, *fruitless* encodes a set of putative transcription factors that are sex-specifically spliced. Male-specific variants are necessary and sufficient to elicit male courtship behaviour. *Fruitless* sequences have been reported in other insect species, but there are no data available on their functional role. In the present work, we cloned and sequenced *fruitless* in males of the German cockroach, *Blattella germanica*, and we studied its expression in male brain and testes. *B. germanica fruitless* encodes a 350-amino acid protein with BTB and Zinc finger domains typical of *fruitless* sequences. Upon RNAi-mediated knockdown of *fruitless* in *B. germanica*, males no longer exhibit courtship behaviour, thus implying that *fruitless* is necessary for male sexual behaviour in our cockroach model. This suggests that the role of *fruitless* as master regulator of male sexual behaviour has been conserved along insect evolution, at least from cockroaches to flies.

**Keywords** *Blattella germanica* · Courtship behaviour · *Drosophila* · *Fruitless* · Insect · RNAi

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Elke Clynen and Laura Ciudad contributed equally to the work.

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## Introduction

Mating in animals, from insects to humans, is often preceded by elaborate courtship by the male. The genetic and molecular mechanisms underlying this complex sex-specific behaviour are well studied in the fruit fly *Drosophila melanogaster*. In a male fruit fly, the sequential stereotyped behaviours that lead to copulation are very characteristic. Briefly, the male orients himself towards the female, taps her with his forelegs, extends and vibrates one wing to sing a courtship song, licks her genitalia and finally attempts copulation (Hall 1994). Thanks to the advanced genetic tools available for *D. melanogaster*, more than 30 genes that affect some behavioural step in male courtship have been identified, but the gene *fruitless* (*fru*) appears to be the master regulator. Male flies lacking the *fru* gene are sterile, given that the last courtship steps, from singing to copulation, are abnormal or absent (Villella and Hall 2008).

In *D. melanogaster*, *fru* encodes a set of putative transcription factors containing a common broad complex,

tramtrack, bric à brac (BTB) N-terminal domain involved in protein–protein interactions and one of four possible C-terminal Zinc finger DNA-binding domains (A, B, C and D). A number of transcripts are generated through the use of four promoters (P1–P4) and alternative splicing at both the 5′ and 3′ ends. Only those transcripts generated by the most distal promoter P1 are spliced in a sex-specific way: in males by default and in females by the control of the sex-determination proteins Transformer (Tra) and Tra2 (Ito et al. 1996; Ryner et al. 1996). Male-specific *fru* transcripts appear to be necessary and sufficient for male sexual behaviour, which was demonstrated by activating the *fru* gene in neural cells in the female fly's brain and sensory organs, resulting in masculinised females, directing at other females a sexual display resembling that of their male counterparts (Demir and Dickson 2005).

In *D. melanogaster*, male-specific *fru* transcripts are expressed in ca. 2% of neurons in the male central nervous system, which are organised into 21 distinct clusters that are (all) interconnected in a circuit that is directly and specifically involved in male sexual behaviour. This circuit includes central, sensory and motor components. Among the sensory neurons are olfactory neurons that may be specialized for detection of female sex pheromones whereas the motor neurons innervate the penis and ejaculatory bulb. Recent reports (Cachero et al. 2010; Yu et al. 2010) have described significant differences between male and female *D. melanogaster* brains, not only anatomically but also in terms of wiring, which could explain, at least in part, the differences observed in sexual behaviour.

*D. melanogaster* is a much derived species within the evolutionary history of insects, and functional genomics data obtained in this fly cannot always be extrapolated to other species, especially to less derived ones. In this respect, our goal was to study the possible role of *fru* in male courtship behaviour in a phylogenetically basal insect, the German cockroach *Blattella germanica*. In *B. germanica*, courtship behaviour is also robust and easy to study. In an encounter, the male touches the female with the antennae, raises the wings upward and then it turns around 180° thus exposing the tergal gland to the female. The secretion of these glands stimulates the female to mount the male and feed, and while the female feeds on the tergal gland, the male pushes the abdomen under the female and clasps her genitalia with his left phallomere to accomplish genital connection (Roth and Willis 1952). This innate courtship behaviour is very different with respect to that of *D. melanogaster* commented above, and the question that arises is, are such evolutionary divergent male courtship behaviour in flies and cockroaches governed by the same master regulator *fru*?

