Molecular Cloning and Expression of an Estrogen Receptor-Related Receptor Gene in *Apis mellifera* (Hymenoptera: Apidae)

by

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ABSTRACT

Estrogen receptor-related receptors (ERRs) belong to a subfamily of orphan nuclear receptors where the proteins are closely related to the estrogen receptors (ERs) in structure. ERR homologs have been found in many animals and play an important role in the regulation of physiologic processes. In our study, the estrogen receptor-related receptor homolog gene (referred to as AmERR) was cloned from *Apis mellifera* Linnaeus. The full-length cDNA of the AmERR gene is 1779 bp, containing a 5'- untranslated region (5'-UTR) of 197 bp and a 3'-UTR of 277 bp. The open reading frame of 1,305 bp encodes a 434-amino acid protein. Using real-time quantitative RT-PCR to study AmERR mRNA expression patterns indicated that this gene is differentially expressed during honeybee development. There is a remarkable increase of relative expression of AmERR mRNA from pupa to adult , which may indicate that the gene plays an important role in the bee's differentiation of tissues and development. A different expression in castes and abundance expression in adults of AmERR mRNA may suggest it is relevant to functions in social communities.

Key words: molecular cloning, estrogen receptor-related receptors(ERR), *Apis mellifera* , real-time quantitative RT-PCR

INTRODUCTION

Nuclear receptors (NR) constitute a large family of proteins that can be considered as ligand-inducible transcription factors, which bind directly to DNA and regulate expression of down stream target genes (Ouyang & Xi

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2009). All members of this superfamily are composed of two main domains, the DNA-binding domain (DBD) and the ligand-binding domain (LBD), which show varying degrees of similarity. However, putative receptor molecules for the ligands have not been found, and they are classified as orphan nuclear receptors (Blumberg & Evans 1998). The orphan receptors are distributed in all protein subfamilies (Escriva *et al*. 2000). The estrogen-related receptors (ERRα, ERRβ, and ERRγ) belong to a subfamily of orphan nuclear receptors, share the similar structure with ERs, bind to the steroid receptor coactivator family without any ligands, and drive transcription activity of the target genes (Giguere *et al*.1988; Giguere 2002; Pettersson *et al*.1996). The results from various laboratories have suggested mammalian ERRs collaborate with estrogen signaling, and are involved in many physiological and developmental processes, as well as the proliferation and differentiation of cells (Cheung *et al*. 2005; Yang *et al*. 1996; Zhang *et al*. 2000; Vanacker *et al*.1999a; Vanacker *et al*. 1999b ;Kraus *et al*. 2002).

In this study, for the first time, we have cloned the full-length cDNA of an estrogen-related receptor homolog gene named AmERR from the eusocial insect, *Apis mellifera*. The expression pattern of AmERR mRNA was studied by using real-time RT-PCR. The results show that this gene is expressed differentially at distinct development stages and in different castes.

MATERIAL AND METHODS

Experimental Insects

The colonies of *Apis mellifera* were obtained at the apiary of Huarun honeybee breeding base in Shaanxi Province, China. Embryos, larvae, pupae and adults (workers, queens, and drones) were collected from the colonies, immediately immersed in liquid nitrogen, and stored at -80°C for RNA extraction (Lü *et al*. 2008; Guo *et al*. 2010).

RNA preparation and cDNA synthesis

Total RNA was extracted from pooled samples of 2 frozen drones (selected randomly) with RNAiso Plus® (Takara Bio Inc.), and then immediately reverse-transcribed for the generation of cDNA using the First-Strand cDNA Synthesis Kit® with oligo(dT) primer (Fermentas Life Sciences, Burlington Ontario). All procedures were performed according to the manufacturer's instructions.

The cDNA templates of different developmental stages and different castes for the real-time quantitative RT-PCR experiments were performed by the same methods described above.

Molecular cloning and Sequencing of AmERR

The full-length cDNA of AmERR was cloned based on the scheme shown in Fig.1. Initially, partial cDNA fragment of AmERR was amplified by reverse-transcription chain reaction (RT-PCR) using degenerated primers A1and A2 (Table 1), which were designed by PrimerPremier 5.0, Oligo 6.0. The primers A1and A2 located at the conserved DNA binding domain and ligand binding domain of the published AmERR homologues from other insect species (*Drosophila melanogaster*, *Culex quinquefasciatus* ,*Nasonia vitripennis, Tribolium castaneum*). PCR products were cloned into PMD19-T Vector (TaKaRa, Dalian, China), and individual clones were sequenced. Based on the first obtained fragment, further specific and degenerated primers (B1 and B2; C1 and C2) (Table 1) were designed and used to obtain most of the AmERR gene coding sequence.

