Neotrop Entomol (2014) 43:362–372
DOI 10.1007/s13744-014-0216-y

SYSTEMATICS, MORPHOLOGY AND PHYSIOLOGY

Three Heat Shock Protein Genes from *Bactrocera* (*Tetradacus*) *minax* Enderlein: Gene Cloning, Characterization, and Association with Diapause

ZC Lü[1], LH Wang[1,3], GF Zhang[1], FH Wan[1,2], JY Guo[1,2], H Yu[3], JB Wang[3]

[1]State Key Lab for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Science, Beijing, China

[2]Center for Management of Invasive Alien Species, Ministry of Agriculture, Beijing, China

[3]Dept of Entomology, Henan Institute of Science and Technology, Xinxiang, Henan Province, China

Keywords

Diapause, gene characterization, gene expression profile, heat shock protein

Correspondence
FH Wan, State Key Lab for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Science, Beijing 100081, China; wanfanghao@caas.cn

Edited by Anne-Nathalie Volkoff – INRA / Univ of Montpellier

Z. C. Lü and L. H. Wang contributed equally to this work.

Received 28 March 2013 and accepted 31 March 2014
Published online: 13 June 2014

© Sociedade Entomológica do Brasil 2014

Abstract

Bactrocera (*Tetradacus*) *minax* Enderlein is a major pest to wild and cultivated species of citrus. *Bactrocera minax* produces one generation per year with a long pupal diapause period of over 6 months, which hinders efforts to obtain vast numbers of insects under standard room conditions. Determining the mechanisms of diapause is significantly important for obtaining large quantities of these insects. To characterize the heat shock protein (Hsp) genes of *B. minax* and to unravel their potential contribution to diapause, we performed 3′ and 5′ RACE to isolate the complementary DNA (cDNA) sequences, bioinformatics to examine the phylogenetic relationships, and real-time quantitative PCR to detect the expression patterns of three Hsp genes during various developmental stages. These results represent the first characterization of the three Hsp genes of *B. minax*; the open reading frames of *Bmhsp23*, *Bmhsp70*, and *Bmhsp90* were 510, 1,911, and 1,089 bp, encoding 170, 636, and 363 amino acids, respectively. BmHsp70 and BmHsp90 displayed high identity to previously identified Hsp70 and Hsp90 genes, respectively. BmHsp23 displayed varying similarity, from 28 to 83%, to previously identified small Hsps. *Bmhsp23* messenger RNA (mRNA) expression was found to be upregulated during diapause initiation, maintenance, and termination. *Bmhsp70* mRNA expression peaked during diapause initiation. *Bmhsp90* mRNA expression remained at a relatively low level during deep diapause. Our present results suggest that *Bmhsp70* might play an important role in diapause initiation, while *Bmhsp23* in diapause initiation and maintenance and *Bmhsp90* in diapause regulation. These results improve our understanding of the mechanism of diapause in *B. minax* at the molecular level.

Introduction

Bactrocera (*Tetradacus*) *minax* Enderlein (Diptera: Tephritidae; Chinese citrus fruit fly) is unique with respect to its feeding behavior and its reproductive rate of one generation per year (van Schoubroeck 1999). *Bactrocera minax* has been causing considerable losses in commercial citrus for more than half a century in China (Wang & Luyi 1995). A series of strategies, including spraying of chemical pesticides and food attractants (Yang *et al* 1994, Wang & Luyi 1995, van Schoubroeck 1999), has been used to manage this pest. However, these measures have not effectively reduced its population density. Studies have revealed that the sterile insect/male technique (SIT) (Wang & Zhang 1993) and the genetic transformation technique play important roles in the prevention and control of fruit fly pests (Wimmer 2003, Vreysen *et al* 2007). The SIT and the genetic transformation technique have good potential to prevent and control

B. minax, as the pest is unique with respect to its feeding behavior and its reproductive rate of one generation per year. However, *B. minax* exhibits a long period for pupa development (more than 6 months), which hinders the production of large quantities of these insects. Therefore, understanding and breaking diapause is a key step to obtain vast numbers of insects by producing two or more generations per year, which is important for the development of new techniques to control this pest.

Diapause is a common developmental strategy used by insects to survive winter and other periods of seasonal adversity (Xu 2008, Denlinger *et al* 2007). *Bactrocera minax* survives winter via pupa diapause from late October to late April or even early May (Wang & Luyi 1995). The mechanism of insect diapause is complex, and understanding the molecular mechanism involved in diapause initiation and regulation will provide the basic information required to break diapause. Insect diapause may be regulated by environmental, hormonal, and molecular factors, and understanding this molecular regulation remains in its infancy (Denlinger 2002, Xu 2008).

A number of diapause-specific genes have been identified based on their expression patterns during early, mid- and/or late diapause (Denlinger 2002, Xu 2008). Regulation of a subset of heat shock protein (Hsp) genes may be related to different types of insect diapause (Denlinger 2002, MacRae 2010, Xiao *et al* 2011). Hsps are a highly conserved superfamily of molecular chaperones that facilitate appropriate protein folding and localization while preventing protein aggregation (Feder & Hofmann 1999, Hartl & Hayer-Hartl 2002). Previous studies have demonstrated that Hsps play a major role in diapause regulation in a wide range of organisms (Yuan *et al* 1996, Denlinger *et al* 2001, Qiu & MacRae 2008a, b, MacRae 2010). During diapause, Hsps are thought

Table 1 Primer sequences used for cDNA cloning and real-time quantitative PCR.

Gene		Primer sequence (5′➞3′)	Fragment length (bp)
PCR			
hsp23		GCATGGACGTGCAGCARTTYAARCC CGGGGCCCACCTGYTGDATYTG	286
hsp70		GATGCAGTCATCACAGTTCCAGC AACAGAGATCCCTCGTCGATGGT	239
hsp90		GCGGCGGGTGTTCATHATGGAYAA GGTGCTGGATCACGTAYTCRTCDAT	504
3′RACE			
hsp23	GSP1 GSP2	AATCCGAGTGAGTTGGCTGTGAAGG TCGCTACGCTTTGCCGAAGGGATT	368
hsp70	GSP1 GSP2	GTATTGCTGGTTTGAATGTTCTGCG ATCTTCGATTTGGGCGGTGGTAC	1,518
hsp90	GSP1 GSP2	TGGAATTGATCGAAGAGCTTACTG CTTCTGGTGATGATGCCGCTTCCTT	1,154
5′RACE			
hsp23	GSP1 GSP2 GSP3	CACGCTCGTTGGACTTAT CCATCCGAAGATAATGTGGAAACC CAAAGCGTAGCGACGTACAAAGT	461
hsp70	GSP1 GSP2 GSP3	CTCGTCGATGGTCAAGAT AGTACCACCGCCCAAATCGAAGA GATTCGCAGAACATTCAAACCAG	691
hsp90	GSP1 GSP2 GSP3	AGTTGCTTACTTGCTCCTTC ATAAGGAAGCGGCATCATCACCAG CAGTAAGCTCTTCGATCAATTCCA	474
Real-time quantitative PCR			
hsp23		AATCCGAGTGAGTTGGCTGTG ATCCGAAGATAATGTGGAAACC	168
hsp70		TGCCAAGAACATCACCATCA GTTCATCTTCGTCGGCATAA	101
hsp90		TTTTGCGTTACCATACCTCG AGTTGCTTACTTGCTCCTTC	129
α-TUB		CGCATTCATGGTTGATAACG GGGCACCAAGTTAGTCTGGA	184

to contribute to cell cycle arrest and increased stress resistance (Denlinger *et al* 2001, Rinehart *et al* 2007, MacRae 2010). Hsp gene expression patterns during diapause may be highly variable between species (Rinehart & Denlinger 2000, Denlinger *et al* 2001, Yocum 2001, Tungjitwitayakul *et al* 2008). Different classes of Hsps can play distinct roles in diapause within a species (Goto *et al* 1998, Rinehart & Denlinger 2000, Rinehart *et al* 2000, 2007, Goto & Kimura 2004, Aruda *et al* 2011). The characteristics of Hsp genes and their mRNA expression profiles in *B. minax*, as well as the relationship between Hsp gene expression and diapause, remain unknown.