## Materials and methods

### Insects

Staged males of *B. germanica* were obtained from a colony reared in the dark at 30±1°C and 60–70% relative humidity and fed on dog chow (Panlab 125) and water ad libitum. All dissections and treatments were carried out on carbon dioxide-anaesthetized specimens.

### Cloning and sequencing

Total RNA was isolated from whole male body using the GenElute Mammalian Total RNA kit (Sigma, Madrid, Spain). Degenerate primers based on conserved *fru* sequences from *Tribolium castaneum*, *Apis mellifera*, *Nasonia vitripennis*, *Chorthippus brunneus*, *D. melanogaster* and *Drosophila pseudoobscura* were used to obtain a *B. germanica* orthologue complementary DNA (cDNA) fragment by polymerase chain reaction (PCR) amplification. Primers used are indicated in Table S1. The PCR parameters were: 95°C (180 s), 45 cycles of 95°C (30 s), 50°C (30 s), 72°C (40 s) and a final extension of 72°C (300 s). The amplified fragment (303 base pairs) was subcloned into the pSTBlue™-1 vector (Novagen, Madison, WI, USA) and sequenced. By Basic Local Alignment Search Tool (BLAST) analysis, the sequence was confirmed to correspond to *fru*. The sequence was completed by 3′- and 5′- rapid amplification of cDNA ends (RACE) experiments (Invitrogen, Paisley, UK) according to the manufacturer's instructions. The PCR products were analysed by agarose gel electrophoresis, subcloned into the pSTBlue™-1 vector and sequenced. Primers used are indicated in Table S2.

### PCR studies

Total RNA was isolated from brain, testes, femoral muscle and accessory glands of adult males and from ovaries of adult females using the GenElute Mammalian Total RNA kit (Sigma). Samples represent a pool of five specimens. An aliquot of 200 ng from each RNA extraction was DNAse-treated (Promega, Madison, WI, USA) and reverse transcribed with Superscript II reverse transcriptase (Invitrogen) and random hexamers (Promega). Genomic DNA was removed by treatment with RNase-free DNase I (Invitrogen). To measure messenger RNA (mRNA) levels, quantitative real-time PCR (qRT-PCR) determinations were carried out using the cDNA products as templates and SYBR Premix Ex Taq™ (iQ SYBR Green supermix, Bio-Rad, Hercules, CA, USA). The iQ5 optical system software version 2.0 was used for detection (Bio-Rad). The PCR primer sequences used to amplify the *fru* transcript are indicated in Table S3. Determinations were carried out in triplicate and normalized

to the internal control of BgActin-5c (Accession number AJ862721) mRNA for each sample. The amplification protocol used for all genes was: initial denaturation at 95°C for 3 min followed by an amplification program for 45 cycles of 10 s at 95°C and 60 s at 60°C, with a final melting curve analysis at 95°C for 60 s, 60°C for 60 s and 81 cycles of 55°C for 30 s. Genomic control and no template were used as negative controls in duplicate. Statistical analysis of gene expression values was carried out using the relative expression software tool (REST) 2008 program (Pfaffl et al. 2002). This program calculates changes in gene expression between two groups, control and treated, using the corresponding distributions of Ct values as input. The program makes no assumptions about the distributions, evaluating the significance of the derived results by pair-wise fixed reallocation randomization test tool in REST.

### Courtship and mating behaviour

Individual adult males of chosen ages (from freshly emerged to 10-day-old) were placed in a cylindrical glass jar (4.5×8.0 cm) with two 5-day-old virgin females and observed for 30 min, during which we recorded whether the presence of the females elicited courtship behaviour (wing raising).

