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Full Length Article

Molecular Cloning, Sequence Feature and Expression Pattern of Acetylcholinesterase (AChE) Gene from *Apis cerana cerana*

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Abstract

The acetylcholinesterase gene ace2 was cloned from head tissue of *Apis cerana cerana* worker bees by RT-PCR and RACE. Full-length cDNA of this gene is 2062 bp with an open read frame of 1887bp that encodes 628 amino acids. The encoded protein is predicted to have a molecular weight of 70.71 kD and isoelectric point 5.49. The protein has a signal peptide precursor of 22 amino acids and is predicted to be a secretory protein. Phylogenetic analyses are congruent with a basal split of the order into Rhabditida, Ixodida, Diptera, Hemiptera, Lepidoptera, Coleoptera and Hymenoptera, which shows a closer relationship of the *Apis cerana cerana* (family Apidae) to Pteromalidae than to Formicidae, Ichneumonidae, Braconidae. We conclude that these two species are part of a molecular clade that is different from the recent studies, in which Tenthredinidae and Vespidae appears as a sister group. Our results show Braconidae appearing as the most basal extant, and Apidae appearing as a sister group of Formicidae, Ichneumonidae and Braconidae. The Hymenoptera shows close phylogenetic relationship to the Hemiptera. Real-time PCR analysis shows that ace2 gene is expressed in head, hemolymph, fat body, and midgut. The highest expression level is in head. Among various developmental stages of *Apis cerana cerana*, transcriptional level of ace2 is obviously higher at adult stage. The lowest transcriptional level is at the larval stage. © 2018 Friends Science Publishers

Keywords: *Apis cerana cerana*; Acetylcholinesterase; Gene cloning; Sequence feature; Real-time quantitative PCR

Introduction

Insects acetylcholinesterase (AChE) is the key enzyme in the central nervous system. It can terminate nerve impulse by hydrolysis acetylcholine. It is the main function of target of organophosphate and carbamate pesticides (Corbett, 1974; Fournier and Mutero, 1994). The increased amount of its expression and structure changes will make the insect resistant to insecticides (Fournier *et al.*, 1992, 1993). In vertebrate and invertebrate animals nerve conduction process, if the neurotransmitter acetylcholine completed the required information on following the physiological role but no hydrolysis in time, participate in the information transmission of nerve and muscle will not be able to return to flabby condition, can lead to excessive excitement, spasm, paralysis and even death (Silman and Futerman, 1987). The role of organophosphorus insecticides disruptsupt the signal transmission of AChE in insects, leading to death of their poisoning (Smissaert, 1964; Voss and Matsumra, 1964).

Numerous studies have shown that AChE has other physiological functions except that it took part in the cholinergic neurotransmitter transmitting. In some non-neural tissues such as red blood cells, platelets and macrophage, the content of AChE is high, suggesting that AChE may have some unknown non-cholinergic effect. Biochemical analysis and histochemical studies have shown that many non-cholinergic nerve cells of the brain which they have a lot of AChE but reject cholinergic neurotransmitter transmitting, such as the substantia nigra and locus coeruleus of monoamine neurons and cells, hypothalamic peptide nerve cell and molecular layer cells of cerebellum (Bigbee and Sharma, 2004). The expression of AChE in many areas of the brain during development is different, but these regions do not contain cholinergic delivery. In addition, the role of AChE in nerve growth and cell apoptosis also that captured people's attention (Duysen *et al.*, 2002).

Hall and Spierer (1986) first cloned AChE coding genes from the *Drosophila melanogaster*. Some insects have two different types of acetylcholinesterase in the body, they can produce mutation types. This is one of the reasons for that different insects have different sensitivity to insecticides. *Drosophila melanogster* and *Musca domestica* only one type AChE in the genome of the encoding gene, named ace2 (Mutero *et al.*, 1994; Walsh *et al.*, 2001). There are two types of acetylcholinesterase encoding genes in the *Culex pipiens* genome, and the ace2 gene has a high degree of similarity to the *Drosophila melanogaster* (Malcolm *et al.*, 1998).

This study took advantage of reverse transcription polymerase chain reaction (RT-PCR) and rapid-

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amplification of cDNA ends (RACE) technologies cloning the bee's ace2 gene cDNA sequence. The characteristics of the gene sequence and system evolution were analyzed, and through real time quantitative PCR to detect expression of the gene in the bee's different development periods and larva different tissues. The study laid a solid foundation for further research the function of the gene.

