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Research Article

Genome-wide comparison of genes involved in the biosynthesis, metabolism, and signaling of juvenile hormone between silkworm and other insects

Daojun Cheng, Meng Meng, Jian Peng, Wenliang Qian, Lixia Kang and Qingyou Xia

State Key Laboratory of Silkworm Genome Biology, Southwest University, Chongqing, China.

Abstract

Juvenile hormone (JH) contributes to the regulation of larval molting and metamorphosis in insects. Herein, we comprehensively identified 55 genes involved in JH biosynthesis, metabolism and signaling in the silkworm (*Bombyx mori*) as well as 35 in *Drosophila melanogaster*, 35 in *Anopheles gambiae*, 36 in *Apis mellifera*, 47 in *Tribolium castaneum*, and 44 in *Danaus plexippus*. Comparative analysis showed that each gene involved in the early steps of the mevalonate (MVA) pathway, in the neuropeptide regulation of JH biosynthesis, or in JH signaling is a single copy in *B. mori* and other surveyed insects, indicating that these JH-related pathways or steps are likely conserved in all surveyed insects. However, each gene participating in the isoprenoid branch of JH biosynthesis and JH metabolism, together with the *FPPS* genes for catalyzing the final step of the MVA pathway of JH biosynthesis, exhibited an obvious duplication in Lepidoptera, including *B. mori* and *D. plexippus*. Microarray and real-time RT-PCR analysis revealed that different copies of several JH-related genes presented expression changes that correlated with the dynamics of JH titer during larval growth and metamorphosis. Taken together, the findings suggest that duplication-derived copy variation of JH-related genes might be evolutionarily associated with the variation of JH types between Lepidoptera and other insect orders. In conclusion, our results provide useful clues for further functional analysis of JH-related genes in *B. mori* and other insects.

Keywords: juvenile hormone, biosynthesis, metabolism, signaling, gene duplication.

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Introduction

In insects, the sesquiterpenoid juvenile hormone (JH) is synthesized and released from the corpora allata (CA) and cooperates with the steroid 20-hydroxyecdysone (20E) that is synthesized and released from the prothoracic glands to orchestrate insect molting, growth, metamorphosis, via stage-specific changes in the titers of these two endocrine hormones (Dubrovsky, 2005). JH activity is elevated early in each larval instar to maintain larval shape and characteristics, whereas the titer of 20E is always increased at the end of each larval instar to trigger the transition instar from larva to larva (Bownes and Rembold, 1987; Riddiford, 1994; Wyatt and Davey, 1996; Futahashi and Fujiwara, 2008). In the final larval instar, the JH titer is remarkably decreased and the 20E titer is increased to a very high level enough to initiate a metamorphic transition from larva to pupa. In addition, JH has been showed to regulate aging and reproduction in insects (Riddiford, 2012; Yamamoto *et al.*, 2013; Zou *et al.*, 2013).

The dynamic change in JH titer is mainly modulated through biosynthetic and metabolic pathways that are catalyzed by different sets of endogenous enzymes (Li *et al.*, 2004; Minakuchi *et al.*, 2006; Noriega *et al.*, 2006; Kinjoh *et al.*, 2007). As summarized in Figure S1, JH biosynthesis involves the mevalonate (MVA) pathway and the isoprenoid branch. The MVA pathway includes eight enzymatic steps and uses acetyl-CoA to generate farnesyl diphosphate (FPP) as a JH precursor. Then, the isoprenoid branch converts the FPP into JH through several continuous steps of oxidization and epoxidation (Kinjoh *et al.*, 2007). In addition, allatotropin (AT) and allatostatin (AS), which belong to the neuropeptide hormone family, promotes and inhibits, respectively, the JH biosynthesis by affecting CA activity (Kataoka *et al.*, 1989; Kramer *et al.*, 1991; Bogus and Scheller, 1996). Furthermore, proper nutritional signals can affect the release of AT and AS by the brain and further results in the activation or inhibition of JH biosynthesis (Noriega, 2004). Moreover, the decrease in JH titer is controlled through its metabolism, which is mainly catalyzed by JH esterase (JHE), JH epoxide hydrolase (JHEH), and JH diol kinase (JHDK). Particularly, several genes involved in JH biosynthesis, metabolism, and signaling have been previously analyzed in insects (Noriega *et al.*, 2006; Kinjoh *et al.*, 2007; Hua-Jun *et al.*, 2011).

Deciphering JH signaling in insects has been attracting increasing attention worldwide. Although the nature of

Send correspondence to Qingyou Xia. State Key Laboratory of Silkworm Genome Biology, Southwest University, No. 1 Tiansheng Road, Beibei District, 400715 Chongqing, China. E-mail: xiaqy@swu.edu.cn.

JH signaling has not been completely uncovered, some molecules have been demonstrated to be involved in JH signaling, including JH binding protein (JHBP), methoprene-tolerant (Met), the 20E receptor EcR, Ultraspiracle (USP), Krüppel homolog 1 (Kr-h1), FKBP39, and Chd64 (Riddiford, 2008; Jindra *et al.*, 2013). Notably, Met protein is a bHLH-PAS transcription factor and has been confirmed as a potential JH receptor in some insect species (Jindra *et al.*, 2013). In *Drosophila melanogaster* and *Bombyx mori*, Met paralog, germ-cell expressed (Gce) or Met2 protein functions as a partner of Met in modulating JH signaling (Abdou *et al.*, 2011; Guo *et al.*, 2012).

Intriguingly, there are eight types of natural JHs characterized in insects, including JH0, JHI, JHII, JHIII, JHIII skipped bisepoxide (JHSB3), JHIII bisepoxide (JHB3), methyl farnesoate (MF), and iso JH0 (Minakuchi *et al.*, 2006; Daimon and Shinoda, 2013). Currently, only JHIII is ubiquitous in all insects. The other four JH variants, including JH0, iso JH0, JHI, and JHII, have been found exclusively in Lepidoptera (Furuta *et al.*, 2013). JHSB3 and JHB3 are specific to Hemiptera and Diptera, respectively. MF is found to be present in both Diptera and Coleoptera. However, little is known about how genes involved in JH biosynthesis, metabolism, and signaling vary among insects.

As noted above, some of the genes (referred to as JH-related genes) participating in JH-related pathways (biosynthesis, metabolism, and signaling) have been characterized in various insect species. However, with the availability of whole-genome sequence, the number, structure, and evolution of all JH-related genes have not yet been investigated and compared systematically at a genome-wide level among insects. The silkworm (*B. mori*) belongs to the order Lepidoptera. In this study, based on the current *B. mori* genome assembly (Xia *et al.*, 2008), we performed a genome-wide identification of genes involved in JH biosynthesis, metabolism, and signaling in *B. mori*; we then evolutionarily compared the *B. mori* genes with their orthologs in other insects.

Materials and Methods

Gene identification and phylogenetic analysis

The *B. mori* genome sequences and the predicted proteins were downloaded from the SilkDB database and were used in our analysis. The predicted protein sets for *D. melanogaster*, *Anopheles gambiae*, *Apis mellifera*, *Tribolium castaneum*, and *Danaus plexippus* were downloaded from NCBI, Ensembl, or specific databases such as FlyBase for *D. melanogaster*, BeeBase for *A. mellifera* (Munoz-Torres *et al.*, 2011), MonarchBase for *D. plexippus* (Zhan and Reppert, 2013), and the Butterfly Genome Database for *Heliconius melpomene* (Heliconius Genome, 2012).

We used protein sequences or conserved domains of known JH-related genes to query against the predicted protein sets of *B. mori* and of other insects, using the BLAST program with an E-value threshold of less than 1e-6. In addition, an online SMART program was used to search for the functional domains of predicted proteins. Multiple candidate members of a JH-related gene were identified on the basis of agreement with both the E-value threshold and the existence of typical domains. Regarding the nomenclature, candidate genes were defined with an original name if the biological properties (such enzymatic activities) had been confirmed or if the genes had only one copy in each insect surveyed. If a JH-related gene had multiple copies and no functional attributes, we refer them as original-like genes.

The chromosomal distribution of JH-related genes in *B. mori* was determined using a *B. mori* genetics linkage map constructed using single-nucleotide polymorphism (SNP) markers (Yamamoto *et al.*, 2008). The multiple alignments of the complete amino acid sequences or functional domains of JH-related genes were performed using ClustalX (Thompson *et al.*, 1997). Based on the multiple sequence alignment results, neighbor-joining phylogenetic trees for JH-related genes from all surveyed insects were constructed using MEGA4.0 (Tamura *et al.*, 2007) with a bootstrap of 1000 replicates.

Microarray-based gene expression analysis

The spatio-temporal expression patterns of JH-related genes in *B. mori* were first surveyed using the microarray method. Microarray gene expression data of multiple larval tissues from *B. mori* larvae at the third day of the fifth instar and related analytical methods from our previous report (Xia *et al.*, 2007) were used to profile the tissue-specific expression of JH-related genes. In nine larval tissues, including the A/MSG (anterior/median silk gland), PSG (posterior silk gland), testis, ovary, fat body, midgut, integument, hemocyte, Malpighian tubule, and head (containing the brain and the associated glands in the retrocerebral complex, corpora allata (CA), corpus cardiacum (CC)), a total of 10,393 genes (transcripts) have been estimated to be activated based on an intensity threshold of 400 (Xia *et al.*, 2007). We retrieved the microarray data for *B. mori* JH-related genes from the active gene selection to examine their tissue expression profiles.