### RNAi experiments

Two different double-stranded RNAs (dsRNAs) were used in RNA interference (RNAi) studies, the first one encompassing a 302-bp fragment located between nucleotide 56 and 358 (dsBgFru-1), covering most of the BTB domain, and the second one encompassing a 287-bp fragment located between nucleotide 637 and 924 (dsBgFru-2), that is, between the BTB and the Zinc finger domains (Fig. S1). Both were amplified by PCR and subcloned into the pSTBlue$^{TM}$-1 vector. As control dsRNA (dsMock), we used a 92-bp noncoding sequence from the pSTBlue$^{TM}$-1 vector. dsRNA synthesis was performed as described earlier (Ciudad et al. 2006). The dsRNAs were re-suspended in diethyl pyrocarbonate-treated water, and 1 μl of the solution (at the concentration of 1 μg/μl of dsBgFru-1, dsBgFru-2 or dsMock) was injected into the abdomen of freshly emerged male fifth instar nymphs. To analyse the RNAi effect on target transcript levels, brain and testes of 5-day-old treated and control adult males were dissected and analysed individually by qRT-PCR as described above. RNAi effects in terms of courtship behaviour were studied at day 9 of the adult stage. Two 5-day-old virgin females were added to the jar, as in the experiments, to observe courtship and mating behaviour (see "Courtship and mating behaviour" Section), and the specimens were observed during 1 h. After that, the three specimens were left together in the same jar, and the presence of sperm in the spermathecae was examined in the two females 10 days later.

## Results and discussion

### Molecular cloning of *fru* in *B. germanica*

Cloning of *fru* cDNAs in *B. germanica* was accomplished by a RT-PCR approach using degenerate primers designed on the basis of conserved motifs of known *fru* sequences. Using cDNA from 5- to 7-day-old adult males of *B. germanica* as a template, a 303-bp PCR fragment was obtained, which was highly similar to the equivalent region in known insect *fru* sequences. 5′-RACE and 3′-RACE experiments using the same template gave a full-length cDNA of 1,175 bp. In the sequence (accession number FN429764), a putative start codon is preceded by in-frame stop codons, thus, suggesting that we obtained a full-length open reading frame. Database BLAST searches indicated that it encoded a *B. germanica* orthologue of Fru, which we called BgFru. It encodes a 350-amino acid protein, with a BTB and $C_2H_2$ Zinc finger domain (Fig. S1), organised as in other Fru sequences. Moreover, maximum-likelihood phylogenetic analysis indicated that BgFru has orthologous relationships with known Fru sequences of other insects (Fig. S2).

In *D. melanogaster*, male-specific Fru sequences have a ca. 110-amino acid N-terminal extension upstream of the BTB domain, which contain a stretch of 12 histidines alternating with neutral residues followed by a proline-rich stretch (Demir and Dickson 2005). The functional significance of these motifs is currently unknown, but several other *Drosophila* transcriptional regulators contain a similar histidine repeat (Ryner et al. 1996). However, this extension is absent in BgFru. The question whether *fru* sex-specific splicing occurs in hemimetabolan insects remains unanswered. Transcripts showing the typically male N-terminal extension have been reported in *D. melanogaster* (Demir and Dickson 2005), in another Diptera, the mosquito *Anopheles gambiae* (Gailey et al. 2006), and in the Hymenoptera *N. vitripennis* (Bertossa et al. 2009), the three species being holometabolan. Conversely, detailed studies on *fru* variants carried out in males of three Orthoptera species of the genus *Chorthippu*s, which is hemimetabolan, revealed only two different transcripts differing in the 5′ region, but having the coding sequences identical and starting immediately upstream the BTB domain, thus without having any extension typical of male-specific *fru* as in *D. melanogaster*, *A. gambiae* and *N. vitripennis* (Ustinova and Mayer 2006). Instead, the authors found a number of intraspecific *fru* paralogous sequences that slightly diverged from each other by single nucleotide substitutions, and they proposed that in *Chorthippu*s spp., different functions of *fru* are accomplished