To unravel the potential contribution of Hsp genes to diapause in *B. minax*, we hypothesized that different Hsp genes play distinct roles in regulating *B. minax* diapause. In this study, we isolated cDNA sequences from three Hsp genes, and examined the expression patterns of these Hsp genes during various developmental stages. We also examined the phylogenetic relationships between these Hsps by comparing them with Hsps from other insect taxa. This study represented the first characterization of Hsp genes in *B. minax* and their expression patterns during different stages of diapause. The present results improve our understanding of the mechanism of diapause in *B. minax* at the molecular level.

Material and Methods

Insects

Bactrocera (*Tetradacus*) *minax* eggs were collected from citrus plants in Zhangjiachong cun Jingzhou, Hubei province in August 2011. The eggs in the citrus plants were placed at 26±1°C with a 12L:12D photoperiod for hatching. Newly hatched larvae were reared in citrus plants in the laboratory. The late third instars jumped into bottles filled with fine sand containing a moisture content of 10–15%. The third late instars pupated in the fine sand and were then transferred to 17±1°C with a 12 L:12D photoperiod until adult eclosion.

Sampling at various developmental stages

Diapause is generally separated into three stages: prediapause, diapause, and postdiapause (Denlinger 2002, Xu 2008). Prediapause includes the induction and preparation phases. First, it was generally considered based on our observations that prediapause in *B. minax* corresponded to organisms from second instars to 30-day pupa. We regarded the period from about the second instar to the first pupal day as the induction phase and the period from about the first to the 30th pupal day as the preparation phase. Diapause included the initiation, maintenance, and termination phases. Diapause of *B. minax* was about from 30- to 150-day pupa. It was considered that diapause initiation occurred at about day 30, diapause maintenance occurred from day 30 to day 120, and diapause termination occurred from day 120 to day 150. Based on these considerations, we choose every tenth day during the diapause termination phase to measure the exact termination time based on the dynamics of Hsp gene expression. Finally, postdiapause comprised organisms from approximately 150- to 160-day-old pupae. Collectively, based on the various possible time points corresponding to each diapause stage, and to reveal the exact timing of each diapause stage at the molecular level, the following time points were chosen to examine the expression profile of Hsp genes: eggs and first, second, and third instars; 1, 7, 30, 60, 90, 120, 130, 140, 150, and 160-day-old pupae; and newly emerged adults (<24 h). Three replicates were performed for each time point.

Reverse transcription PCR and rapid amplification of cDNA ends

Total RNA was isolated using the RNeasy Mini Kit (QIAGEN, Valencia, CA, USA), and 2 μg RNA was used to generate the cDNA using the oligo(dT)$_{15}$ primer according to the instructions provided with the reverse transcription system (Invitrogen Life Technologies, Burlington, ON, Canada). Degenerate primers (Table 1) were used to amplify partial segments of the Hsp genes. Then, 5′ and 3′ rapid amplification of cDNA ends (RACE) were performed to obtain full-length cDNAs according to the manufacturer's instructions (Rapid Amplification of cDNA Ends System, version 2.0, Invitrogen, Carlsbad, CA, USA) using gene-specific primers corresponding to GSP1 and GSP2 (Table 1). To ensure that the 5′ and 3′ fragments were derived from the same gene, specific primer sets flanking the open reading frames (ORFs) were designed and used to amplify the full-length cDNAs.

Sequence analysis of hsp cDNA

The *hsp* cDNAs from other species were used as query sequences to search for alternative insect Hsp genes in the GenBank database using the BLAST software available on the NCBI website (http://www.ncbi.nlm.gov/BLAST/). Sequence alignment and identity analyses were performed using DNAMAN (version 5.0, Lynnon BioSoft, Quebec, Canada). The ORFs were identified using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The amino acid sequences and the molecular weight of the proteins were calculated using DNASTAR. The accession numbers of *Bmhsp23*, *Bmhsp70*, and *Bmhsp90* are KJ541737, KJ541738, and KJ541739, respectively.

Homology and phylogenetic analyses

To evaluate the molecular evolutionary relationship of Hsps from various insects, phylogenetic trees were constructed based on their protein sequences. Sequence homology searches were performed using BLAST, and all sequences were retrieved from GenBank using BLAST-N and BLAST-X. The retrieved sequences were aligned using the multiple alignment tool of the ClustalX program. Gaps and missing data were excluded from the data analysis. MEGA 5.1 was used to perform the tree calculations. The tree constructions were performed using the maximum parsimony method. Support for the nodes was assessed as a proportion of 1,000 bootstrap replicates to derive the confidence values of the phylogeny analysis.

Real-time quantitative PCR

Total RNA from the samples was extracted using the RNeasy Mini Kit (QIAGEN, Valencia, CA, USA), and the RNase-Free Set (QIAGEN, USA) was used to remove genomic DNA. The quantity and quality of the RNA were assessed via spectrophotometry (Beckman Du 650 spectrophotometer, Fullerton, CA, USA), and the A260/A280 ratios were typically above 1.8. The RNA quality was also evaluated via 1% agarose gel electrophoresis. According to the manufacturer's instructions, 2 μg total RNA was used to synthesize cDNAs using the SuperScriptTM III Reverse transcriptase kit (Invitrogen Life Technologies, Burlington, ON, Canada). The cDNA was stored at −80°C until further analysis.

The mRNA expression levels of *hsp23*, *hsp70*, and *hsp90* from eggs, larvae, pupae, and adults were examined via quantitative real-time PCR analysis. The sequences of the primers are listed in Table 1. The reactions were performed using an iQTM 5 real-time PCR detection system (BioRad, Foster City, CA, USA). The amplification volume was 20 μL, including 0.5 μL of the forward primer (10 mM/μL), 0.5 μL of the reverse primer (10 mM/μL), 10.0 μL of SYBR Mix, 0.4 μL of Rox, 1.0 μL of the cDNA sample, and 7.6 μL of ultra-pure water. The PCR cycle conditions were as follows: 94°C for 5 min, followed by 40 cycles of amplification consisting of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min, and then 72°C for 10 min. After the amplification phase, a dissociation curve was generated to ensure that there was only one product. A control without any template was included in all batches. The amplification efficiency of each gene was validated by constructing a standard curve using five serial dilutions of cDNA. The data were analyzed based on the C_p method according to the mathematical model of Pfaffl (2001), simplified to $2^{-\Delta\Delta Ct}$ as follows:

$$^{-\Delta\Delta}Ct = (C_p\text{target}-C_p\text{reference})_{\text{treatment}}-(C_p\text{target}-C_p\text{reference})_{\text{control}}.$$

Eggs were used as a control, and alpha-tubulin (α-TUB) was used as the reference gene based on our preliminary experiments, which revealed that α-TUB was stably expressed throughout the various developmental stages (unpublished data). The relative expression level of each *hsp* mRNA was defined as the fold-change normalized to the amount of α-TUB. Each sample was assessed in triplicate.