The developmental expression pattern was analyzed using microarray data of *B. mori* gene expression during metamorphosis (unpublished data), including 19 developmental time points during the larva-pupa-adult transitions, namely, V4 (fourth day of the fifth larval instar), V5, V6, V7, W0 (beginning of wandering), W12 (12 hours after wandering), W24, W36, W48 (completing spinning), W60 (immediately after pupation), W72, W96, W120, W144, W168, W192, W216, W240, and adulthood. Gene expression in *B. mori* larvae at V3 (third day of the fifth larval instar) was set as the common reference. The ratio between

the experimental and reference intensities for a JH-related gene was used to evaluate expression changes during the larva-pupa-adult transitions of *B. mori*. The related analytical method was based on our previous reports (Xia *et al.*, 2007; Huang *et al.*, 2009). Tissue and developmental expression patterns from the microarray analysis were visualized using the GeneCluster2.0 program (Reich *et al.*, 2004).

Real-time quantitative RT-PCR examination of gene expression

We also used a real-time quantitative RT-PCR approach to examine expression patterns of JH-related genes during the larval growth of *B. mori*. The *B. mori* strain *Dazao* was reared under a temperature of 25 °C. *B. mori* larvae were collected at ten time points during the larval feeding and molting stages from the fourth instar to the fifth instar, including IV0 (0 hour after the third larval molt, namely, the beginning of the fourth instar), IV1 (day 1 after the third molt), IV2 (day 2 after the third molt), IV3 (day 3 after the third molt), IV4 (day 4 after the third molt), IVM (just initiating the fourth larval molt), V0 (0 hours after the fourth molt, namely, the beginning of the fifth instar), and V1 (day 1 of the fifth instar).

Total RNA was extracted using TRIzol reagent (Invitrogen, USA) and was reverse transcribed into cDNA with M-MLV reverse transcriptase (Promega, USA). Real-time RT-PCR was conducted as described in our previous study (Wang *et al.*, 2008). The *B. mori* ribosomal protein L3 (*RpL3*) was used as an internal control. All primers used are listed in Table S1.

Results

Inventory of genes involved in JH biosynthesis, metabolism, and signaling in *B. mori* and other insects

We used the amino acid sequences of known insect JH-related genes to search against the predicted *B. mori* proteins using the BLAST program. Initially, 55 JH-related genes were identified, including 34 for JH biosynthesis, 13 for JH metabolism, and eight for JH signaling (Table 1). Several JH-related genes exhibited multiple copies, including *farnesyl diphosphate synthase* (*FPPS*), *farnesyl*

Table 1 - Inventory of JH-related genes in the *B. mori* genome.

Pathway	Gene	Symbol	Gene ID	ORF (aa)	Scaffold	Chromosome
MVA pathway of JH biosynthesis	Acetoacetyl CoA thiolase	Acat	BGIBMGA011029	405	nscaf3015	23
	Hydroxymethylglutaryl-CoA synthase	HMGS	BGIBMGA004001	456	nscaf2767	19
	Hydroxymethylglutaryl-CoA reductase	HMGR	BGIBMGA003229	785	nscaf2623	2
	Mevalonate kinase	MevK	BGIBMGA013075	410	nscaf3058	16
	Phosphomevalonate kinase	MevPK	BGIBMGA001556	186	nscaf2136	21
	Diphosphomevalonate decarboxylase	MevPPD	BGIBMGA007459	383	nscaf2886	3
	Isopentenyl-diphosphate delta-isomerase	IPPI	BGIBMGA004904	251	nscaf2822	25
	Farnesyl diphosphate synthase 1	FPPS1	BGIBMGA001926	427	nscaf2204	19
	Farnesyl diphosphate synthase 2	FPPS2	BGIBMGA001927	382	nscaf2204	19
	Farnesyl diphosphate synthase 3	FPPS3	BGIBMGA014635*	385	nscaf2204	19
Isoprenoid branch of JH biosynthesis	Farnesyl phosphatase-like protein 1	FPPase-l1	BGIBMGA011595	251	nscaf3028	-
	Farnesyl phosphatase-like protein 2	FPPase-l2	BGIBMGA011596	247	nscaf3028	-
	Farnesol dehydrogenase	FOHSDR	BGIBMGA005248	254	nscaf2827	8
	Aldehyde dehydrogenase-like protein 1	ALDH-l1	BGIBMGA001966	750	nscaf2204	19
	Aldehyde dehydrogenase-like protein 2	ALDH-l2	BGIBMGA001965	440	nscaf2204	19
	Juvenile hormone acid methyltransferase	JHAMT	BGIBMGA010391	250	nscaf2993	12
	Juvenile hormone acid methyltransferase-like protein 1	JHAMT-l1	BGIBMGA008032	136	nscaf2889	9
	Juvenile hormone acid methyltransferase-like protein 2	JHAMT-l2	BGIBMGA010392	266	nscaf2993	12
	Juvenile hormone acid methyltransferase-like protein 3	JHAMT-l3	BGIBMGA010393	168	nscaf2993	12
	Juvenile hormone acid methyltransferase-like protein 4	JHAMT-l4	BGIBMGA010563	137	nscaf2993	12
	Juvenile hormone acid methyltransferase-like protein 5	JHAMT-l5	BGIBMGA010564	121	nscaf2993	12
	Juvenile hormone acid methyltransferase-like protein 6	JHAMT-l6	BGIBMGA014014	267	nscaf3099	28
	Farnesoic acid O-methyltransferase-like protein 1	FAMeT-l1	BGIBMGA002604	443	nscaf2529	5
	Farnesoic acid O-methyltransferase-like protein 2	FAMeT-l2	BGIBMGA002314	248	nscaf2330	26
	Farnesoic acid O-methyltransferase-like protein 3	FAMeT-l3	BGIBMGA006513	229	nscaf2853	6
	Farnesoic acid O-methyltransferase-like protein 4	FAMeT-l4	BGIBMGA002684	206	nscaf2529	5
	Farnesoic acid O-methyltransferase-like protein 5	FAMeT-l5	BGIBMGA002605	338	nscaf2529	5

Table 1 (cont.)

Pathway	Gene	Symbol	Gene ID	ORF (aa)	Scaffold	Chromosome
	Farnesoic acid O-methyltransferase-like protein 6	FAMeT-l6	BGIBMGA006319	224	nscaf2852	6
	Farnesoic acid O-methyltransferase-like protein 7	FAMeT-l7	BGIBMGA006318	829	nscaf2852	6
	Cytochrome P450 15C1	Cyp15 C1	BGIBMGA011708	288	nscaf3031	11
Neuropeptide regulation of JH biosynthesis	Allototropin	AT	BGIBMGA011850	291	nscaf3031	11
	Allatostatin	AS	BGIBMGA014377	150	scaffold416	14
	Allatotropin receptor	ATR	BGIBMGA004429	255	nscaf2795	20
	Allatostatin receptor	ASR	BGIBMGA005708	362	nscaf2830	-
JH metabolism	Juvenile hormone esterase	JHE	BGIBMGA000772	567	nscaf1705	25
	Juvenile hormone esterase-like protein 1	JHE-l1	BGIBMGA000774	566	nscaf1705	25
	Juvenile hormone esterase-like protein 2	JHE-l2	BGIBMGA000775	560	nscaf1705	25
	Juvenile hormone esterase-like protein 3	JHE-l3	BGIBMGA000776	572	nscaf1705	25
	Juvenile hormone epoxide hydrolase	JHEH	BGIBMGA013930	461	nscaf3099	28
	Juvenile hormone epoxide hydrolase-like protein 1	JHEH-l1	BGIBMGA011468	401	nscaf3027	23
	Juvenile hormone epoxide hydrolase-like protein 2	JHEH-l2	BGIBMGA009211	510	nscaf2943	14
	Juvenile hormone epoxide hydrolase-like protein 3	JHEH-l3	BGIBMGA013994	637	nscaf3099	28
	Juvenile hormone epoxide hydrolase-like protein 4	JHEH-l4	BGIBMGA013793	395	nscaf3097	28
	Juvenile hormone epoxide hydrolase-like protein 5	JHEH-l5	BGIBMGA013929	355	nscaf3099	28
	Juvenile hormone diol kinase	JHDK	BGIBMGA008814	183	nscaf2925	3
	Juvenile hormone diol kinase-like protein 1	JHDK-l1	BGIBMGA008813	182	nscaf2925	3
	Juvenile hormone diol kinase-like protein 2	JHDK-l2	BGIBMGA008815	179	nscaf2925	3
JH signaling	Juvenile hormone binding protein	JHBP	BGIBMGA011549	243	nscaf3027	23
	FKBP39	FKBP39	BGIBMGA001490	402	nscaf2136	21
	Chd64	Chd64	BGIBMGA007092	174	nscaf2865	17
	Methoprene-tolerant 1	Met1	BGIBMGA005416	455	nscaf2828	8
	Methoprene-tolerant 2	Met2	BGIBMGA000657	661	nscaf1690	1
	Ecdysone receptor	EcR	BGIBMGA006767	496	nscaf2855	10
	Ultraspiracle	USP	BGIBMGA006183	270	nscaf2847	4
	Kruppel homolog 1	Kr-h1	BGIBMGA003160	348	nscaf2589	4

Note: * indicates new gene assembly. – indicates unknown.

phosphatase (*FPPase*), *aldehyde dehydrogenase* (ALDH), *JH acid methyltransferase* (*JHAMT*), *farnesoic ac*id *O-methyltransferase* (*FAMeT*), *JHE*, *JHEH*, and *JHDK*. All JH-related genes for *B. mori* mapped to different chromosomes except for the *FPPase* gene and *allatostatin receptor* (*ASR*) gene (Figure 1 and Table 1). Intriguingly, the copies of several JH-related gene with multiple copies were distributed on the same chromosome in a tandem manner, for example, *FPPS* on chromosome 12, *JHDK* on chromosome 3, *FAMeT* on chromosomes 5 or 6, *JHAMT* on chromosome 12, *JHE* on chromosome 25, and *JHEH* on chromosome 28.