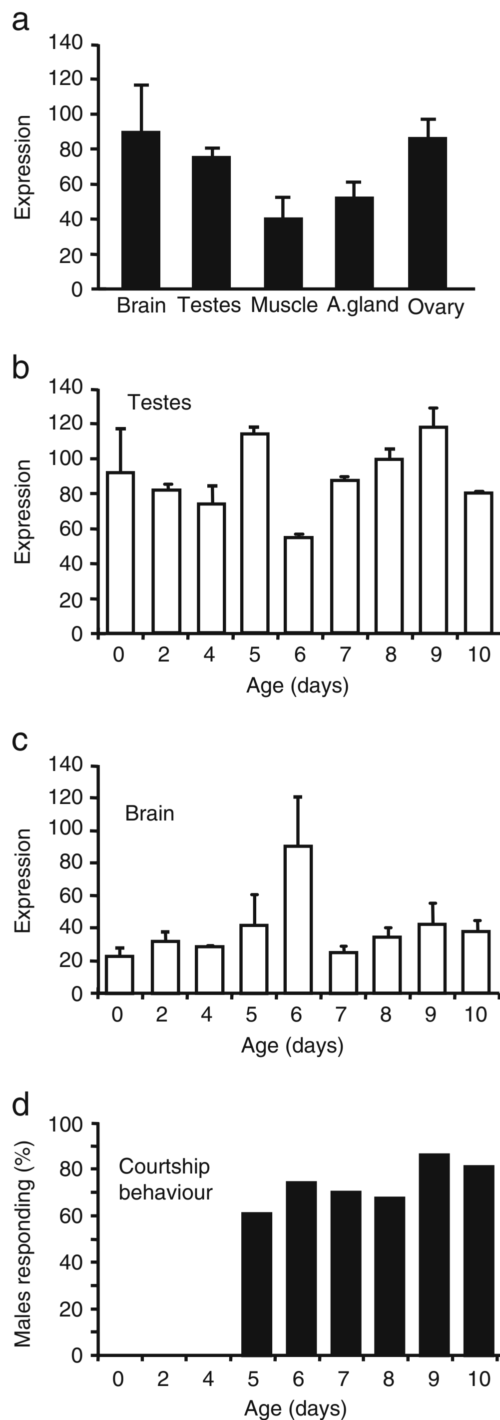


**Fig. 1** *Fruitless* in *Blattella germanica*. (**a**) Expression of BgFru mRNA in different male (brain, testes, femoral muscle and accessory glands) and female (ovaries) tissues obtained from 5- to 7-day-old adult specimens. Expression of BgFru mRNA in testes (**b**) and brain (**c**) of adult males measured by qRT-PCR; in all mRNA determinations, data represent three biological replicates (mean ± SEM) and are indicated as copies of BgFru mRNA per 1,000 copies of BgActin-5c. **d** Percentage of males of different adult ages that exhibited courtship behaviour after being exposed to two mature females ($n$=20–24)

by different paralogues whereas in *D. melanogaster*, they are regulated by alternative transcripts of the *fru* gene (Ustinova and Mayer 2006).

## Expression of BgFru and courtship behaviour in males of *B. germanica*

We first studied the expression of BgFru in different male and female tissues. qRT-PCR analyses revealed that BgFru is expressed in all tissues tested: brain, testes, femoral muscle and accessory glands from adult males and ovaries from adult females (Fig. 1a). Highest BgFru mRNA levels were found in male brain, testes and ovaries. The expression in ovaries shows that females express a *fru* transcript that could be the BgFru that we isolated in males or a different isoform containing the region amplified by the primers used (Fig. S1). The function of *fru* in *B. germanica* females was not studied in the present work.

We focused our study of BgFru in males by monitoring its expression in testes and brain tissues of adult specimens from emergence to day 10. In testes, mRNA levels oscillate between ca. 50 to ca. 120 copies of BgFru per 1,000 copies of BgActin-5c, but they do not show regular and dramatic fluctuations (Fig. 1b). The mRNA expression pattern in brain tissues showed low values (ca. 25 copies of BgFru per 1,000 copies of BgActin-5c) until day 4 of adult life, they then increased on day 5 (ca. 50 copies), and peaked on day 6 (ca. 100 copies; Fig. 1c). Possibly, the mRNA produced in this peak of transcription is translated into the protein necessary during the following sexually active days (see "RNAi of BgFru abolishes courtship behaviour in *B. germanica*" Section).

Interestingly, the expression pattern of BgFru in male brains is correlated with the temporal development of courtship capabilities. The study of courtship behaviour from adult emergence until day 10 (Fig. 1d) showed that males do not respond at all to the presence of mature females during the first 4 days of adult life. On day 5, 61% of the tested males responded to the presence of females by raising the wings and thus exposing the tergal glands. The percentage of response increased on day 6 (74%), slightly decreased on days 7 and 8 (70% and 67%, respectively), and increased again on days 9 and 10 (>80%; Fig. 1d). The males responded by raising the wings 3 to 11 min after adding the two females to the jar. The coincidence between the peak of expression in the brain and the onset of courtship suggests that BgFru has a role in this behaviour.