Materials and Methods

Bees Tissue Sampling, RNA Extraction and RT-PCR

The bees were provided by Zunyi Normal College. Take 8 worker bees' heads, in accordance with the EasyPure RNA Kit (TransGen Biotech) extract total RNA, using 1% agarose gel electrophoresis detection. RT-PCR reactions were conducted using Trans ScriptTM Two-Step RT-PCR Super Mix (TransGen Biotech). Detailed procedures followed the instructions of the manufacturer. The reaction included the primers of CDSIII, SMARTIV and L1 (Table 1), serving as an internal control. The PCR conditions were shown in Table 2. The RT-PCR products were analyzed by electrophoresis on a 1.5% agarose gel and documented with a Gel Documentation System (Nucleotech Corp., San Mateo, CA).

The Intermediate Fragment cDNA Amplification

We designed 1 pair of degenerate primers F1 and R1 (Table 1) using primer premier 5.0. These primers were designed based on the *Apis mellifera* (Elsik *et al.*, 2014) sequence and worked for the intermediate fragment cDNA amplification of the *Apis cerana cerana*. Then, we proceeded the middle of fragment amplification cloning obtained cDNA. The PCR conditions were shown in Table 2. The PCR product was separated by 1% agarose gel electrophoresis, the expected size of DNA fragment was recovered. The PCR products were connected pMD18-T vector, then were transformed into *E.coli DH5α* competent cells. The positive clones by microbial PCR testing (primer for F1 and R1, Table 1) were sequenced the Sangon biological engineering (Shanghai) Co., Ltd.

3´RACE Amplification of cDNA

We amplified 3´end cDNA by reference 3´-Full RACE Core Set Ver. 2.0 (TaKaRa) instructions. According to the sequence information of *Apis cerana cerana* ace2 gene middle fragments obtained by cloning, the specific primers F2 and F3 were designed by Primer Premier 5.0 (Table 1). We used same RT-PCR step reverse transcription of cDNA template, CDSIII and F2 as primer for the first PCR reaction. The PCR conditions were shown in Table 2. The first PCR reaction product of 40 times dilution as a template for the second round of the reaction. The PCR product was separated by 1% agarose gel electrophoresis, the expected size of DNA fragment was recovered. The PCR products were connected pMD18-T vector, then were transformed into *E. coli DH5α* competent cells. The positive clones by microbial PCR testing (primer for M13-F and M13-R, Table 1) were sequenced the Sangon biological engineering (Shanghai) Co., LTD.

5´RACE Amplification of cDNA

We amplified 5´end cDNA by reference 5´-Full RACE Kit (TaKaRa) instructions. According to the sequence information of *Apis cerana cerana* ace2 gene middle fragments obtained by cloning, the specific primers R2 and R3 were designed by Primer Premier 5.0 (Table 1). We used same RT-PCR step reverse transcription of cDNA template, UPM and R2 as primer for the first PCR reaction. The PCR conditions were shown in Table 2. The first PCR reaction product of 40 times dilution as a template for the second round of the reaction. The PCR product was separated by 1% agarose gel electrophoresis, the expected size of DNA fragment was recovered. The PCR products were connected pMD18-T vector, then were transformed into *E. coli DH5α* competent cells. The positive clones by microbial PCR testing (primer for M13-47 and RV-M, Table 1) were sequenced the Sangon biological engineering (Shanghai) co., Ltd.

Data Analysis

DNA sequences were corrected and analyzed using Sequencing Analysis v3.4.1 (Applied Biosystems, USA) and Seqman v5.05 (DNASTAR Inc., USA). Coding of gene amino acids with sequin sequence editing software; using Signal P4.1 Server online tool (http://www.cbs.Dtu.dk/services/SignalP) is the prediction of protein signal peptide (Thomas *et al.*, 2011). Through the expasy - protparam online tools (http://web.Expasy.org/protparam/) prediction of molecular weight and isoelectric point of protein. In combination with the acetylcholinesterase gene sequences of other insects (Table 4), 24 species phylogenetic relationships in the order Insecta were analyzed. We reconstructed phylogenetic relationships of the order Insecta with the Neighbor-Joining of MEGA 5 the evolutionary relationship of homologous protein sequence system was analyzed (Tamura and Peterson, 2011), and 1000 repetitions were used for self-test.

The Real-Time Quantitative PCR Detection of Ace2 Gene Expression

We took in different development stage worker bee's heads, larvae's head, larvae's blood, larvae's fat body and larvae's midgut. We extracted total RNA and reverse transcriptase cDNA, they were as template for the real-time quantitative PCR. Using Primer Premier 5.0 software, according to middle fragment we designed specific primers F4 and R4 (Table 1).