To determine comprehensively the evolution of JH-related genes among insects, we further identified JH-related genes in other insects, namely, 35 in *D. melanogaster*, 35 in *A. gambiae*, 36 in *A. mellifera*, 47 in *T. castaneum*, and 44 in *D. plexippus* (Table 2, Table S2). Some JH-related genes in these five insects had at least two copies, including three genes (*Farnesol dehydrogenase* (*FOHSDR*), *JHE*, and *JHEH*) in *D. melanogaster*, three (*FOHSDR*, *JHE*, and *JHEH*) in *A. gambiae*, four (*FPPase*, *FOHSDR*, *FAMeT*, and *JHE*) in *A. mellifera,* five (*FPPase*, *FOHSDR*, *ALDH*, *JHAMT*, and *JHEH*) in *T. castaneum*, and six (*FPPS*, *FPPase*, *ALDH*, *FAMeT*, *JHEH*, and *JHDK*) in *D. plexippus*. Moreover, the copies of several JH-related genes in these insects were distributed in tandem on a chromosome, including the *JHEH*s in *D. melanogaster;* the *JHEHs* and *FOHSDRs* in *A. gambiae*; the *JHAMTs* and *JHEHs* in *T. castaneum*; and the *FPPSs*, *FPPases*, *FAMeTs*, *JHEHs*, and *JHDKs* in *D. plexippus*. On the basis of these and similar observations in *B. mori*, we speculate that the JH-related genes with multiple copies were duplicated during the evolution of *B. mori* and other insects.

The MVA pathway of JH biosynthesis

The upstream mevalonate (MVA) pathway of JH biosynthesis is responsible for producing the JH precursor farnesyl diphosphate (FPP). As shown in Figure S1, the MVA pathway involved eight enzymatic steps. Interestingly, each of the enzyme-encoding genes involved in the first seven steps of the MVA pathway, which produce the

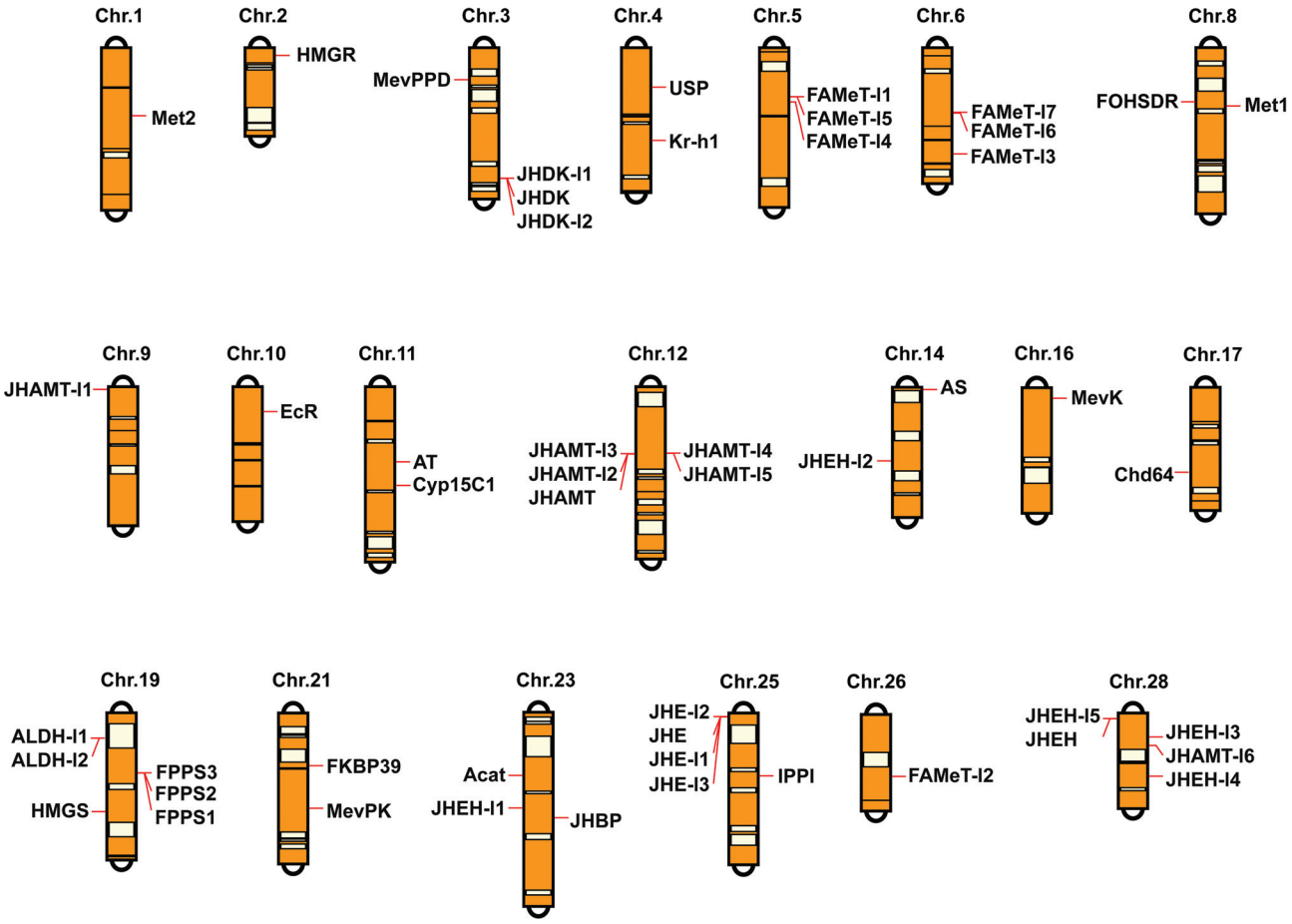

Figure 1 - Chromosomal distribution of JH-related genes in *B. mori.* Based on the assembly of the whole-genome sequence and single-nucleotide polymorphism (SNP) markers linkage map for *B. mori*, a total of 52 JH-related genes were mapped on the different chromosomes of *B. mori*. Several different copies of each of the JH-related genes with multiple copies are clustered in tandem on the chromosomes.

FPP precursor isopentenyl diphosphate (IPP), existed as a single copy in *B. mori* and five other surveyed insects and showed a 1:1:1:1:1:1 orthologous relationship (Table 2).

Farnesyl diphosphate synthase (FPPS) catalyzes the final reaction of the MVA pathway, converting IPP into the JH precursor FPP. Three transcripts of the *FPPS* gene have been identified in *B. mori* (Kinjoh *et al.*, 2007). We noted that only two *FPPS* transcripts matched well with two predicted genes for *B. mori*, namely, *FPPS1* for BGIBMGA001926 and *FPPS2* for BGIBMGA001927. We further used the complete cDNA sequence of the *FPPS3* transcript to search against the *B. mori* genome assembly using the BLASTn algorithm. In this search, we found that the *FPPS3* transcript matched the downstream genomic region of the *FPPS2* gene; therefore, we defined *FPPS3* as BGIBMGA014635 for a supplement of the *B. mori* predicted genes. Strikingly, a comparative analysis revealed a copy number variation in the *FPPS* genes among the surveyed insects. In addition to three copies in *B. mori*, there were six copies in *A. mellifera*, two in *D. plexippus*, and one each in *D. melanogaster*, *A. gambiae*, and *T. castaneum*. From the phylogenetic tree of the *FPPS* and *FPPS*-like genes that were identified here and collected from online resources for other insects (Figure 2), we observed that all *FPPS* and *FPPS*-like genes from *B. mori* and other lepidopterans, including *D. plexippus*, *Heliconius melpomene*, *Choristoneura fumiferana*, *Mythimna unipuncta*, and *Agrotis ipsilon*, clustered into two groups, and different copies from each insect were separately grouped, indicating that the *FPPS* duplication in lepidopterans may have occurred after their separation from other insect species and before their separation from each other. However, all six *FPPS*-like genes in *A. mellifera* grouped well together, suggesting that the *FPPS* genes from *A. mellifera* may have been duplicated after the separation of *A. mellifera* from other insects. The *FPPS* genes from other five insect species, including *Aedes aegypti*, *Culex quinquefasciatus*, *Anthonomus grandis*, *Dendroctonus jeffreyi*, and *Nasonia vitripennis*, also existed as a single copy and clustered together with the *FPPS* genes from *D. melanogaster*, *A. gambiae*, and *T. castaneum*.

The isoprenoid branch of JH biosynthesis

The downstream isoprenoid branch pathway of JH biosynthesis converts FPP to JH (Minakuchi *et al.*, 2006).