## RNAi of BgFru abolishes courtship behaviour in *B. germanica*

The role of BgFru in male *B. germanica* was studied by RNAi. For this purpose, freshly emerged fifth instar

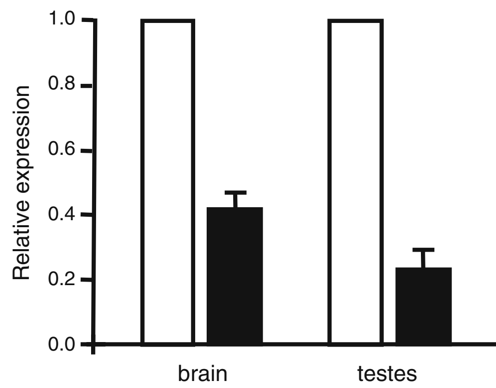


**Fig. 2** RNAi of *fruitless* in *Blattella germanica*. Expression of *fruitless* mRNA in testes and brain from 5-day-old adult males. qRT-PCR data were normalized against BgActin-5c expression, and data represent values of dsBgFru-1-treated specimens (*black columns*) normalized with respect to dsMock expression levels (*white columns*), and indicated as the mean ± SEM (*n*=3–4). According to REST software (Pfaffl et al. 2002), the expression in brain and testes is downregulated by a factor of 0.42 and 0.23, respectively

nymphs were treated with 1 µg of dsBgFru-1 (*n*=22) or dsMock (*n*=29). Both experimental groups moulted to sixth instar nymphs 6 days later and then to adults 8 days later. On day 6 of adult life, we measured the mRNA levels of BgFru in testes and brain of both groups and found that transcript levels were significantly lower in dsBgFru-1-treated specimens with respect to those treated with dsMock (Fig. 2). On day 9 of the adult stage, when males are fully responsive to the presence of females (Fig. 1d), courtship behaviour was examined in dsBgFru-1 and dsMock groups. Results (Table 1) indicated that ca. 80% of the 29 dsMock-treated males touched the antennae of one or the other female partners and raised the wings after 12 min of interaction, on average. Then, as expected, they turned around 180°, thus exposing the tergal gland to the females, and different attempts of copulation followed. Conversely, none of the 22 dsBgFru-1-treated males exhibited any wing raising behaviour, despite that they had a number of physical contacts with the two mature female partners (Table 1). Ten days later, the females used in the experiments were dissected to assess the presence of spermatozoids in the spermathecae. Only 2 out of the 44 females used in the experiments with dsBgFru-1-treated males had formed an ootheca (in *B. germanica*, the formation of unviable oothecae by virgin females is not unusual), and none of these 44 females had spermatozoids in the spermathecae. Conversely, all the 56 females that had been in contact with dsMock-treated males formed an ootheca and had spermatozoids in the spermathecae, thus indicating that all them achieved mating.

To assess the specificity of the behavioural effects, a second dsRNA, dsBgFru-2, was tested following the same procedures used for dsBgFru-1. The results were similar to those obtained with dsBgFru-1. Fifteen out of 18 males (ca. 83%) treated with dsMock in this set of experiments showed the typical sequence of courtship behaviour, starting with antennae touching and wing raising within the first 15 min of observations. On the contrary, none of the 16 dsBgFru-2-treated males raised the wings or showed any sign of courtship behaviour during the observations.

This is the first functional evidence for a role of *fru* in sex-specific behaviour outside dipterans and indicates that *fru* regulates courtship in males also in *B. germanica*, as it occurs in *D. melanogaster*, in spite of the disparate courtship behaviours shown by these two species. The fact that *fru* regulates male courtship in cockroaches, which is a considerably basal insect group from a phylogenetical point of view, suggests that this function might be one of the most ancestral of *fru*.

Inspired by the formidable success of forward genetics in the identification of key regulators of morphogenetic processes, a number of authors predicted that single genes might have the role of master regulators of complex innate behaviours (Dulac 2005; Lorenz 1981). Several years ago, *fru* was proven to be one of such genes, in this case, regulating male courtship behaviour, in the evolutionarily derived dipteran *D. melanogaster* (Demir and Dickson 2005). Our present results on a cockroach model show that *fru* not only plays such a role of master regulator of a complex innate behaviour, but also that this role has been conserved along insect evolution, at least from the phylogenetically basal order Blattaria to the most distal Diptera.