Statistical analyses

Statistical analyses were performed using the SPSS software package (version 13). Prior to all statistical analyses, the data were examined with respect to assumptions of normality using the Kolmogorov-Smirnov test. The Hsp gene expression levels on the different development stages were analyzed using one-way ANOVA followed by the least significant difference (LSD) test (p values ≤0.05).

Results and Discussion

Cloning, characterization and homology, phylogenetic, and expression profile analyses of Bmhsp23

The full-length cDNA of *B. minax hsp23* (*Bmhsp23*) is 805 bp, including a 5′-terminal UTR of 136 bp, a 3′-terminal UTR of 164 bp containing a poly(A) tail, and an ORF of 510 bp encoding a polypeptide of 170 amino

```
1    GGGGGGGGGAAATTAGAAGTCGTTGAACAGGCCACGACAAATTCGAAAACAAAACTTTTA
61   CTAAAAATCAAAGCGAAAGAACTGTTTTCTATTTTTGTATTCTTGAGAAGTGAATAAATT
121  AAATACAGAAGTGAAAATGTCGACTCTACCATTAATCTTGAGTTTAACTAATGATCTTGG
1                     M  S  T  L  P  L  I  L  S  L  T  N  D  L  G
181  TCGCTTGACACCATTCTATGAACCTGGGTTCTACACTCAATGGCCAGCAATCACAACTTC
16      R  L  T  P  F  Y  E  P  G  F  Y  T  Q  W  P  A  I  T  T  S
241  ACCTAGTGGCCGGTTGCGGAAACTTGAGAAAGATTTACCCCTAGCCGCTATTGGAAAGGA
36      P  S  G  R  L  R  K  L  E  K  D  L  P  L  A  A  I  G  K  D
301  CGGCTTCCGCCCTTGCATGGACGTGCAGCATTTTAATCCGAGTGAGTTGGCTGTGAAGGT
56      G  F  R  P  C  M  D  V  Q  H  F  N  P  S  E  L  A  V  K  V
361  GGTCGATGATCATATCGTGGTCGAGGGTAAACACGAGGAACGTGAAGATGATCATGGTTA
76      V  D  D  H  I  V  V  E  G  K  H  E  E  R  E  D  D  H  G  Y
421  TATCTCACGACACTTTGTACGTCGCTACGCTTTGCCGAAGGGATTCGAAGCCGATAAAGT
96      I  S  R  H  F  V  R  R  Y  A  L  P  K  G  F  E  A  D  K  V
481  GGTTTCCACATTATCTTCGGATGGTGTTTTAACAGTTAGTGTACCAAAACCGGCCATCGA
116     V  S  T  L  S  S  D  G  V  L  T  V  S  V  P  K  P  A  I  E
541  AGATAAGTCCAACGAGCGTGTGATCCAAATTCAACAAACTGGACCAGCTCATTTGAATGT
136     D  K  S  N  E  R  V  I  Q  I  Q  Q  T  G  P  A  H  L  N  V
601  GAAGGAGAATCCCGAGGAAACTACCAAGGAAGAGAAGTCAAAAGCTTAAAAGTTGTGCAT
156     K  E  N  P  E  E  T  T  K  E  E  K  S  K  A  *
661  TGATAACTTAATCAGTCACTATTTCATTATTTCGTACAATGTTTTGTTCAGTATTTATAA
721  GCGTTAATTAATTCGTAGGAGAAGAAGAGTCTGTTCACAATTTAAGTTTGAAATAAAAT
781  TTCATTGAAAATTGAAAAAAAAAAA
```

Fig 1 The full-length cDNA sequence of *Bactrocera* (*Tetradacus*) *minax hsp23* (*Bmhsp23*) and its deduced amino acid sequence. The character shading (ATG) indicates the translational start codon. The *asterisk* indicates the translational termination codon (TAA). The termination signal is in *bold*, and the poly(A) tail is *underlined*. The typical sequences characteristic of the sHspα crystal domains are *double-underlined*.

acids with a predicted molecular mass of 19.03 kDa and a theoretical isoelectric point of 6.10 (Fig 1). The typical α crystal domains of sHsps were located from amino acid positions 49 to 131. In addition, homology analysis revealed that the deduced amino acid sequence of BmHsp23 displayed varying similarity, from 28 to 83%, to previously identified sHsps (Supplementary Material Table 1). Furthermore, as shown in Fig 2, Hsps in the order Diptera are differentiated into two clusters, and an orthologous cluster contained several sHsps from different insect orders, which suggested that these sHsps evolved prior to species divergence (Kokolakis *et al* 2008, Liu *et al* 2012).

The small heat shock protein gene is an important diapause regulatory gene. A possible role for sHsp genes in diapause is their involvement in the regulation of cell cycle arrest (Tammariello & Denlinger 1998). It was reported that sHsps were upregulated during diapause in a variety of insect species (Rinehart *et al* 2007), which suggested that sHsps are key players in the overwintering response of many insects (Gkouvitsas *et al* 2008). The sHsp mRNA expression level peaked in diapause pupae of *Plutella xylostella* Linnaeus and *Lactuca sativa* Linnaeus (Sonoda *et al* 2006, Huang *et al* 2009). Aruda *et al* (2011) found that the *hsp22* expression level was elevated during deep diapause in *Calanus finmarchicus* Gunnerus. The Hsp23 gene was highly upregulated during *Sarcophaga crassipalpis* diapause and was implicated in diapause entry (Rinehart *et al* 2007). Different sHsp genes displayed distinct functions in *Sesamia nonagrioides* Lefèbvre, and it was found that SnoHsp19.5 mRNA was consistently expressed throughout diapause, whereas SnoHsp20.8 mRNA was downregulated during