Table 2 - Copy number of JH-related genes in *B. mori* and the other insects.

Pathway	Gene	Symbol	*B. mori* (Lepidoptera)	*D. melanogaster* (Diptera)	*A. gambiae* (Diptera)	*A. mellifera* (Hymenoptera)	*T. castaneum* (Coleoptera)	*D. plexippus* (Lepidoptera)
MVA pathway of JH biosynthesis	Acetoacetyl CoA thiolase	ACAT	1	1	1	1	1	1
	Hydroxymethylglutaryl-CoA synthase	HMGS	1	1	1	1	1	1
	Hydroxymethylglutaryl-CoA reductase	HMGR	1	1	1	1	1	1
	Mevalonate kinase	MevK	1	1	1	1	1	1
	Phosphomevalonate kinase	MevPK	1	1	1	1	1	1
	Diphosphomevalonate decarboxylase	MevPPD	1	1	1	1	1	1
	Isopentenyl-diphosphate delta-isomerase	IPPI	1	1	1	1	1	1
	Farnesyl diphosphate synthase	FPPS	3	1	1	6	1	2
Isoprenoid branch of JH biosynthesis	Farnesyl phosphatase	FPPase	2	1	1	2	3	2
	Farnesol dehydrogenase	FOHSDR	1	6	6	2	7	1
	Aldehyde dehydrogenase	ALDH	2	1	1	1	6	2
	Juvenile hormone acid methyltransferase	JHAMT	7	1	1	1	3	1
	Farnesoic acid O-methyltransferase	FAMeT	7	1	1	2	1	5
	Cytochrome P450 15A1	Cyp15A1	-	-	-	1	1	-
	Cytochrome P450 15C1	Cyp15C1	1	-	-	-	-	1
Neuropeptide regulation of JH biosynthesis	Allototropin	AT	1	-	1	-	1	1
	Allatostatin	AS	1	1	1	1	1	1
	Allototropin receptor	ATR	1	1	1	1	1	1
	Allatostatin receptor	ASR	1	1	1	1	1	1
JH metabolism	Juvenile hormone esterase	JHE	4	2	2	2	1	1
	Juvenile hormone epoxide hydrolase	JHEH	6	3	3	1	5	8
	Juvenile hormone diol kinase	JHDK	3	1	1	1	1	2
JH signaling	Juvenile hormone binding protein	JHBP	1	1	1	1	1	1
	FKBP39	FKBP39	1	1	1	1	1	1
	Chd64	Chd64	1	1	1	1	1	1
	Methoprene-tolerant/Methoprene-tolerant 1	Met/Met1	1	1	1	1	1	1
	Germ cell expressed/Methoprene-tolerant 2	Gce/Met2	1	1	-	-	-	1
	Ecdysone receptor	EcR	1	1	1	1	1	1
	Ultraspiracle	USP	1	1	1	1	1	1
	Kruppel homolog 1	Kr-h1	1	1	1	1	1	1

Note: - represents no identification.

Recently, in insects, three types of catalytic enzymes, including farnesyl pyrophosphate phosphatase (FPPase), farnesol dehydrogenase (FOHSDR), and aldehyde dehydrogenase (ALDH), have been successfully identified as being responsible for the sequential conversion of FPP into farnesol, farnesal, and farnesoic acid (FA) (Mayoral *et al.*, 2009; Nyati *et al.*, 2013; Rivera-Perez *et al.*, 2013). In addition to the previous prediction of these genes in several insects, we identified two *FPPas*e-like genes, one *FOHSDR* gene, and two *ALDH*-like genes in *D. plexippus*. Although each of these genes exhibited a different number of copies among the surveyed insects, the copy number of each gene was the same in *B. mori* and *D. plexippus* as well as in *D. melanogaster* and *A. gambiae* (Table 2 and Table S2).

The conversion of FA into JH is completed via two catalytic reactions in insects, namely, epoxidation and methyl esterification. Notably, this conversion can occur in two ways (Figure S1). One involves FA oxidation by Cyp15C1 to form JH acid (JHA), after which JHA is methylated by juvenile hormone acid methyltransferase (JHAMT) to synthesize JH. Another way is that FA is methylated to form methyl farnesoate (MF) by farnesoic

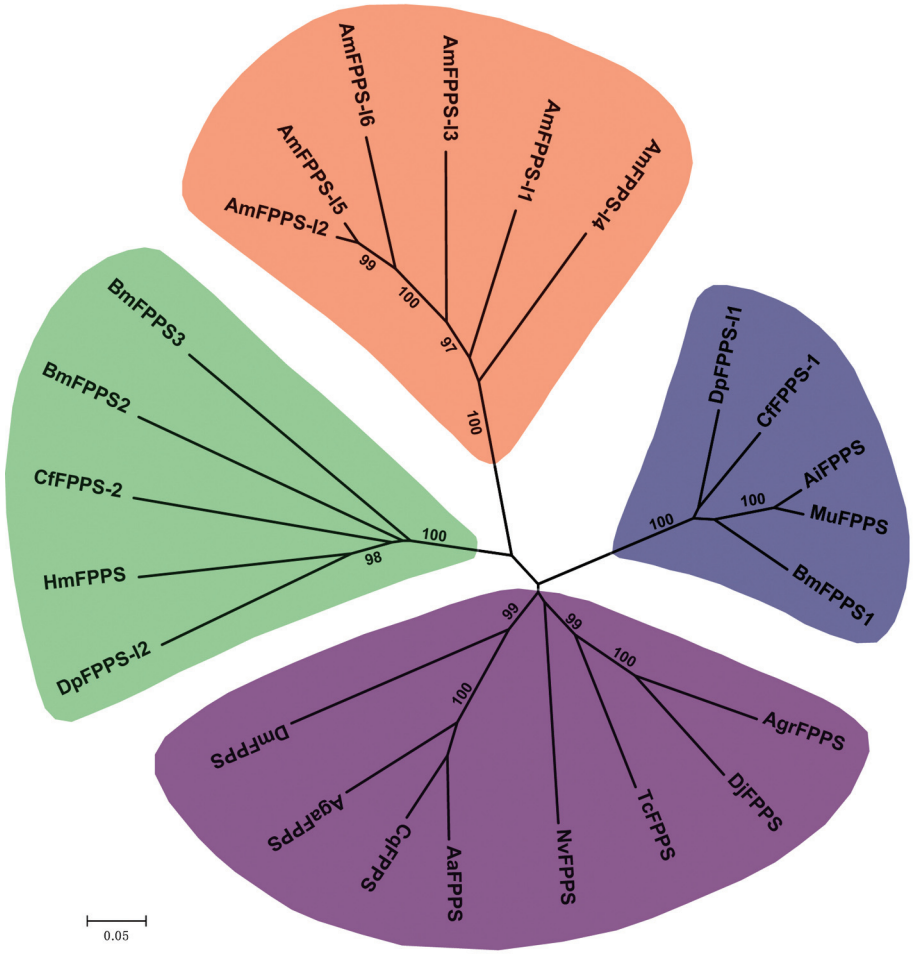

Figure 2 - Phylogenetic tree of the *FPPS* genes from *B. mori* and other insects. Based on the multiple alignments of the amino acid sequences of FPPS proteins from *B. mori* and other insects, a phylogenetic tree was constructed using the neighbor-joining method with 1000bootstrap replicates after removing the highly divergent sequences at the N- or C-terminus. Bootstrap values > 60% are marked. Bm, *Bombyx mori*; Dm, *Drosophila melanogaster*; Aga, *Anopheles gambiae*; Am, *Apis mellifera*; Tc, *Tribolium castaneum*; Dp, *Danaus plexippus*; Cf, *Choristoneura fumiferana*; Hm, *Heliconius melpomene*; Mu, *Mythimna unipuncta*; Agi, *Agrotis ipsilon*; Aa, *Aedes aegypti*; Cq, *Culex quinquefasciatus*; Agr, *Anthonomus grandis*; Dj, *Dendroctonus jeffreyi*; Nv, *Nasonia vitripennis*.

acid O-methyltransferase (FAMeT), after which MF is oxidized by Cyp15A1 to produce JH.

Cyp15C1 and *Cyp15A1*, the two genes involved in FA epoxidation, belong to the Cyp15 subfamily of cytochrome P450 enzymes. The *Cyp15C1* gene has been identified in *B. mori* and *D. plexippus*, and the *Cyp15A1* gene has been characterized in *T. castaneum* and *A. mellifera* (Daimon *et al.*, 2012; Daimon and Shinoda 2013). No *Cyp15C1* or *Cyp15A1* was identified in *D. melanogaster*. We performed a BLASTp search against the genome of *H. melpomene* (Lepidoptera) and identified a homolog of the *B. mori Cyp15C1* gene, namely, HMEL006305, further indicating that *Cyp15C1* may be Lepidoptera-specific.