**Table 1** Effects of treatments with dsBgFru-1 and dsMock on male courtship behaviour and mating in *Blattella germanica*. Treatments were carried out in penultimate nymphal instar and effects were studied in 9-day-old adults

| Treatment | N | Males exhibiting courtship | Time elapsed until courtship (min ± SEM) | Othecae formed by the female partners | Presence of spermatozoids in the spermathecae of the female partners |
|---|---|---|---|---|---|
| dsMock | 29 | 23 (79%) | 12.0 ± 1.7 | 58 females, 58 oothecae | Present in the 58 females used |
| dsBgFru-1 | 22 | 0 (0%) | – | 44 females, 2 oothecae | Absent in the 44 females used |

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## SUPPLEMENTARY TABLES

**Table S1.** Degenerate primers used to obtain a *fruitless* orthologue cDNA fragment in *Blattella germanica*.

| Forward/Reverse | Primer sequence |
|---|---|
| Forward | 5’-TGYYTNMGNTGGAAYAAYCAYCC-3’ |
| Reverse | 5’-TCNGCNGTYTTNARRAACATNGG-3’ |

**Table S2.** Primers used in 3’- and 5’-RACE experiments to complete the *fruitless* sequence of *Blattella germanica*.

| Experiment | Forward/Reverse | Primer sequence |
|---|---|---|
| 3’-RACE | Forward 1 | 5'-CCCATCCTCATCCGATAATATTT-3' |
| 3’-RACE | Nested forward 2 | 5'-GTATGAAGGAGAAGTTAATGTTAAGCC-3' |
| 5’-RACE | Reverse 1 | 5'-GCTTGATAGGAGGTGGTGATGG-3' |
| 5’-RACE | Nested reverse 2 | 5’-CTTCTTCGATCTCGGGGTGTTATGG-3’ |

**Table S3.** Primer sequences used in qRT-PCR to amplify the BgFru transcript in *Blattella germanica*.

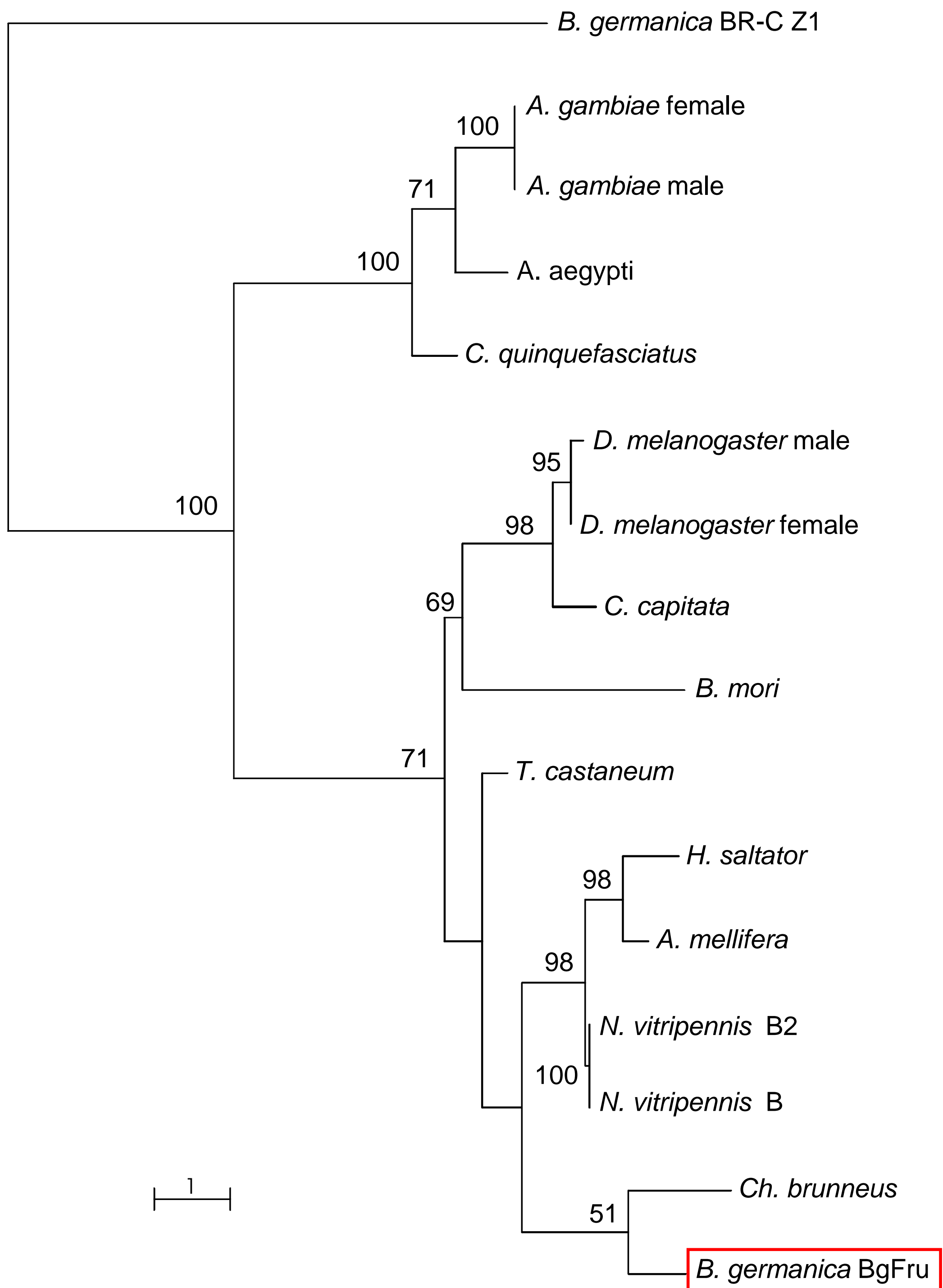
| Forward/Reverse | Primer sequence |
|---|---|
| Forward | 5’-GTGGTGCAGGTGATCGGTTT-3’ |
| Reverse | 5’-GCTTGATAGGAGGTGGTGATGG-3’ |