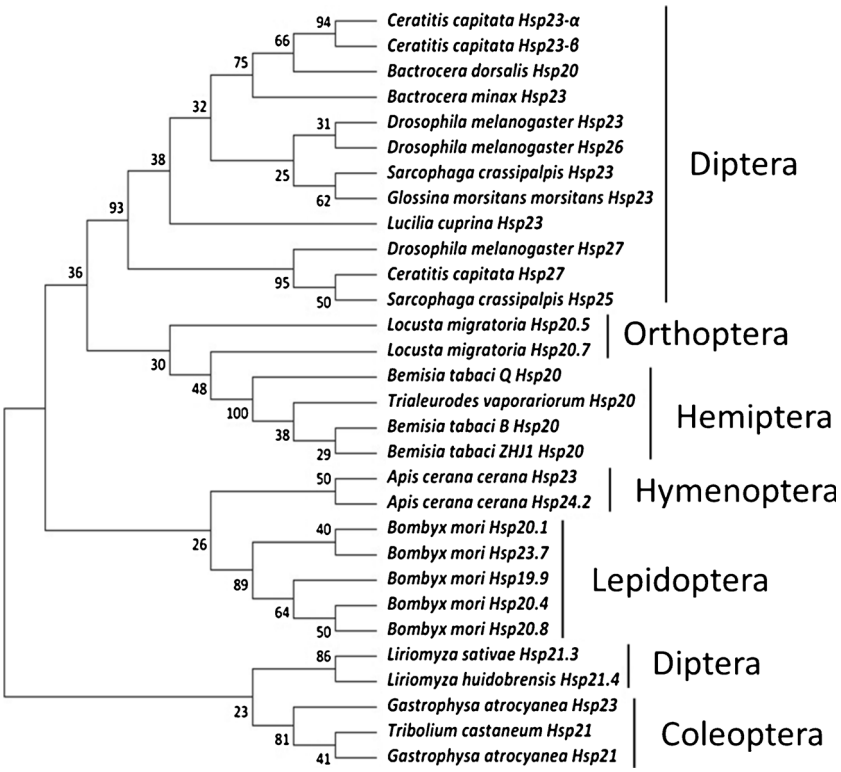

Fig 2 A phylogenetic tree based on the known amino acid sequences of sHsp was generated via maximum parsimony analysis, and this tree was used to determine the relationships between different insects. The *numbers* above the branches indicate the percentages of bootstrap replicates in which each species was grouped together. The *scale bar* indicates the number of substitutions per site for each unit branch length. The bootstrap values of 1,000 replicates are displayed for each branch. *Apis cerana* Hsp23 (AEH05930); *Apis cerana* Hsp24.2 (AEH05929); *Bactrocera dorsalis* Hsp20 (AEJ88464); *Bactrocera minax* Hsp23 (KJ541737); *Bemisia tabaci B* Hsp20 (ACH85196); *Bemisia tabaci Q* (ADG03464); *Bemisia tabaci ZHJ1* Hsp20 (ADG03467); *Bombyx mori* Hsp19.9 NP_001036984; *Bombyx mori* Hsp20.1 (NP_001036941); *Bombyx mori* Hsp20.4 (NP_001037038); *Bombyx mori* Hsp20.8 (NP_001091794); *Bombyx mori* Hsp23.7 (BAD74198); *Ceratitis capitat a*Hsp23-α (ACG58883); *Ceratitis capitata* Hsp23-β (ACG58884); *Ceratitis capitata* Hsp27 (EU700493); *Drosophila melanogaster* Hsp23 (AAA28637); *Drosophila melanogaster* Hsp26 (AAF50288); *Drosophila melanogaster* Hsp27 (AAF50285); *Gastrophysa atrocyanea* Hsp21 (BAD91164); *Gastrophysa atrocyanea* Hsp23 (BAD91165); *Glossina morsitans* Hsp23 (ADD18977); *Liriomyza huidobrensis* Hsp21.4 (DQ452370); *Liriomyza sativae* Hsp21.3 (ABE57138); *Locusta migratoria* Hsp20.5 (ABC84492); *Locusta migratoria* Hsp20.7 (ABC84494); *Lucilia cuprina* Hsp23 (AFA36667); *Sarcophaga crassipalpis* Hsp23 (AAC63387); *Sarcophaga crassipalpis* Hsp25 (ABL06941); *Trialeurodes vaporariorum* Hsp23 (ACH85200); *Tribolium castaneum* Hsp21 (XP_974390).

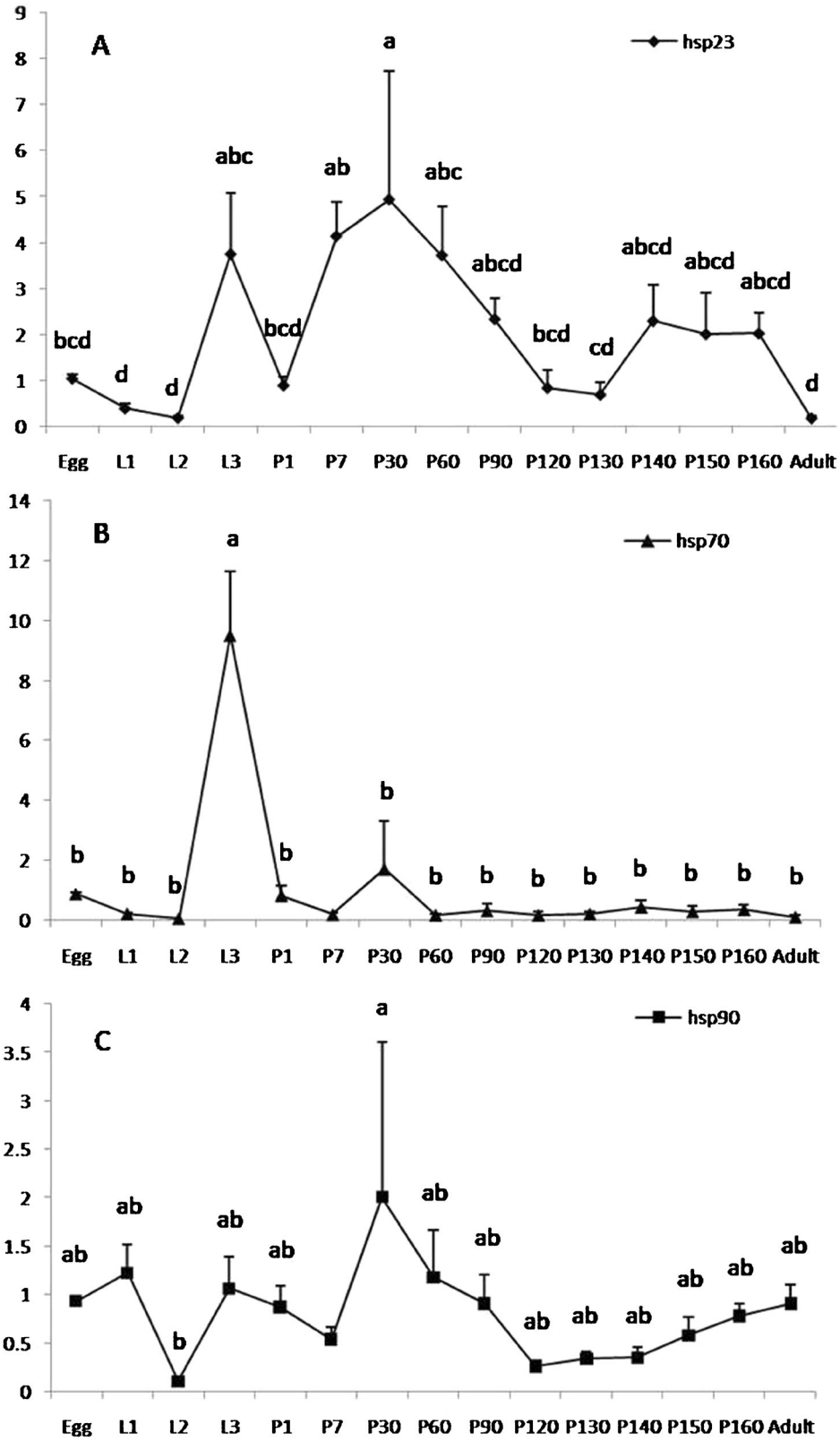

Fig 3 Hsp gene (*a* hsp23, *b* hsp70, and *c* hsp90) mRNA expression during each developmental stage in *Bactrocera* (*Tetradacus*) *minax*. L1, L2, and L3 represent the first, second, and third instars, respectively. P1, P7, P30, P60, P90, P120, P130, P140, P150, and P160 represent 1, 7, 30, 60, 90, 120, 130, 140, 150, and 160-day old pupae, respectively. The results are expressed as means + SEM. The differences were considered significant for p values ≤ 0.05.

mid-diapause and was upregulated upon diapause termination (Gkouvitsas *et al* 2008). In the present study, *Bmhsp23* mRNA expression was significantly different among the developmental stages of *B. minax* studied ($F_{14,71}$=2.519, p=0.007) (Fig 3 *a*). *Bmhsp23* expression was upregulated during diapause initiation, maintenance, and termination, suggesting it plays a key role during diapause.