JHAMT and FAMeT are considered two catalytic enzymes for the methyl esterification of FA. One authentic JHAMT enzyme was first characterized in *B. mori* (Shinoda and Itoyama, 2003). As listed in Table 1, the gene (BGIBMGA010391) encoding the authentic JHAMT and six *JHAMT*-like genes (from *JHAMT-l1* to *JHAMT-l6*) were found in the *B. mori* genome, and each contains a methyltransf_12 domain. Furthermore, three *JHAMT* copies (including authentic *JHAMT*, *JHAMT-l1*, and *JHAMT-l2*) were predicted in *T. castaneum*, whereas only one copy of *JHAMT* gene was identified in four other insects (Table 2 and Table S2). A phylogenetic tree revealed that the different copies of the *JHAMT* gene in *B. mori* and *D. plexippus* grouped well together, and that the three *JHAMT* copies in *T. castaneum* also grouped into a clade (Figure S2), suggesting that the *JHAMT* gene in *B. mori* or *T. castaneum* was duplicated after their separation from other insect species.

FAMeT has been found to exist in insects and crustaceans (Hui *et al.*, 2010), and evidence from *D. melanogaster* has suggested that FAMeT has a minor role in JH biosynthesis, but it may play a major role in JH signaling (Burtenshaw *et al.*, 2008; Zhang *et al.*, 2010). Our data revealed that in the *B. mori* genome, there are seven *FAMeT*-like genes, each of which contains two typical

Methyltransf_FA and DM9 domains. Comparatively, *FAMeT* exists as a single copy in *T. castaneum* as well as in two Diptera insects but as two copies in *A. mellifera* and five copies in *D. plexippus* (Table 2 and Table S2). A phylogenetic tree of insect *FAMeT* or *FAMeT*-like genes was constructed using their coding sequences containing two functional domains (Figure S2). In this tree, with the exception of *A. mellifera*, one copy of the *FAMeT* gene from each of the five remaining insects grouped into a clade. Other copies of the *FAMeT* gene from *B. mori*, *A. mellifera*, and *D. plexippus* grouped together into an additional clade in an irregular manner. This phylogenetic relationship indicates that the duplication of the different *FAMeT* copies may have occurred after insect radiation and subsequently undergone a rapid sequence diversification during insect evolution.

Neuropeptide regulation of JH biosynthesis

Insect JH biosynthesis is also modulated by two neuropeptides, namely, allatotropin (AT) and allatostatin (AS). AT and AS play antagonistic roles during JH biosynthesis, with the former being a stimulator and the later being an inhibitor of CA activity (Stay, 2000). As listed in Table 1, from the *B. mori* genome, we retrieved four genes that respectively encode AT, AS, the AT receptor (ATR), and the AS receptor (ASR), which have been previously reported in other insects (Secher *et al.*, 2001; Park *et al.*, 2002; Roller *et al.*, 2008; Yamanaka *et al.*, 2008; Horodyski *et al.*, 2011). In addition, *AS*, *ASR*, and *ATR* were all identified in five other insects, whereas *AT* gene was predicted only in *A. gambiae*, *T. castaneum*, and *D. plexippus* in our analysis. Notably, each of the identified genes related to the neuropeptide regulation of JH biosynthesis appears to exist as a single copy in *B. mori* and the other surveyed insects (Table 2).

Enzymes involved in JH metabolism

The metabolic degradation of JH contributes to the reduction of JH titer and is catalyzed by three enzymes, namely, JHE, JHEH, and JHDK. As shown in Figure S1, JHE catalyzes the conversion of JH into JH acid (JHa) or the conversion of JH diol (JHd) into JH acid diol (JHad). A previous report characterized one *JHE* gene encoding an authentic JHE enzyme in *B. mori* (Hirai *et al.*, 2002). Here, in addition to the known *JHE* gene, three *JHE*-like genes (namely, *JHE-l1*, *JHE-l2*, and *JHE-l3*) were identified from the *B. mori* genome (Table 1). Moreover, we retrieved different copies of the *JHE* gene in five other insects, namely, 2, 2, 2, 1, and 1 for *D. melanogaster*, *A. gambiae*, *A. mellifera*, *T. castaneum*, and *D. plexippus*, respectively (Table 2 and Table S2). All the identified *JHE* genes contain a COesterase domain. A phylogenetic analysis showed that the *JHE* genes in Lepidoptera, Hymenoptera, Diptera, or Coleoptera separately grouped well together (Figure S3), consistent with the classical phylogeny of these insect species. Notably, different copies of the *JHE* gene in *D. melanogaster* grouped first together, as did those in *A. gambiae* and *A. mellifera*, suggesting that *JHE* duplication in these three species occurred after their separation. Nevertheless, in Lepidoptera, *JHE* from *B. mori* grouped first with *JHE* from *D. plexippus* and then with the grouping clade of *JHE-l1* and *JHE-l2* from *B. mori*, suggesting that *JHE* in both *B. mori* and *D. plexippus* may have a common ancestor, and the other three *JHE*-like genes in *B. mori* may have undergone a great sequence diversification after their duplication from *JHE*.

JHEH catalyzes the conversion of JH into JHa or the conversion of JHa into JHad. According to previous reports in *B. mori* (Zhang *et al.*, 2005; Seino *et al.*, 2010),in addition to an authentic *JHEH*, there are five *JHEH*-like genes in the *B. mori* genome (Table 1). The copy numbers of the *JHEH* genes vary, with three in *D. melanogaster*, three in *A. gambiae*, one in *A. mellifera*, five in *T. castaneum*, and seven in *D. plexippus* (Table 2 and Table S2). On the phylogenetic tree of the *JHEH* genes (Figure S3), different copies of the *JHEH* gene from each of four insect species (*D. melanogaster*, *A. gambiae*, *A. mellifera*, and *T. castaneum*) grouped first together, indicating that the *JHEH* gene from these four species may have undergone species-specific duplication after their separation from the other insects. Moreover, the copies of the *JHEH* genes from two lepidopterans, *B. mori* and *D. plexippus*, grouped into four clades, and each clade contained different copies from both of these species, suggesting that *JHEH* duplication in Lepidoptera may have occurred before their radiation.

JHDK is required for the conversion of JHd into JH diol phosphate (JHdp). To date, JHDK has been functionally characterized in two Lepidoptera insects, namely, *B. mori* and *Manduca sexta* (Maxwell *et al.*, 2002; Li *et al.*, 2005). Here, three copies of the *JHDK* gene were identified in the *B. mori* genome (Table 1), including one authentic *JHDK* and two *JHDK*-like genes, each of which contains an EF-hand domain. Furthermore, as listed in Table 2 and Table S2, there were two copies of the *JHDK* gene in another lepidopteran, *D. plexippus*, but only one copy was identified in four other surveyed insects. Phylogenetic analysis showed that the *JHDK* copies from Lepidoptera grouped into two clades, and the *JHDK* genes from other insects grouped well together (Figure S3), suggesting that *JHDK* duplication in Lepidoptera may have also occurred before their separation.

Genes involved in the JH signaling pathway

Recently, additional evidence has shown that the bHLH-PAS transcription factor Met is a potential receptor for JH signaling (Jindra *et al.*, 2013). *Met* and its paralogous gene *Gce* were first identified in *D. melanogaster* (Wilson and Fabian, 1986; Baumann *et al.*, 2010b), in which they were partially redundant in mediating JH signaling (Baumann *et al.*, 2010a; Abdou *et al.*, 2011). Two copies of the

Met gene, *Met1* and *Met2*, have been characterized in *B. mori* (Li *et al.*, 2010; Guo *et al.*, 2012; Kayukawa *et al.*, 2012). Intriguingly, our analysis also identified two copies of the *Met* gene in two other lepidopteran insects, *D. plexippus* and *H. melpomene*, namely *DpMet-l1* and *DpMet-l2* in *D. plexippus* (Table 2 and Table S2) and *HmMet-l1* (HMEL011931) and *HmMet-l1* (HMEL009818) in *H. melpomene*. However, only one copy of the *Met* gene was identified in *A. gambiae*, *A. mellifera*, and *T. castaneum* (Table 2 and Table S2). Phylogenetic analysis revealed that *Met* and *Gce* from *D. melanogaster* grouped together (Figure 3). In Lepidoptera, *Met1* and *Met-l1* grouped into one clade, whereas *Met2* and *Met-l2* grouped into another, indicating that the *Met* duplication in Lepidoptera may have occurred before their separation.

In addition, some molecules, including JHBP, FKBP39, Chd64, EcR, USP, and Kr-h1, have been demonstrated to be involved in JH signaling (Dubrovsky, 2005; Li *et al.*, 2007; Jindra *et al.*, 2013). Our results showed that each of these six genes was identified as having one copy in *B. mori* and five other insect species (Table 2 and Table S2).

Expression profiles of JH-related genes in multiple tissues of *B. mori* larvae

Using microarray data of gene expression in multiple tissues of *B. mori* larvae on the third day of the fifth instar when JH titer is present (Sakurai and Niimi, 1997; Xia *et al.*, 2007), we investigated the tissue expression of JH-related genes. As a result, 36 of 55 JH-related genes were detected in at least one tissue (Figure 4). Among JH biosynthesis-related genes, *Acat*, *MevPK*, *IPPI*, *FPPS2*, *ALDH-l1*, *JHAMT-I6*, *FAMeT-I4*, and *Cyp15C1* were expressed in the head (mainly containing CA, CC, and brain), which may be directly involved in JH biosynthesis. In particular, several JH biosynthesis-related genes showed either high expression in other larval tissues or ubiquitous expression, such as *Acat* and *MevPK* in all surveyed tissues; *FAMeT-l1*, *FAMeT-l5*, and *FAMeT-l7* in the midgut; and *JHAMT-l4*, *JHAMT-l5*, and *JHAMT-l6* in the fat body and integument. This indicates that these genes may function in other biological processes.