Table 1: Primers and their sequences used in this study

Objective	Primer	Sequences (5'-3')	Primer	Sequences (5'-3')
RT-PCR	CDSIII	AAGCAGTGGTATCAACGCAGAGTACTTTTTTTTTTTTTTTTTTTTT TTTTTTTTVN	SMARTIV	AAGCAGTGGTATCAACGCAGAGTG GCCATTACGGCCGG
	L1	CTAATACGACTCACTATAGGGCAAGC AGTGGTATCAACGCAGAGT		
Middle	F1	TACAGGCTCAGGCACAAG	R1	GACCTCATACACGCCATCACC
3´RACE	F2	GGATACTGGTGGACGATTGCG GAT	M13-F	TGTAA AACGACGGCCAGT
	F3	ATCTTCCTTCCCAAGCATCCTCTC	M13-R	CAGGAAACAGCTATGAC
5´RACE	R2	CCCTTGTGCCTGAGCCTGT	RV-M	GAGCGGATAACAATTTCACACAGG
	R3	TGTTCGGGTTCCACATCTCC	M13-47	CGCCAGGGTTTTCCCAGTCACGAC
	UPM	CTAATACGACTCACTATAGGGC		
qPCR	F4	CCTGACAAACATGGACTTGT	F5	ACTGTATCTTAATGTACTGC
	R4	CAGGAGGCCGAACGCGCCCA	R5	TTCGTAATACCTTCATTGCA

Note. F means forward and R means reverse

Table 2: The polymerase chain reaction conditions

Methods	Steps	PCR Conditions
RT-PCR	first	72°C 2 min, 0°C 2 min, then 42°C 90 min, 72°C 10 min
	second	94°C 2 min, then 40 cycles with 94°C 15 s, 55°C 30 s, 68°C 1 min,68°C an additional 5 min.
Middle		94°C for 5 min, then 40 cycles with 94°C 30 s, 55°C 30 s, 72°C 1 min., 72°C an additional 5 min
3´RACE	first	94°C 5 min, then 25 cycles with 94°C 30 s, 64°C 30s, 72°C 1.5min, an additional72°C 10 min.
	second	94°C 5 min, then 35 cycles with 94°C 30 s, 64°C 30s, 72°C 1.5min, an additional 72°C 10 min.
5´RACE	first	94°C for 5 min, then 30 cycles with 94°C 30 s, 61°C 30s, 72°C 1.5 min, an additional 72°C 10 min.
	second	94°C 5 min, then35 cycles with 94°C 30 s, 62°C 30s, 72°C 1.5min, an additional 72°C 10 min.

According to the information of bee's ribosomal protein s6 kinase (GenBank accession No. FJ966885), we designed internal reference primers F5 and R5 (Table 1). We proceeded the real-time quantitative PCR based on SYBR Premix Ex TaqTM (Perfect Real Time) preparation reaction system, repeat each sample set 3, according to the ABI 7300 fast real-time PCR System operating methods for test operation, using ABI 7300 SDS Software ct value and base line automatically analysis for test result. We analyzed the results of the use of excel Software, the use of $2^{-\Delta\Delta ct}$ method to analyze gene mRNA transcription level difference, Δct=ct (purpose gene)- ct(internal gene), ΔΔct=Δct-Δct(maximum).

Results

RNA Extraction and Gene Cloning

The extraction total RNA quality is good. The gene fragments were consistent with the expected fragment size. They are shown in Fig. 1.

Sequence Characteristic of Ace2 Gene and the Encoded Protein

The gene cDNA sequence analysis found that the gene cDNA sequence is 2062bp, including 5´end 169bp of untranslated region (5´-UTR) and an open reading frame (ORF) of 1887bp, number of amino acids are 628. Using signal P 4.1 application Server online tools to predict protein contains consists of 22 amino acids residues of precursor signal peptide (Fig. 2), the protein is secreted proteins. Through the expasy-protparam online tools that predict molecular weight is 70.71KD, and isoelectric

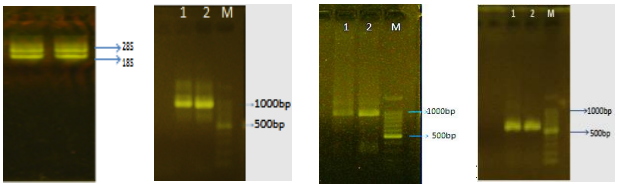

Fig. 1: Electrophoretograms of the total RNA (A), the middle fragment (B), 3'-RACE amplification product (C) and 5'-RACE amplification product (D) of ace2 gene