## SUPLEMENTARY FIGURES

## Figure S1

```
   1   TTCACGCAAGGTGTCAGTCAAGTTGTAATAGAATTAAATATGGACCAACAATTCTGTCTGCGATGGAACAAC
   1                                            M  D  Q  Q  F  C  L  R  W  N  N

  73   CACCAAAAGAACTTAACTGATGTACTGAGTGGCTTACTGCAAAGAGAAGTTTTAGTAGATGTAACTCTTGCT
  12    H  Q  K  N  L  T  D  V  L  S  G  L  L  Q  R  E  V  L  V  D  V  T  L  A

 145   TGTGATGGTGAAACATTTAGAGCACATCAGACAATTTTATCAGCTTGCAGCCCTTATTTTGAAAGTATTTTC
  36    C  D  G  E  T  F  R  A  H  Q  T  I  L  S  A  C  S  P  Y  F  E  S  I  F

 217   CTTCAAAATACCCATCCTCATCCGATAATATTTTTAAGAGATGTGAATTATACGGAAATGAAGGCTCTTTTA
  60    L  Q  N  T  H  P  H  P  I  I  F  L  R  D  V  N  Y  T  E  M  K  A  L  L

 289   CAGTTTATGTATGAAGGAGAAGTTAATGTAAGCCAAAACTTGCTTCCAATGTTTTTAAAGACTGCAGAGGCT
  84    Q  F  M  Y  E  G  E  V  N  V  S  Q  N  L  L  P  M  F  L  K  T  A  E  A

 361   CTTCAAATTCGTGGACTTGCTGACAATGCAGTTAGTAGTAAAAAGTCTGATGACCAAATGTCACCAGCAGTG
 108    L  Q  I  R  G  L  A  D  N  A  V  S  S  K  K  S  D  D  Q  M  S  P  A  V

 433   AACTCTCCAGCAAGAAATTCAGAACACAGTAGGCCCAGTAGTCCAATGCCAGAAAAACGAAAACGAAAATCT
 132    N  S  P  A  R  N  S  E  H  S  R  P  S  S  P  M  P  E  K  R  K  R  K  S

 505   TCTGGGAATTGTGATATGTCTGGTGGTGCAGGTGATCGGTTTCATTCGGATTCTCAGACATCACAGTGCAGT
 156    S  G  N  C  D  M  S  G  G  A  G  D  R  F  H  S  D  S  Q  T  S  Q  C  S

 577   TTCAAATCCAACCCTGCCTCCCTCCCAAAATTAAATCCCATAACACCCGAGATCGAAGAAGTTGTGGATTTA
 180    F  K  S  N  P  A  S  L  P  K  L  N  P  I  T  P  E  I  E  E  V  V  D  L

 649   CCACCATCACCACCTCCTATCAAGCAAGAGGTCGATGCAAGCCATTCAGAATATAAAGAGCCTTACATGAAT
 204    P  P  S  P  P  P  I  K  Q  E  V  D  A  S  H  S  E  Y  K  E  P  Y  M  N

 721   ATGTCAGAATCACTTGCACTACCTTCGGGTGGTGTTGGTATTCTGAACCCCAGCGATATGAACTCTCTTCAT
 228    M  S  E  S  L  A  L  P  S  G  G  V  G  I  L  N  P  S  D  M  N  S  L  H

 793   GGACCAAGTTCTTTGGATGCTAGTGACCAGGAGCAGGGACCAGCTTCACAGGACACCTTGGATGGCCTTGAT
 252    G  P  S  S  L  D  A  S  D  Q  E  Q  G  P  A  S  Q  D  T  L  D  G  L  D

 865   GGCTCGTATACACCACACAAGTCAGGACATTTGAGAATGTATGATAAACTTCCTATGGGTTTGGCTCAATGC
 276    G  S  Y  T  P  H  K  S  G  H  L  R  M  Y  D  K  L  P  M  G  L  A  Q  C

 937   AAAATGTGCGGAAAAACTGTGACAAACATGAGAAATCATTATTTATCTCATTTTCCTGAAAATCATCAATGT
 300    K  M  C  G  K  T  V  T  N  M  R  N  H  Y  L  S  H  F  P  E  N  H  Q  C

1009   AACATATGCTTGAAATTTTTTTCAAGATCAGATAGTCTCAAACTGCATTATCGTAGGAAGCATTTAAATCAA
 324    N  I  C  L  K  F  F  S  R  S  D  S  L  K  L  H  Y  R  R  K  H  L  N  Q

1081   ACATATGAATAATGGTTATAAGAATTGTGATACACAAAAGACAGGAGACATTGTTAAAATAAAATTAGATAC
 348    T  Y  E  *

1153   AAAATTAAAAAAAAAAAAAAAAA 1175
```