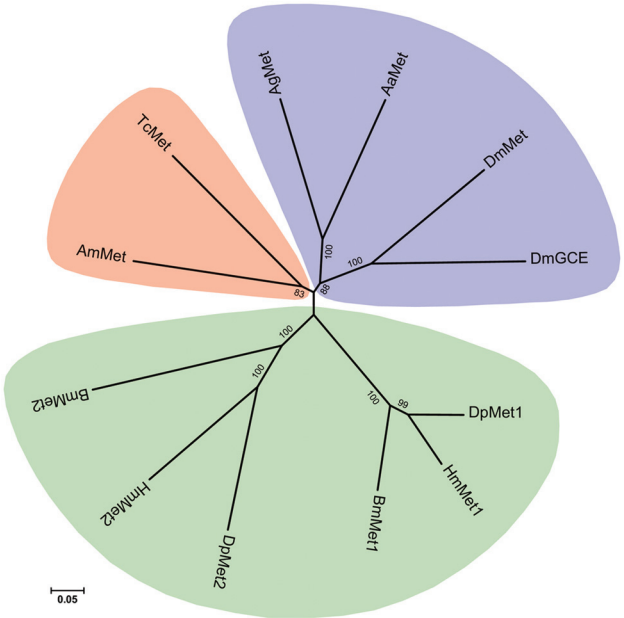

Figure 3 - Phylogenetic tree of the *Met* genes in *B. mori* and other insects. The amino acid sequences encoding by *Met* genes from seven insect species, including *Bombyx mori* (Bm), *Drosophila melanogaster* (Dm), *Anopheles gambiae* (Aga), *Apis mellifera* (Am), *Tribolium castaneum* (Tc), *Danaus plexippus* (Dp), and *Heliconius melpomene* (Hm), were used to build a phylogenetic tree. See Figure 2 for a detailed description of the approaches for constructing phylogenetic tree.

Some genes involved in JH metabolism are mostly expressed in the midgut or Malpighian tubule (Figure 4). For example, five genes, namely, *JHE-l1*, *JHEH*, *JHEH-l4*, *JHDK*, and *JHDK-l1*, were expressed in both the midgut and Malpighian tubule. *JHEH-l1* and *JHDK-l2* were expressed in the midgut and Malpighian tubule, respectively. *JHE-l3* and *JHEH-l2* expression was enriched in both the A/MSG and PSG, whereas *JHEH6* was highly expressed in all tissues with the exception of hemocytes. *JHEH-l2* was specifically expressed in the A/MSG. In addition, among the genes involved in the JH signaling pathway, *JHBP*, *Met1*, *EcR*, *FKBP39*, and *Chd64* were all weakly expressed in at least one larval tissue. In particular, *FKBP39* and *Chd64* were expressed in all analyzed tissues. However, from the microarray data, the expression of three genes, *USP*, *Met2*, and *Kr-h1*, was not observed in any tissue. It is possible that the expression levels for these three transcription factors may be too low at day 3 of the fifth larval instar and were therefore difficult to be detected.

Expression profiles of JH-related genes during *B. mori* metamorphosis

Based on microarray data for gene expression at 19 developmental points during *B. mori* metamorphosis (unpublished data), we found that 42 of 55 JH-related genes were expressed during at least one developmental point (Figure 5). Among the genes involved in the MVA pathway of JH biosynthesis the majority were highly expressed during the pupa-adult transition, with the exception of *MevK*, whose expression was detected in the female on the fourth day of the fifth larval instar (V4). In particular, *Acat* and *IPP1* displayed a high expression in males, whereas *FPPS1* and *FPPS3* were highly expressed in females. Among the genes involved in the isoprenoid branch of JH biosynthesis, only *ALDH-l1*, *Cyp15C1*, and several copies of *JHAMT* and *FAMeT* were detected during metamorphosis, and they were highly expressed before wandering and during the pupa-adult transition (Figure 5). In particular, *FAMeT-l2* and *FAMeT-l4* exhibited a male-specific expression pattern during the pupa-adult transition. Further studies will be re-

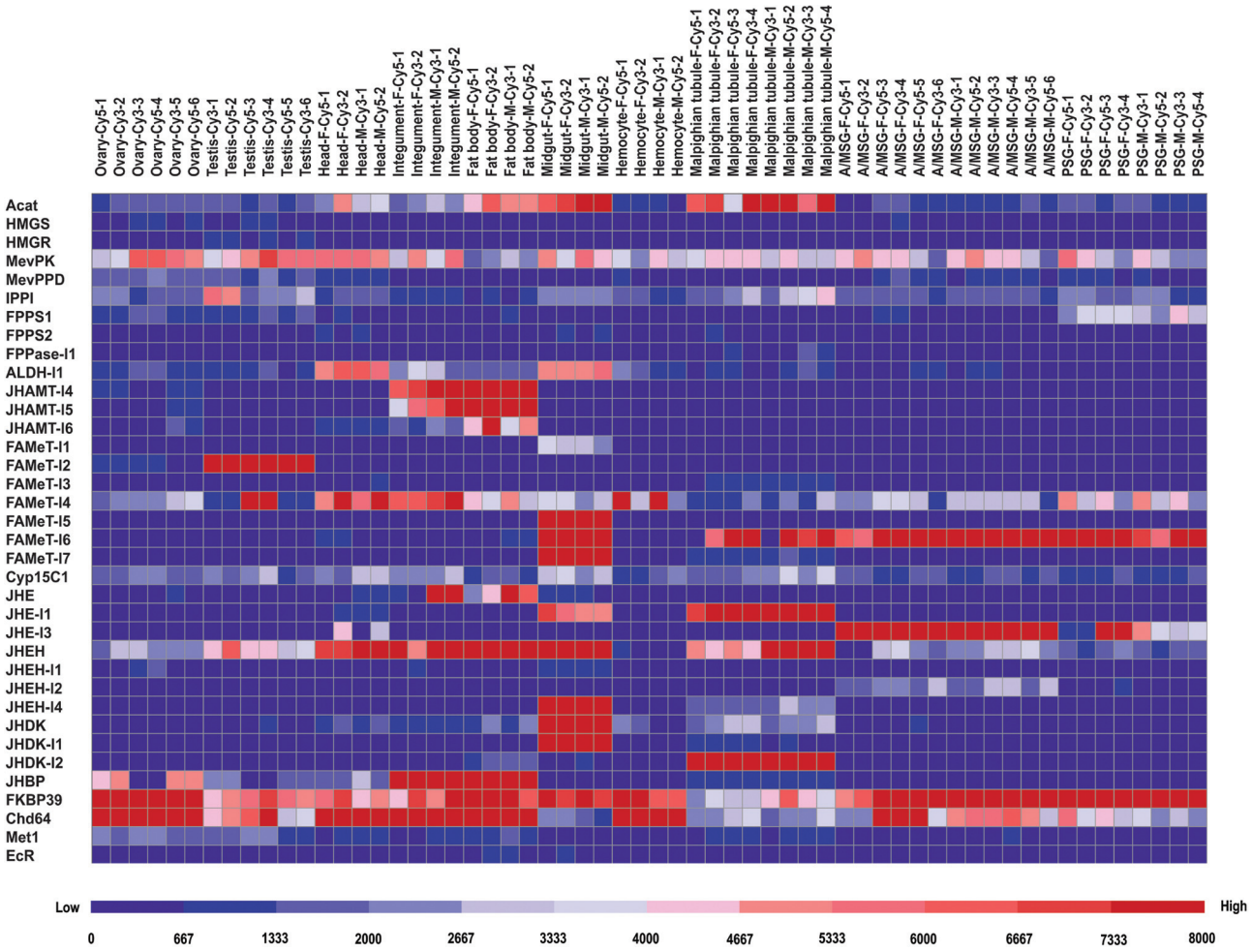

Figure 4 - Expression pattern of JH-related genes in multiple tissues from *B. mori* larvae. Microarray data of genome-wide gene expression in multiple tissues from *B. mori* larvae on the third day of the fifth instar were downloaded from the *B. mori* genome database. Each tissue sample was analyzed with at least two biological repeats. If a JH-related gene was estimated to have an average expression signal intensity of more than 400 in any tissue, it was considered to be expressed in that tissue. F, female; M, male. A/MSG, anterior/median silk gland; PSG, posterior silk gland.

quired to decipher the roles of these two *FAMeT* genes during the development of *B. mori* males.

Microarray data of gene expression during *B. mori* metamorphosis also revealed that among the genes involved in JH metabolism, *JHE* and *JHE-l1* were highly expressed during pupation. *JHEH*, *JHEH-l2*, *JHEH-l4*, *JHEH-l5*, *JHDK-l1*, *JHDK*, and *JHDK-l2* were mainly expressed before wandering. *JHEH-l1* and *JHEH-l3* displayed a high expression in females during pupa-adult transition. Among the genes involved in JH signaling, *JHBP*, *FKBP39*, *Chd64*, and *Met1* exhibited ubiquitous expression during *B. mori* metamorphosis. *Met2* expression was detected only in males during pupa-adult transition, and *EcR* and *USP* were highly expressed before wandering. *Kr-h1* presented a high expression during the late stage of the pupa-adult transition.