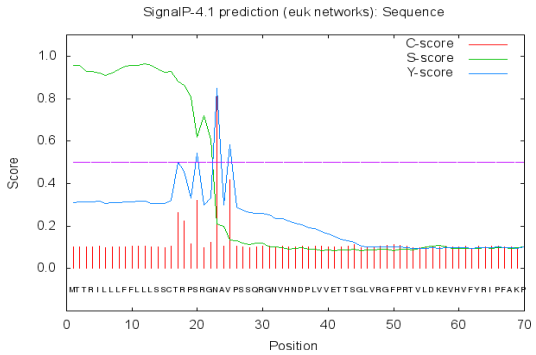

Fig. 2: Signal peptide predicted by Signal P 4.1 Server

point (PI) is 5.49. Total number of negatively charged residues (Asp + Glu) are 69. Total number of positively charged residues (Arg + Lys) are 57. The N-terminal of the sequence considered is M (Met). The estimated half-life is 30 hours (mammalian reticulocytes, in vitro). The instability index (II) is computed to be 41.61. This classifies the protein is unstable.

Table 3: Information of acetylcholinesterase gene of insects were used in this study

Classify	Species	GenBank accession No.	Source
Hymenoptera	*Apis mellifera*	NM-001040230	Elsik *et al.* (2014)
	Apis mellifera	AF213012	Shapira *et al.* (2001)
	Apis cerana	JR037618	Sun *et al.* (2013)
	Apis cerana cerana	MF523539	Wang *et al.* (this study)
	Nasonia vitripennis	NC_015869	Werren *et al.* (2010)
	Polyrhachis vicina	JF742990	Fan and Xi (2011)
	Diadegma fenestrale	KM272210	Kim *et al.* (2014)
	Microplitis demolitor	NW_014463818	Burke *et al.* (2014)
	Athalia rosae	KB467497	Kim *et al.* (2014)
	Polistes dominula	XM_015320137	Lowe and Eddy (2016)
Hemiptera	*Sitobion avenae*	AY707319	Chen and Han (2006)
	Myzus persicae	AF287291	Nabeshima *et al.* (2002)
	Bemisia tabaci	AJ576072	Javed *et al.* (2016)
	Nilaparvata lugens	JN688930	Li *et al.* (2012)
	Aphis gossypii	AF502082	Li and Han (2002)
Coleoptera	*Tribolium castaneum*	NM_001293605	Kim *et al.* (2010)
Lepidoptera	*Plutella xylostella*	JX404027	Lai and Xu (2012)
	Helicoverpa armigera	AF369793	Ren *et al.* (2002)
	Helicoverpa assulta	AY817736	Lee *et al.* (2006)
	Bombyx mandarina	EF166089	Li *et al.* (2015)
	Bombyx mori	DQ115793	Shang *et al.* (2007)
Diptera	*Musca domestica*	AF287291	Kozaki *et al.* (2002)
	Culex tritaeniorhynchus	AB122152	Nabeshima *et al.* (2004)
Ixodida	*Boophilus microplus*	AJ278345	Baxter and Barker (2002)
Rhabditida	*Caenorhabditis elegans*	AF025378	Grauso *et al.* (1998)

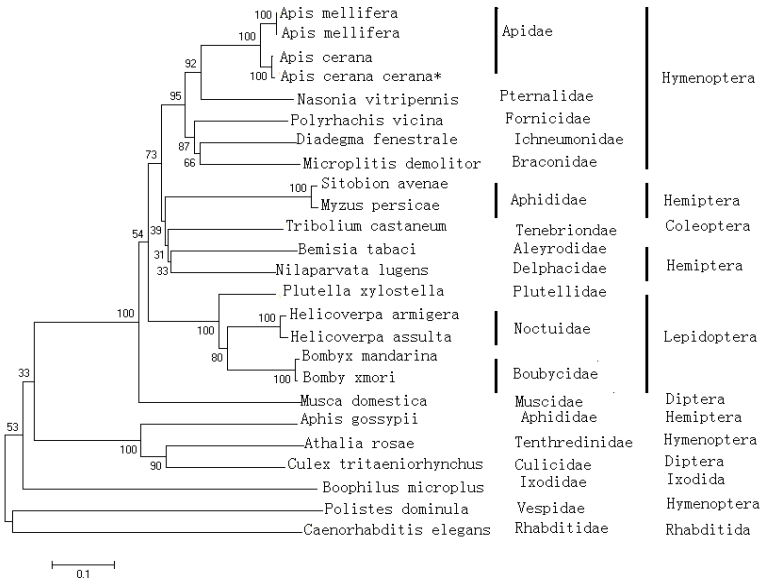

Fig. 3: Phylogenetic relationships among insects by Neighbor-Joining inference analysis based on protein code data. Branch lengths are mean estimates. Numbers in the nodes are from highest to lowest bootstrap value. The scale in the left lower corner indicates relative branch lengths