Cloning, characterization and homology, phylogenetic, and expression profile analyses of Bmhsp70

The full-length cDNA of the Bmhsp70 was 2262 bp long, including a 5′-UTR of 178 bp, a 3′-UTR of 180 bp containing a poly(A) tail, and an ORF of 1,911 bp encoding a polypeptide of 636 amino acids with a predicted molecular mass of 69.42 kDa and a theoretical isoelectric point of 5.36 (Fig 4). The IDLGTTYS and DLGGGTFD motifs were located at amino acid positions 6–13 and 196–203, respectively. The nonorganellar conserved motif RARFEEL was located at amino acid positions 297 to 303. The end of BmHsp70 was characteristic of the cytosolic Hsp70-specific EEVD motif. The predicted ATP-GTP binding domain was AEAYLGTT, which was located at amino acid positions 128 to 135 (Fig 4). Additionally, homology analysis revealed that the deduced amino acid sequence of BmHsp70 displayed high identity, from 70 to 96%, to the previously identified inducible Hsp70 (Supplementary Material Table 2) and that the sequence was highly conserved. Furthermore, as shown in Fig 5, the inducible Hsp70 from insects of the same order were clustered into the same group, which was consistent with traditional taxonomy.

The role of hsp70 in mediating diapause varied greatly among insect taxa. For example, changes in hsp70 expression were not a factor in *Lucilia sericata* Wiedemann larval diapauses (Tachibana *et al* 2005), *Helicoverpa zea* Boddie pupal diapause (Zhang & Denlinger 2009) or *Drosophila triauraria* Bock & Wheeler adult diapause (Goto *et al* 1998).

Hsp70 mRNA expression decreased upon diapause initiation in *Omphisa fuscidentalis* Hampson and remained lower at the pupal stage, which indicated that *hsp70* was not associated with *O. fuscidentalis* diapause (Tungjitwitayakul *et al* 2008). In *S. nonagrioides*, *hsp70* expression was downregulated during larval diapause (Gkouvitsas *et al* 2009). However, *hsp70* expression was strongly induced during *Megachile rotundata* Fabricius pupal diapause (Yocum *et al* 2005). In the present study, we found that *Bmhsp70* expression was significantly different between various developmental stages ($F_{14,69}$=12.225, p<0.001) (Fig 3 *b*). *Bmhsp70* expression peaked during diapause initiation in third instars, which suggested that *Bmhsp70* might play an important role in diapause induction in *B. minax*.

Cloning, characterization and homology, phylogenetic, and expression profile analyses of Bmhsp90

The partial cDNA sequence of *B. minax hsp90* (*Bmhsp90*) was 1,720-bp long (Fig 6). It was found that the present Hsp90 sequence may represent the second half of the full-length cDNA (including the 3′ end) and consisted of about half of the

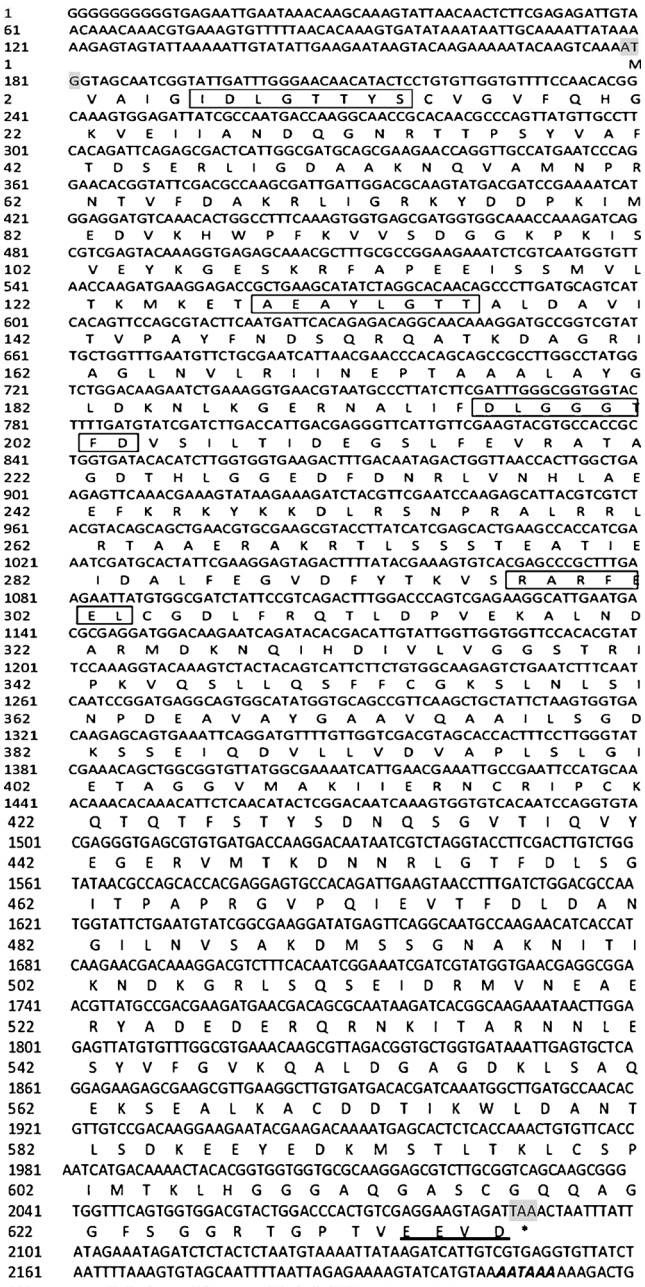

Fig 4 The full-length cDNA sequence of *Bactrocera* (*Tetradacus*) *minax hsp70* (*Bmhsp70*) and its deduced amino acid sequence. The character shading (ATG) indicates the translational start codon. The *asterisk* indicates the translational termination codon (TAA). The termination signal is in *bold*, and the poly(A) tail is *underlined*. The characteristic Hsp70 motifs are *boxed*, and the cytosolic Hsp70-specific motif is *double-underlined.*

full-length cDNA. The present Hsp90 cDNA sequence included a 3′-terminal UTR of 180 bp containing a poly(A) tail and an ORF of 1,089 bp encoding a polypeptide of 363 amino acids. The end motif of MEEVD was identified. One characteristic Hsp90 family sequence, GVVDSEDLPLNISRE, was detected. In addition, homology analysis revealed that compared to previously identified Hsp90 genes, the identity of the deduced amino acid sequence of BmHsp90 varied from 76 to 98% (Supplementary Material Table 3), indicating that the Hsp90 sequence is highly conserved. Hsp90 from Orthoptera, Hymenoptera, Hemiptera, Diptera, and Coleoptera were clustered into the same large group, which revealed that Hsp90 from these orders is highly conserved (Fig 7). However, the genetic distance of Hsp90 from Lepidoptera indicated several evolutionary divergences.