Because JH biosynthesis and JH metabolism are initiated during larval feeding and molting, respectively, we used real-time quantitative RT-PCR experiments to further check the consistency between JH titer and the expression profiles of several JH-related genes with multiple copies during *B. mori* larval growth. As shown in Figure 6, *FPPS1* expression was highly during *B. mori* larval molting and gradually decreased during larval feeding, which was consistent with the changes in JH titer. Interestingly, *FPPS1* expression at the beginning of the feeding stages was higher in the fifth instar larvae than in the fourth instar larvae. Several copies of the genes involved in the isoprenoid branch of JH biosynthesis, including *JHAMT-l3*, *JHAMT-l4*, *FAMeT-l2*, and *FAMeT-l5*, exhibited a high expression during larval feeding and subsequently decreased during larval molting, also indicating consistency with the changes in the JH titer. Moreover, four copies of the genes involved in JH metabolism, including *JHE-l1*, *JHE-l3*, *JHDK*, *JHDK-l1*, *JHDK-l2*, *JHEH-l1*, *JHEH-l3*, and *JHEH-l5*, showed an increased expression during larval feeding and were obviously decreased during larval molting. The ex-

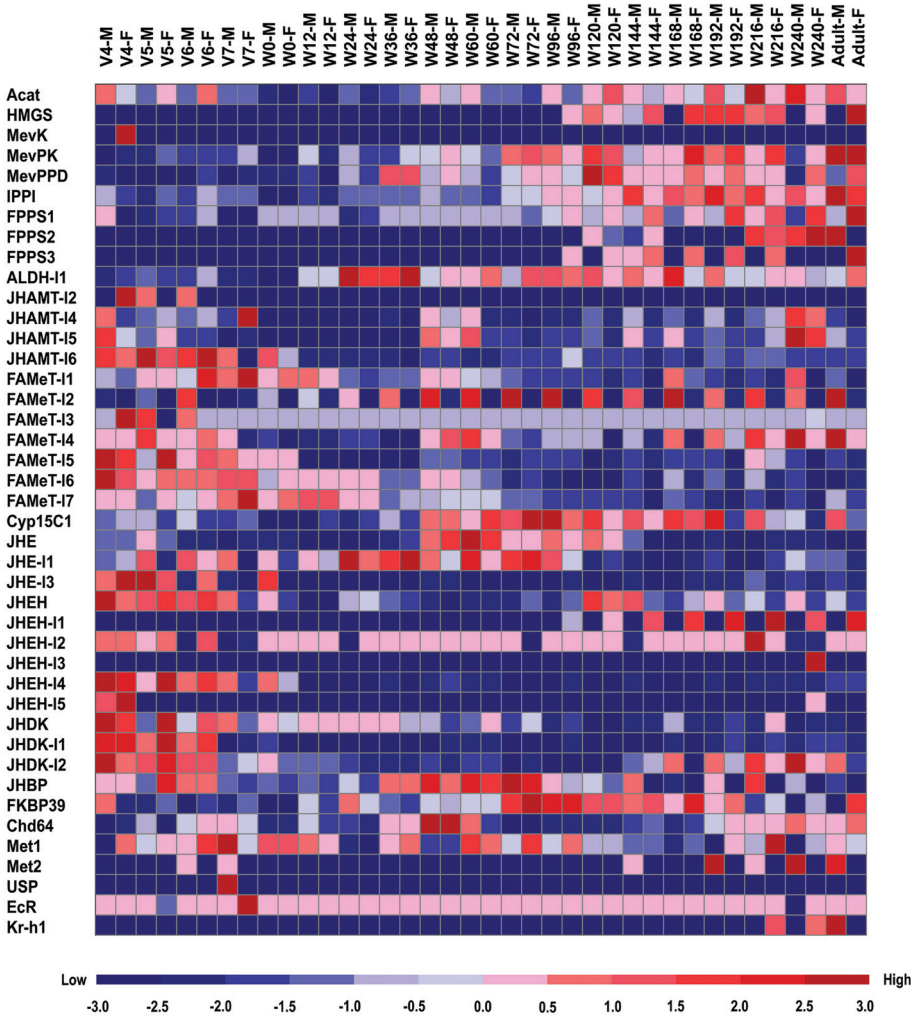

Figure 5 - Expression profiles of JH-related genes during metamorphosis in *B. mori*. The developmental expression profiles of JH-related genes for *B. mori* were analyzed using microarray data of genome-wide gene expressions at 19 time points during *B. mori* metamorphosis. The ratio was calculated by comparing the signal intensity of the mRNA expression level for each JH-related gene at each time point to that in the common reference of larvae on the third day of the fifth instar in *B. mori.* Subsequently, the expression change of each JH-related gene was evaluated by this expression ratio and visualized using the GeneCluster2.0 program. Plus and minus denote up- and down-regulation, respectively.

pression of these genes was also consistent with the change in JH titer during *B. mori* larval feeding and molting.

Discussion

JH plays key roles in the regulation of various aspects of insect growth and development. Previous studies have reported the genome-wide identification of genes involved in JH biosynthesis, metabolic degradation, or signaling in several insect species, such as the JH biosynthesis-related genes in *B. mori* (Kinjoh *et al.*, 2007; Xia *et al.*, 2008), *JHE*s in *A. aegypti* (Bai *et al.*, 2007), and *JHEHs* in *T. castaneum* (Seino *et al.*, 2010). To understand comprehensively the evolutionary conservation and variation of JH-related genes in insects, we systematically identified and compared JH-related genes in *B. mori* and five other insect species whose whole genomes have been sequenced, including the recently completed *D. plexippus* (Lepidoptera) genome sequence.

The developmental changes in JH titer in insects are mainly controlled by the processes of biosynthesis in the CA and metabolic degradation in the targeting tissues. Here, we observed an evolutionary divergence of the genes involved in JH biosynthesis among *B. mori* and other insects. First, the genes related to the upstream seven steps of the MVA pathway of JH biosynthesis, which is responsible for producing IPP as FPP precursor, all existed as a single copy and displayed a rigorous orthologous relationship among the analyzed insects (Table 2 and Table S2), indicating that the process of IPP production was conserved during insect evolution. Second, the *FPPS* gene involved in the last step of the MVA pathway (production of JH precursor FPP) and all identified genes participating in the isoprenoid branch for producing JH showed a variation in copy num-