The System Evolution of Ace2 Gene Encoding Protein Analysis

In combination with the information of acetylcholinesterase gene of other insects that have been reported in GenBank (Table 3), phylogenetic relationships in the order Hymenoptera were analyzed. Phylogenetic relationships of *Apis cerana cerana* in the order Hymenoptera by ace2 gene encoding protein are shown in Fig. 3.

The Expression of Ace2 Gene in Different Developmental Stages and Larval Tissues

The expression pattern about ace2 gene of *Apis cerana cerana* are shown in Table 4 and Fig. 4.

Discussion

The extraction total RNA from head to by agarose gel electrophoresis, the electrophoresis visible 28S and 18S two

bands, the stripes were clear, and tail phenomenon was not obvious. The result showed that the extracted RNA quality is good, can meet the requirements of subsequent molecular biology experiment, as shown in Fig. 1A. We used the degenerate primer F1 and R1 to proceed PCR amplification (Table 1). We obtained fragment that is consistent with the expected fragment size (Fig. 1B). The sequencing showed that the fragment size is 982 bp, and it was confirmed that this fragment was highly homologous with ace2 genes of other insects. According to degenerate primers amplified fragment sequence, we designed specifically a pair primers, the ace2 gene cDNA 3′and 5′end were obtained by RACE technique, the 3′end sequence is 960 bp (Fig. 1C), 5′end sequence is 610 bp (Fig. 1D). By sequence assembly, the cDNA sequence of the bees ace2 gene was got, which length is 2062 bp. The gene sequence was submitted to GenBank (GenBank accession No. MF523539).

Four species of honey bees in the family Apidae form a distinct clade with a high bootstrap value (100). This is consistent with the previously determined morphological classification of the family Apidae and its significant differentiation from other families in the order Hymenoptera. As expected, the monophyly of Hymenoptera, the inclusion of *Apis mellifera* (Shapira *et al.*, 2001), *Apis cerana* (Sun *et al.*, 2013) and *Apis cerana cerana* within this order is highly supported (bootstrap value of 100) (Fig. 3). Apidae is recovered with maximal support as a sister group of *Nasonia vitripennis* (Pteromalidae) (Werren *et al.*, 2010). The families of Formicidae (*Polyrhachis vicina*) (Fan and Xi, 2011), Ichneumonidae (*Diadegm afenestrale*) (Kim *et al.*, 2014) and Braconidae (*Microplitis demolitor*) (Burke *et al.*, 2014), are also recovered as sister groups with a high bootstrap value (95).

In other branches, the families of Aphididae (*Sitobion avenae* and *Myzus persicae)* (Nabeshima *et al.*, 2002; Chen and Han, 2006), Tenebrionidae (*Tribolium castaneum*) (Kim *et al.*, 2010), Aleyrodidae (Bemisiatabaci) and Delphacidae (*Nilaparvata lugens*; Li *et al.*, 2012) formed a distinct group. The families of Plutellidae (*Plutella xylostella*) (Lai and Xu, 2012), Noctuidae (*Helicoverpa armigera* and *Helicoverpa assulta*) (Ren *et al.*, 2002; Lee *et al.*, 2006) and Bombycidae (*Bomby xmori* and *Bombyx mandarina*) (Shang *et al.*, 2007; Li *et al.*, 2015) are strongly supported (bootstrap value of 100) as a sister group. However, *Athalia rosae* (families of Tenthredinidae) and *Polistes dominula* (families of Vespidae) (Kim *et al.*, 2014; Lowe and Eddy, 2016) are excluded from Hymenoptera. The *Aphis gossypii* (families of Aphididae) (Li and Han, 2002), *Athalia rosae* (families of Tenthredinidae) and *Culex tritaeniorhynchus* (families of Culicidae) are strongly supported (bootstrap value of 100) as another sister group (Nabeshima *et al.*, 2004).