Hsp90 displayed a different expression pattern during insect diapause. In the present study, *Bmhsp90* mRNA expression was not significantly different between the various diapause stages ($F_{14,72}$=1.013, p=0.453) (Fig 3 *c*). *Bmhsp90* mRNA was expressed

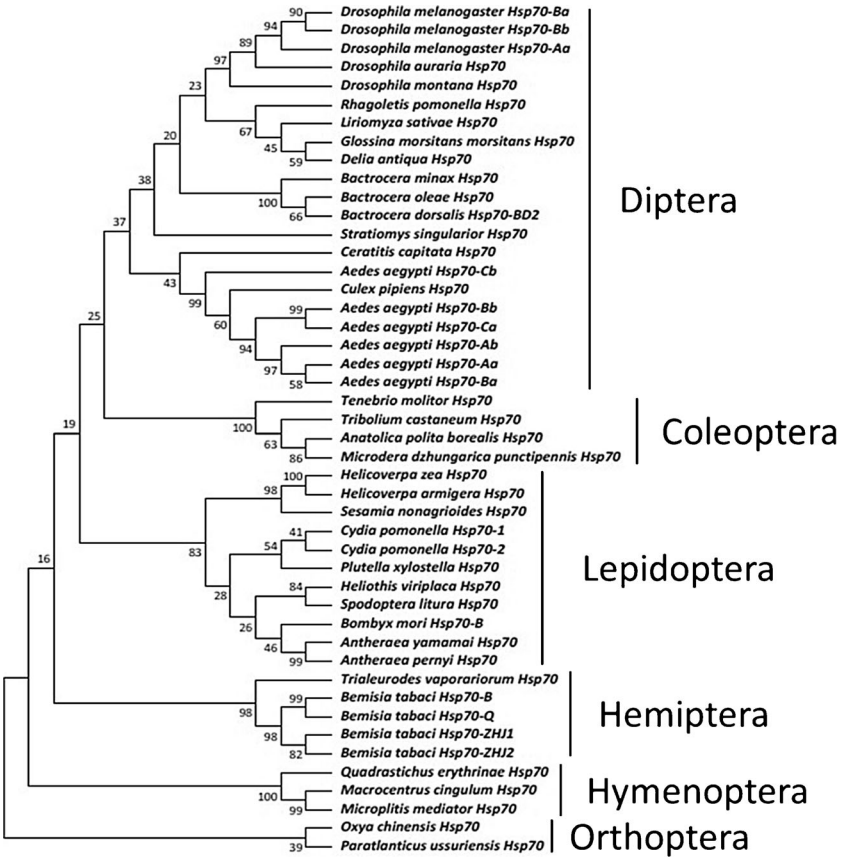

Fig 5 A phylogenetic tree based on the known amino acid sequences of inducible Hsp70 was generated via maximum parsimony analysis, and this tree was used to determine the relationships between different insects. The *numbers* above the branches indicate the percentages of bootstrap replicates in which each species was grouped together. The *scale bar* indicates the number of substitutions per site for each unit branch length. The boots trap values of 1,000 replicates are displayed for each branch. *Aedes aegypti* Hsp70Aa (ACJ64193); *Aedes aegypti* Hsp70Ab (ACJ64194); *Aedes aegypti* Hsp70Ba (ACJ64195); *Aedes aegypti* Hsp70Bb (ACJ64196); *Aedes aegypti* Hsp70Ca (ACJ64197); *Aedes aegypti* Hsp70Cb (ACJ64198); *Anatolica polita borealis* Hsp70 (ABQ39970); *Anopheles albimanus* Hsp70A2 (AAC41543); *Antheraea pernyi* Hsp70 (ADI50267); *Antheraea yamamai* Hsp70 (BAD18974); *Bactrocera dorsalis* Hsp70BD2 (ADQ12986); *Bactrocera minax* Hsp70 (KJ541738); *Bactrocera oleae* Hsp70 (CAI44197); *Bemisia tabaci* Hsp70 (ACH85197); *Bemisia tabaci* Hsp70 (ADG03465); *Bemisia tabaci* ZHJ1 Hsp70 (ADG03468); *Bemisia tabaci* ZHJ2 Hsp70 ADO14473; *Bombyx mori* Hsp70B (AEI58996); *Ceratitis capitata* Hsp70 (AAC23392.1); *Culex pipiens* Hsp70 (AAX84696); *Cydia pomonella* Hsp70-1 (AFK93489); *Cydia pomonell*a Hsp70-2 (AFK93490); *Delia antiqua* Hsp70 (AAY28732); *Drosophila auraria* Hsp70 (CAA55168); *Drosophila melanogaster* Hsp70Aa (AAN13535); *Drosophila melanogaster* Hsp70Ba (AAN13545); *Drosophila melanogaster* Hsp70Bb (AAN13546); *Drosophila Montana* Hsp70 (ACB59072); *Glossina morsitans* Hsp70 (ADD19447); *Helicoverpa armigera* Hsp70 (ADP37711); *Helicoverpa zea* Hsp70 (ACV32640); *Heliothis viriplaca* Hsp70 (ACS72236); *Liriomyza sativae* Hsp70 (AAW32099); *Macrocentrus cingulum* Hsp70 (ACD84944); *Microdera dzhungarica punctipennis* Hsp70 (AEB52075); *Microplitis mediator* Hsp70 (ABV55505); *Oxya chinensis* Hsp70 (AFN08643); *Paratlanticus ussuriensis* (Hsp70 AEP68850); *Plutella xylostella* Hsp70 (ADV58255); *Quadrastichus erythrinae* Hsp70 (AFC76151); *Rhagoletis pomonella* Hsp70 (ABL06948); *Sesamia nonagrioides* Hsp70 (ABZ10939); *Spodoptera litura* Hsp70 (ADV03160); *Stratiomys singularior* Hsp70 (ACB59073); *Tenebrio molitor* Hsp70 (AFE88580); *Trialeurodes vaporariorum* Hsp70 (ACH85201); *Tribolium castaneum* Hsp70 (XP_974442).

throughout life and remained at a relatively low level during deep diapause of *B. minax*. The low expression level of *Bmhsp90* mRNA might be attributed to a reduction in the level of ecdysone, leading to an increase in *Bmhsp23* mRNA expression (MacRae 2010). It was reported that the *hsp90* expression level was downregulated during pupal diapause of *S. crassipalpis* Macquart (Rinehart & Denlinger 2000), while it was upregulated during diapause termination of *L. sericata* (Tachibana *et al* 2005), and it was constantly expressed throughout pupal diapause of *M. rotundata* (Yocum *et al* 2005). Fan *et al* (2013) found that Hsp90, Hsp70, Hsp20.8, and Hsp20.4 were highly expressed in both diapause and nondiapause eggs of *Bombyx mori* Linnaeus and suggested they may play an important role in initial embryonic development regardless of the occurrence of diapause.

In summary, different Hsp genes play distinct roles in *B. mina* during the various diapause stages and that these Hsp genes interact with one another. This study represented the first characterization of Hsp genes in *B. minax* and their mRNA expression profiles during different diapause stages. The unique physiological expression patterns suggested that the Hsp genes play distinct roles in the regulation of diapause in *B. minax. Bmhsp70* might play an important role in initiation diapause, *Bmhsp23* might play a key role in diapause initiation and maintenance, and *Bmhsp90* might play a minor role in the regulation of diapause. Our data improve our understanding of the mechanisms of diapause in *B. minax* at the molecular level. However, the precise physiological function of these Hsp genes during diapause in *B. minax* warrants further investigation.