**Figure S2**



## FIGURE LEGENDS

**Figure S1.** Nucleotide sequence and deduced amino acid sequence of the cDNA corresponding to *B. germanica fruitless*. The BTB domain is highlighted in black with white lettering, and the Zinc finger in highlighted in grey with black lettering. The region encompassed by the dsBgFru-1 is indicated in yellow and that of dsBrFru-2 in blue. The primers used for qRT-PCR experiments are indicted in green.

**Figure S2.** Phylogenetic tree showing the position of *B. germanica fruitless* (BgFru, red square) (FN429764) with respect to other insect *fruitless* sequences obtained from GenBank. These were: *Chortippus brunneus* (ABD85043.1), *Tribolium castaneum* (NP_00157690), *Harpegnathos saltator* (EFN81805.1), *Apis mellifera* (XP_392552.3), *Nasonia vitripennis* isoform B (NP_001157604.1) and isoform B2, which contains a 5' extension (NP_001157607.1), *Bombyx mori* (EU649701.1), *Anopheles gambiae* male-specific isoform C (AAU50567.1), *A. gambiae* female-specific isoform C (AAU50568.1), *Aedes aegypti* (XP_001657625.1), *Culex quinquefasciatus* (XP_001860373.1), *Drosophila melanogaster* male-specific isoform B (NP_732344.1), *D. melanogaster* female-specific isoform H (NP_732346.1) and *Ceratitis capitata* (AAF22527.1). The Z1 isoform of Broad complex (BR-C Z1) of *B. germanica* (CBJ05857.1) (Piulachs et al. 2010), which contains a BTB and a Zinc finger domain, was used as external group. Protein sequences were aligned with ClustalX (Thompson et al. 1997) but with manually adjusting the zinc finger regions. The resulting alignment was analyzed by the PHYML3.0 program (Guindon and Gascuel 2003) based on the maximum-likelihood principle with the amino acid substitution model. Four substitution rate categories with a gamma shape parameter of 1.444 were used. The data were

bootstrapped for 100 replicates using PHYML. Branch lengths are proportional to sequence divergence. The bar represents 1 difference per site. Bootstrap values >50 are shown in the node of clusters. The topology indicates that *B. germanica* BgFru is close to *Ch. brunneus fru*, and that both cluster with the hymenopteran node (*A. mellifera*, *N. vitripennis* and *H. saltator*), whereas the beetle *T. castaneum* is the sister group of all them.

## SUPPLEMENTARY REFERENCES

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Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25 (24):4876-4882.