Even though phylogenetic interrelationships of highly diversified families of the order Hymenoptera, such as Apidae, Pteromalidae, Formicidae, Ichneumonidae and Braconidae are well studied at the molecular level, the main phylogenetic interrelationships of Hymenoptera families remain largely unresolved. *Boophilus microplus* (families of Ixodidae) (Baxter and Barker, 2002), *Polistes dominula* (families of Vespidae), *Caenorhabditis elegans* (families of Rhabditoidea) (Grauso *et al.*, 1998), which are placed as a sister group of all other analyzed insects.

Table 4: mRNA transcriptional levels of ace2 gene at different developmental stages and different tissues of larvae of *Apis cerana cerana* measured by Real-time quantitative PCR

	ACHE	GAPDH	ΔCt	ΔΔCt	$2^{-\Delta\Delta Ct}$
Egg	21.41123	25.06014	-3.64891	0.012166	0.991603
Larva	24.73884	27.27861	-2.53978	1.1213	0.45968
Pupa	23.6036	26.93803	-3.33444	0.326639	0.797392
Worker bees	18.9455	25.1445	-6.199	-2.53792	5.807529
Head	24.73884	27.27861	-2.53978	1.1213	0.45968
Hemolymph	25.15224	26.50374	-1.3515	2.309576	0.20172
Fat body	25.28633	27.54877	-2.26244	1.398637	0.379287
Midgut	25.17874	25.27728	-0.09854	3.562538	0.084639

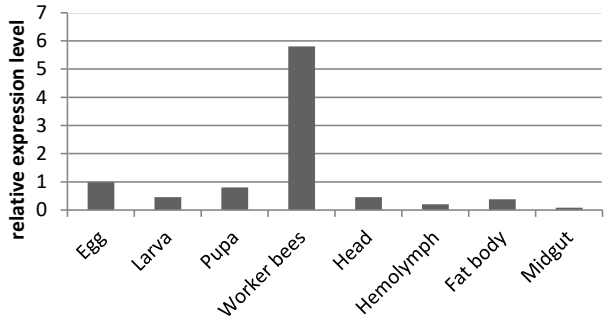

Fig. 4: The mRNA transcriptional levels of ace2 gene at different developmental stages and different tissues of larvae of *Apis cerana cerana* measured by Real-time quantitative PCR

Choosing the bee's ribosomal protein S6 kinase gene as internal reference, using real-time quantitative PCR detecting the ace2 gene transcription level in each development period of bee and different tissues of larvae as the head, hemolymph, fat body and midgut (Table 4). In each development period, the ace2 mRNA in transcription level of worker bee is obviously higher than that of other development periods. The transcription level of worker bee is 12.63 times that of the larval stage. Their eggs and pupal stage are relative higher than larval stage, and larval stage is the lowest (Fig. 4). Ace2 mRNA is expressed in various tissues of the larvae. Its transcription level is relatively high in the head, fat body and hemolymph. Midgut's transcription level is the lowest in various tissues of the larvae (Fig. 4).

Real-time quantitative PCR to detect the bee's ace2 gene is expressed in larvae of tissues such as the head, and express the amount the highest in the head. Ace2 gene have higher expression in fat body, speculated that the fat body may be an important organ detoxification, the gene

abundantly expressed in fat body may be associated with the biological defense function of the bees. Since first isolated from horse serum cholinesterase successively been purification (Stedman and Easson, 1932). Many acetylcholinesterases from different animal origin were separation and purification. Many studies have shown that these animals acetyl cholinesterase mainly concentrated in the brain, liver and hemolymph (Randall, 1994; Mendelson *et al.,* 1998), that animal of acetyl cholinesterase expect as neurotransmitter passing nerve impulses, also have other non-cholinergic effects. By real-time quantitative PCR results showed that ace2 gene in the egg stage, pupal stage and adult stage transcription all exceed larval stage.

In adult period is the highest transcription (Fig. 4). AChE2 whether in the different development period of bees play a different role, needing to further research. Real-time quantitative PCR detection of ace2 in the bee's larvae hemolymph, fat body have a higher level of transcription, also detected in midgut in a lower level of transcription. About whether the ace2 gene expression in these organizations suggests that AChE2 may have the function of the non-cholinergic, has yet to be further in-depth study.

Conclusion

We found that Braconidae appears as the most basal extant, and Apidae appears as a sister group of Formicidae, Ichneumonidae and Braconidae. The Hymenoptera showed a close phylogenetic relationship to the Hemiptera. The lowest transcriptional level was at the larval stage and the highest expression organization was in head.

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