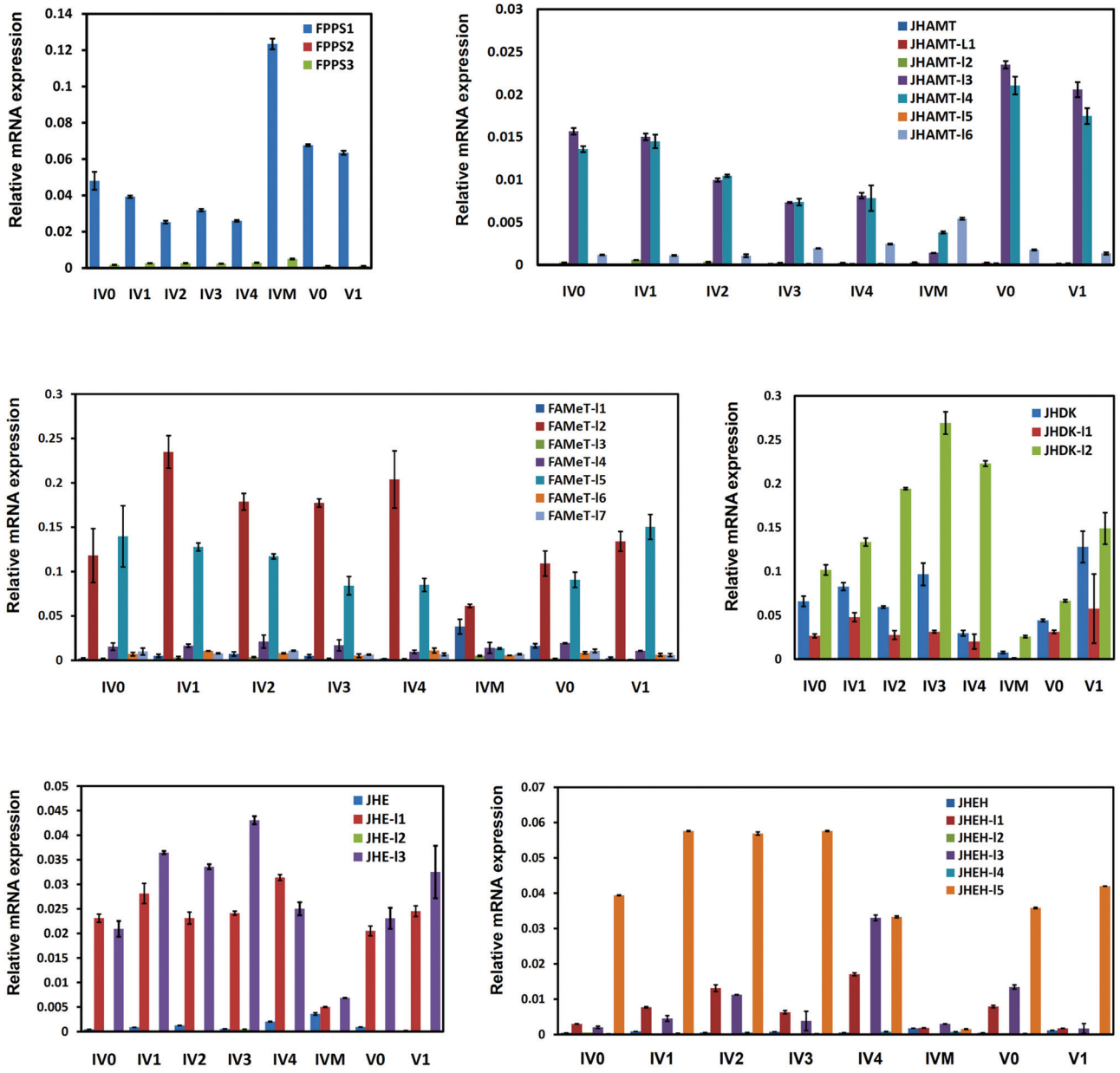

Figure 6 - Expression patterns of JH-related genes with multiple copies during larval growth and molting in *B. mori*. Quantitative analysis of the gene expression of JH-related genes with multiple copies during larval growth and molting in *B. mori* was performed by real-time RT-PCR. The *B. mori* ribosomal protein L3 (*RpL3*) gene was used as an internal control. IV0, immediately after completion of the third larval molting and the beginning of feeding in the fourth larval instar; IV1, one day after feeding in the fourth larval instar; IV2, two days after feeding in the fourth larval instar; IV3, three days after feeding in the fourth larval instar; IV4, four days after feeding in the fourth larval instar; IVM, the fourth larval molting; V0, immediately after completion of the fourth larval molting and the beginning of feeding in the fifth larval instar; V1, one day after feeding in the fifth larval instar.

ber or gene class among *B. mori* and other insects (Table 2 and Table S2), which may be a consequence of gene duplication. In fact, JH types also varied between Lepidoptera and other insect orders, and more types of JH variants are present in Lepidoptera (Minakuchi *et al.*, 2006; Riddiford, 2008; Goodman and Cusson, 2012; Daimon and Shinoda, 2013; Jindra *et al.*, 2013) . Thus, we speculate that the copy variation among the genes involved in the FPP production from IPP and in the isoprenoid branch may reflect the functional divergence of the associated gene products, and is likely linked to the diversity of JH types among insects. Our comparison raised two additional issues regarding the relationship between the variations in JH-related genes and in JH types. One issue is that *FPPS* genes from Lepidoptera may have undergone a species-specific divergence (Figure 2), which agrees with previous findings (Kinjoh *et al.*, 2007). Therefore, we hypothesize that FPPS-mediated enzymatic reactions may be the first step leading to the diversity of JH types among insects. Undoubtedly, whether all the predicted *FPPS* genes are authentically involved in JH

biosynthesis needs to be investigated by more enzymatic experiments. However, JHAMT and FAMeT, two enzymes involved in the methyl esterification of FA, were predicted in *B. mori* and other insects (Table 2 and Table S2). Previous studies in *D. melanogaster* have reported that FAMeT was likely not involved in JH biosynthesis (Burtenshaw *et al.*, 2008; Zhang *et al.*, 2010). However, another analysis speculated that FAMeT-mediated methyl esterification should exist in insects and crustaceans (Hui *et al.*, 2010). Undoubtedly, more evidence is required to resolve the controversial functions of FAMeTs in JH biosynthesis. Furthermore, Cyp15C1 and Cyp15A1 were confirmed to be involved in two routes of FA epoxidation (Daimon and Shinoda, 2013). However, several surveyed insects do not contain either Cyp15C1 or Cyp15A1 (Table 2 and Table S2). Thus, additional experiments are needed to address whether both epoxidation routes occur in insects with no Cyp15C1 or no Cyp15A1 or whether other Cyp genes whose functions have yet to be identified catalyze one or the other route. Finally, all identified genes involved in neuropeptide regulation of JH biosynthesis existed as a single copy, implying that their regulatory mechanism is also conserved among insects.

The metabolic degradation of JH is catalyzed mainly by JHE, JHEH, and JHDK. Our results showed that each of the genes encoding these three enzymes may have undergone duplication in Lepidoptera (Table 2 and Table S2). In particular, *JHDK* has multiple copies in Lepidoptera but one copy in the other surveyed insects. Given the existence of more JH types in Lepidoptera, we propose that, as was true of copy variation among several JH biosynthesis-related genes, the duplication and divergence of JH metabolism-related genes may also be a functionally adaptive evolution to the diversity of JH types in insects.

The nature of JH signaling has been the subject of an increasing number of experimental studies. To date, it has been confirmed that seven genes, namely, *JHBP*, *FKBP39*, *Chd64*, *EcR*, *USP*, *Met*, and *Kr-h1*, are implicated in JH signaling (Li *et al.*, 2007; Suzuki *et al.*, 2011: Jindra *et al.*, 2013) . Except for *Met*, each of the other genes existed as a single copy in *B. mori* and other surveyed insects (Table 2 and Table S2). The Met protein is a bHLH-PAS transcription factor and has been characterized as a JH receptor in insects (Wilson and Fabian, 1986; Jindra *et al.*, 2013). Unlike the single copy of the *Met* gene in *A. gambiae*, *A. mellifera*, and *T. castaneum*, two copies of the *Met* gene have been identified previously in other insects, namely, *Met* and *Gce* in *Drosophila* (Baumann *et al.*, 2010b) and *Met1* and *Met2* in *B. mori* (Li *et al.*, 2010; Guo *et al.*, 2012; Kayukawa *et al.*, 2012). Our analysis also identified two copies (*Met-l1* and *Met-l2*) of the *Met* gene in two other lepidopterans, *D. plexippus* and *H. melpomene*. Although current evidence is not sufficient to elucidate why *Met* was duplicated in these insects, two copies of the *Met* gene have been demonstrated to cooperatively modulate JH signaling via a protein-protein interaction in *D. melanogaster* (Baumann *et al.*, 2010a; Abdou *et al.*, 2011) and in *B. mori* (Guo *et al.*, 2012; Kayukawa *et al.*, 2012). Together with the findings from the phylogenetic tree of insect Met genes (Figure 3) and the functional redundancy of two copies of the *Met* gene, we propose that the JH signaling cascade is also evolutionarily conserved across insects.

Given that multiple copies were predicted for several genes involved in JH biosynthesis and metabolism, it was important to determine whether these copies were functional. Our microarray data and quantitative RT-PCR experiments in *B. mori* showed that some copies of the JH-related genes exhibited moderate expression in the head (mainly containing CA, CC, and brain) or in a dynamic manner that correlated with the temporal changes in JH titer, such as *FPPS1*, *FPPS2*, *JHAMT-l3*, *JHAMT-l4, JHE-l1*, *JHEH-l5*, *JHDK*, and *JHDK-l2*. Previous studies have confirmed the enzymatic activities or physiological functions of several JH-related genes in *B. mori*, such as *JHAMT* (Shinoda and Itoyama, 2003), *JHE* (Tan *et al.*, 2005), *JHEH* (Seino *et al.*, 2010), and *JHDK* (Li *et al.*, 2005). One copy of the *JHAMT* gene has also been verified to be activated functionally in JH biosynthesis in *T. castaneum* (Minakuchi and Riddiford, 2008). Furthermore, most enzyme-encoding genes with multiple copies appear to be expressed in other larval tissues excluding head (mainly containing CA, CC, and brain), during developmental stages with no JH activities, or in one or other sex (Figures 4, 5 and 6). These observations indicate that the copies of the JH-related genes may play roles in other physiological processes. Nevertheless, further enzymatic activity assays are undoubtedly required to assess whether multiple copies of the JH-related genes encode authentic enzymes involved in JH biosynthesis and metabolism or encode proteins involved in other physiological processes.

In summary, we first performed a systematic identification of the genes involved in JH biosynthesis, metabolism, and signaling in insects, including two lepidopterans (*B. mori* and *D. plexippus*), two dipterans (*D. melanogaster* and *A. gambiae*), one hymenopteran (*A. mellifera*), and one coleopteran (*T. castaneum*). A comparative analysis concluded that the early steps of the MVA pathway and neuropeptide regulation of JH biosynthesis, as well as JH signaling, are apparently conserved among *B. mori* and other surveyed insects. However, most genes involved in the last step of the MVA pathway and the isoprenoid branch of JH biosynthesis, as well as JH metabolism, seem to have undergone duplication, resulting in multiple copies in Lepidoptera. This duplication may be functionally and evolutionarily relevant to the variation of JH types among Lepidoptera and other insect species. Although some copies of several JH-related multi-copy genes show a specific spatio-temporal expression correlated to JH activity in *B. mori*, it remains to be confirmed whether their enzymatic activities are associated with JH biosynthesis and metabo-

lism. Taken together, the results of our analysis provide new clues for understanding the genetic basis of JH biosynthesis, metabolism, and signaling in insects.

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Internet Resources

SMART program, http://smart.embl-heidelberg.de/.

Microarray data in *Bombyx mori* genome database, http://www.silkdb.org/microarray/download.html.

Supplementary Material

The following online material is available for this article:

Table S1 - RT-PCR Primers used in this study.

Table S2 - JH-related genes in the other surveyed insects.

Figure S1 - Summary of insect JH-related pathways.

Figure S2 - Phylogenetic tree of the *JHAMT* and *FAMeT* genes.

Figure S3 - Phylogenetic tree of the *JHE*, *JHEH*, and *JHDK* genes.

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