Acknowledgments We thank Fu-Lian Wang for her help in the collection of insects. This study was supported by the National Basic Research and Development Program (Grant No. 2009CB119200), the Ministry of Science and Technology, China, and the Commonwealth Special Fund for the Agricultural Industry (no. 200903047).

Electronic Supplementary Material The online version of this article (doi:10.1007/s13744-014-0216-y) contains supplementary material, which is available users.

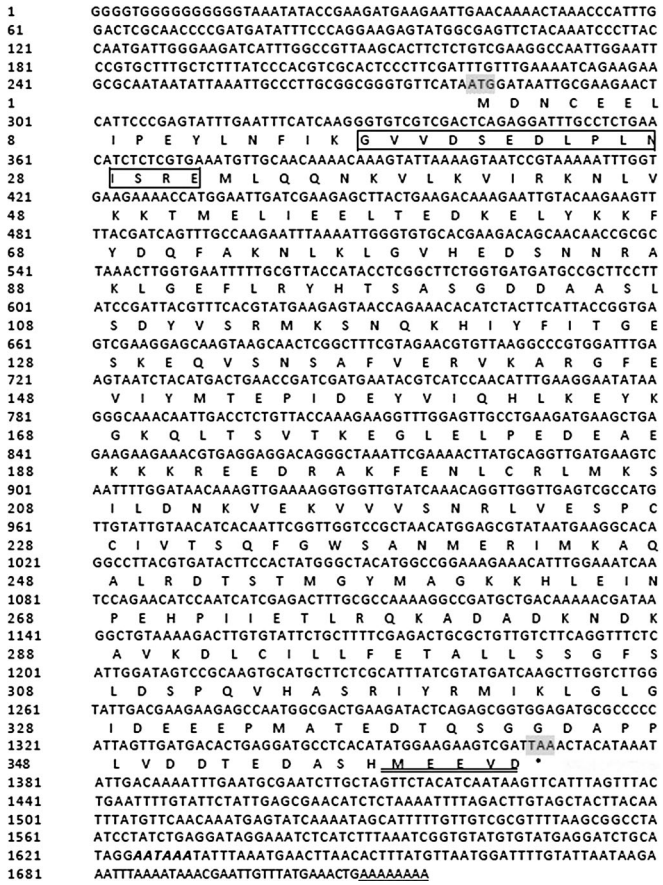

Fig 6 The partial cDNA sequence of *Bactrocera* (*Tetradacus*) *minax hsp90* (*Bmhsp90*) and its deduced amino acid sequence. The character shading (ATG) indicates the translational start codon. The *asterisk* indicates the translational termination codon (TAA). The termination signal is in *bold*, and the poly(A) tail is *underlined*. The C-terminal end motif of Hsp90 is *double-underlined*.

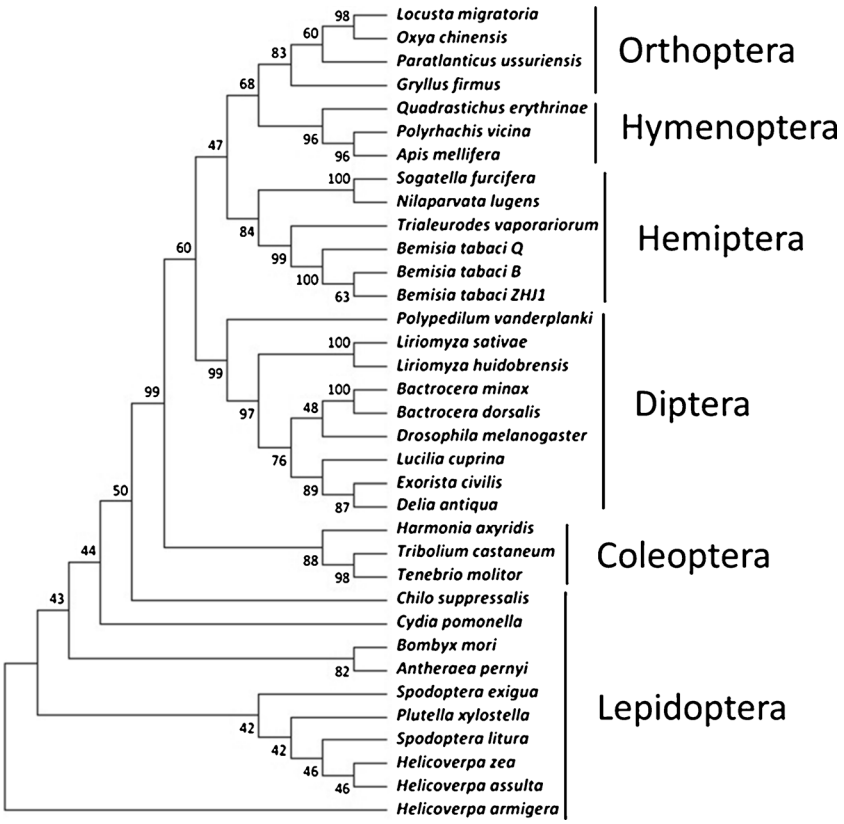

Fig 7 A phylogenetic tree based on the known amino acid sequences of Hsp90 was generated via maximum parsimony analysis, and this tree was used to determine the relationships between different insects. The *numbers* above the branches indicate the percentages of bootstrap replicates in which each species was grouped together. The *scale bar* indicates the number of substitutions per site for each unit branch length. The bootstrap values of 1,000 replicates are displayed for each branch. *Antheraea pernyi* (ADD91573); *Apis mellifera* (NP-001153536); *Bactrocera dorsalis* (AEJ88466); *Bactrocera minax* Hsp90 (KJ541739); *Bemisia tabaci B* (ACH85198); *Bemisia tabaci Q* (ADG03466); *Bemisia tabaci ZHJ1* (ADG03469); *Bombyx mori* (ADG57739); *Chilo suppressalis* (BAE44307); *Cydia pomonella* (AFA35118); *Delia antiqua* (CAI64494); *Drosophila melanogaster* (AAF47734); *Exorista civilis* (ACD63052); *Gryllus firmus* (ADK64952); *Harmonia axyridis* (ACL50550); *Helicoverpa armigera* (ADP37710); *Helicoverpa assulta* (ADM26742); *Helicoverpa zea* (ACV32639); *Liriomyza huidobrensis* (AAW49252); *Liriomyza sativae* (AAW49253); *Locusta migratoria* (AAS45246); *Lucilia cuprina* (ABQ42553); *Nilaparvata lugens* (ADE34169); *Oxya chinensis* (AFN08644); *Paratlanticus ussuriensis* (AFP54306); *Plutella xylostella* (BAE48742); *Polypedilum vanderplanki* (ADM13380); *Polyrhachis vicina* (AEM76721); *Quadrastichus erythrinae* (AFC76152); *Sogatella furcifera* (AFK64820); *Spodoptera exigua* (ACL77779); *Spodoptera litura* (ADM26738); *Tenebrio molitor* (AFN02497); *Trialeurodes vaporariorum* (ACH85202); *Tribolium castaneum* (NP-001094067).

References

Aruda AM, Baumgartner MF, Reitzel AM, Tarrant AM (2011) Heat shock protein expression during stress and diapause in the marine copepod *Calanus finmarchicus*. J Insect Physiol 57:665–675

Denlinger DL (2002) Regulation of diapause. Annu Rev Entomol 47:93–122

Denlinger DL, Rinehart JP, Yocum GD, Denlinger DL, Giebultowicz JM, Saunders DS (2001) Stress proteins: a role in insect diapause? In: Denlinger DL, Giebultowicz JM, Saunders DS (eds) Insect timing: circadian rhythmicity to seasonality. Elsevier Science, Netherlands, pp 155–171

Denlinger D, Li A, Rinehart J (2007) Heat shock proteins are critical for insect winter survival. Comp Biochem Physiol A 146:149–157

Fan LF, Lin JR, Zhong YS, Liu JY (2013) Shotgun proteomic analysis on the diapause and non-diapause eggs of domesticated silkworm *Bombyx mori*, PLoS One doi: 10.1371/journal.pone.0060386

Feder ME, Hofmann GE (1999) Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annu Rev Physiol 61:243–282

Gkouvitsas T, Kontogiannatos D, Kourti A (2008) Differential expression of two small Hsps during diapause in the corn stalk borer *Sesamia nonagrioides* (Lef.). J Insect Physiol 54:1503–1510

Gkouvitsas T, Kontogiannatos D, Kourti A (2009) Cognate Hsp70 gene is induced during deep larval diapause in the moth *Sesamia nonagriodes*. Insect Mol Biol 18:253–264

Goto SG, Kimura MT (2004) Heat-shock-responsive genes are not involved in the adult diapause of *Drosophila triauraria*. Gene 326:117–122

Goto SG, Yoshida KM, Kimura MT (1998) Accumulation of Hsp70 mRNA under environmental stresses in diapausing and nondiapausing adults of *Drosophila triauraria*. J Insect Physiol 44:1009–1015

Hartl FU, Hayer-Hartl M (2002) Protein folding-molecular chaperones in the cytosol: from nascent chain to folded protein. Science 295:1852–1858

Huang LH, Wang CZ, Kang L (2009) Cloning and expression of five heat shock protein genes in relation to cold hardening and development in the leafminer, *Liriomyza sativa*. J Insect Physiol 55:279–285

Kokolakis G, Tatari M, Zacharopoulou A, Mintzas AC (2008) The hsp27 gene of the Mediterranean fruit fly, *Ceratitis capitata*: structural characterization, regulation and developmental expression. Insect Mol Biol 17:699–710

Liu ZH, Xi DM, Kang MJ, Guo XQ, Xu BH (2012) Molecular cloning and characterization of Hsp27.6: the first reported small heat shock protein from *Apis cerana cerana*. Cell Stress Chaperones 17:539–551

MacRae TH (2010) Gene expression, metabolic regulation and stress tolerance during diapause. Cell Mol Life Sci 67:2405–2424

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29(9):e45

Qiu ZJ, MacRae TH (2008a) ArHsp21, a developmentally regulated small heat shock protein synthesized in diapausing embryos of *Artemia franciscana*. Biochem J 411:605–611

Qiu ZJ, MacRae TH (2008b) ArHsp22, a developmentally regulated small heatshock protein produced in diapause-destined Artemia embryos, is stress inducible in adults. FEBS J 275:3556–3566

Rinehart JP, Denlinger DL (2000) Heat-shock protein 90 is down-regulated during pupal diapause in the flesh fly, *Sarcophaga crassipalpis*, but remains responsive to thermal stress. Insect Mol Biol 9:641–645

Rinehart JP, Yocum GD, Denlinger DL (2000) Developmental upregulation of inducible hsp70 transcripts, but not the cognate form, during pupal diapause in the flesh fly, *Ssarcophaga crassipalpis*. Insect Biochem Mol Biol 30(6):515–521

Rinehart JP, Li A, Yocum GD, Robich RM, Hayward SAL, Denlinger DL (2007) Up-regulation of heat shock proteins is essential for cold survival during insect diapause. PNAS 104:11130–11137

Sonoda S, Ashfaq M, Tsumuki H (2006) Cloning and nucleotide sequencing of three heat shock protein genes (hsp90, hsc70, and hsp19.5) from the diamondback moth, *Plutella xylostella* (L.) and their expression in relation to developmental stage and temperature. Arch Insect Biochem Physiol 62:80–90

Tachibana SI, Numata H, Goto SG (2005) Gene expression of heat-shock proteins (Hsp23, Hsp70 and Hsp90) during and after larval diapause in the blow fly *Lucilia sericata*. J Insect Physiol 51:641–647

Tammariello SP, Denlinger DL (1998) G0/G1 cell cycle arrest in the brain of *Sarcophaga crassipalpis* during pupal diapause and the expression pattern of the cell cycle regulator, proliferating cell nuclear antigen. Insect Biochem Mol Biol 28:83–89

Tungjitwitayakul J, Tatun N, Singtripop T, Sakurai S (2008) Characteristic expression of three heat shock-responsive genes during larval diapause in the bamboo borer *Omphisa fuscidentalis*. Zool Sci 25:321–333

Van Schoubroeck F (1999) Learning to fight a fly: developing citrus IPM in Bhutan. PhD thesis. Wageningen University and Research Centre, Wageningen, The Netherlands, p 200

Vreysen MJB, Robinson AS, Hendrichs J (2007) Area-wide control of insect pests: from research to field implementation. Alphey LS: Engineering Insects for the Sterile Insect Technique, pp 51–60

Wang XJ, Luyi L (1995) Research progress in the Chinese citrus fruit fly. Entomol Knowl 32:310–315

Wang H, Zhang H (1993) Control of the Chinese citrus fly, *Dacus citri* (Chen), using the sterile insect technique, pp 505–512. In: Proceedings of an International Symposium on Management of Insect Pests, Vienna, 19–23 October 1992. International Atomic Energy Agency, Vienna

Wimmer EA (2003) Applications of insect transgenesis. Nat Rev 4:225–233

Xiao HJ, Wei ZJ, Xue FS (2011) Progress in heat shock proteins (Hsps) related to insect diapauses. Acta Entomol Sin 54(9):1068–1075

Xu WH (2008) Progress in insect diapauses. Acta Entomol Sin 45(4):512–518

Yang P, Carey JR, Dowell RV (1994) Tephritid fruit flies in China: historical background and current status. Pan Pac Entomol 70:159–167

Yocum GD (2001) Differential expression of two HSP70 transcripts in response to cold shock, thermoperiod, and adult diapause in the Colorado potato beetle. J Insect Physiol 47:1139–1145

Yocum GD, Kemp WP, Bosch J, Knoblett JN (2005) Temporal variation in overwintering gene expression and respiration in the solitary bee *Megachile rotundata*. J Insect Physiol 51:621–629

Yuan Y, Crane DD, Barry CE (1996) Stationary phase-associated protein expression in Mycobacterium tuberculosis: function of the mycobacterial alpha-crystallin homolog. J Bacteriol 178(15):4484–4492

Zhang QR, Denlinger DL (2009) Molecular characterization of heat shock protein 90, 70 and 70 cognate cDNAs and their expression patterns during thermal stress and pupal diapause in the corn earworm. J Insect Physiol 56:138–150