Full-length cDNA of AmERR was obtained by rapid amplication of cDNA ends, using the 3'-Full RACE Core Set® and 5'-Full RACE Kit® (Takara Bio Inc), following the manufacturer's instructions .The gene specific primers used for RACE are shown in Table 1.

Structural and phylogenetic analysis of AmERR

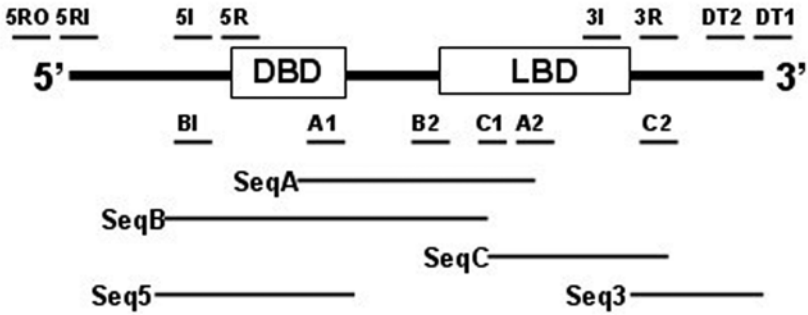

Fig.1. Cloning strategy and map of the primers used for amplifying the full sequence of the AmERR gene: A1&A2 for Seq.A of 385 bp, B1&B2 for Seq.B of 934 bp, C1&C2 for Seq.C of 509 bp, 5RO,5RI, 5I and 5R for Seq.5of 783bp; 3R,3I,DT1 and DT2 for Seq.3 of 385 bp.

Table 1. Oligonucleotide primers used for cDNA cloning and real-time quantitative RT-PCR.

Target	Name	Primer Sequence 5'-3'
A	A1	CAAGGHAAYATCGAGTACA
	A2	ATCTGYTTCGCCCAVCCRAT
B	B1	AGGTAKCCCTSGAASTCC
	B2	CGCTTGTCCTGGTCTTACTGC
C	C1	CATYTCDACRAASAGCTTGTTCAT
	C2	AACAGATTCCAGGATTCAGCA
3'RACE	3R	AAGGCTTCAAAGATTGGGATT
	3I	TTACCCAGTCTCAGGCAGGTC
	oligo(dT)1	GGCCACGCGTCGACTAGTAC$(T)_{17}$
	oligo(dT)2	GGCCACGCGTCGACTAGTAC
5'RACE	5RO	CATGGCTACATGCTGACAGCCTA
	5RI	CGCGGATCCACAGCCTACTGATGATCAGTCGATG
	5R	CGTCAACGTCGTGGTAGGG
	5I	CGGTGGACCTTCTGTATTT
Real-time	F	TCCAGGATTCAGCAGTTTAGCTTT
	R	TCTACCATTATTTGGCTTGCTTCTC
β-actin	S	CCCTCTTCCAGCCATCGTTC
	A	CCACCGATCCAGACGGAGTA

Note: K: G or T; M: A or C; W: A or T; Y: C or T; D: A, T or G; R: A or G

The open reading frame (ORF) of the AmERR gene was searched using the NCBI ORF Finder(http://www.ncbi.nlm.nih.gov/gorf/gorf.html).The identities of AmERR protein and known ERR protein sequences were analyzed using the MegAlign (DNASTAR package). Sequence alignments based on the amino acid sequences of the known ERRs were performed by Clustal X 1.81 (Jeanmougin *et al.*1998), followed by manual inspection. From these alignments, a phylogenetic tree was constructed by MEGA 4.0 (Tamura *et al*. 2007), according to the neighbor-joining method with a bootstrap test calculated with 2000 replicates and a Poisson correction model.

Real-time quantitative RT-PCR

The expression of AmERR was quantified by real-time quantitative RT-PCR . Total RNAs were isolated from honeybees of different developmental periods and different castes. We used 4.5 micrograms of total RNAs for reverse transcription using the method mentioned above. To prevent genomic DNA

contamination, the RNAs for real-time PCR were treated with RNase-free DNase (Roche Applied Science, Mannheim,Germany;http://www.roche-applied-science.com/index.jsp). Reactions were performed using an iQ5® apparatus (Bio-Rad Laboratories, Inc., Hercules, California; http://www.bio-rad.com/) with a SYBR Premix Ex Taq Kit® (Takara Bio Inc). Primers (F,R) of AmERR and primers (S,A) of β-actin were used in real-time RT-PCR (Table 1). The detailed protocol was as follows: 95°C for 1min, 40 cycles of 95°C for 10s and 58°C for 25s, followed by a dissociation-curve program from 55 to 95°C with a heating rate of 0.5°C every step and continuous-fluorescence acquisition. All RT-PCR reactions were performed in triplicate. To obtain precise quantification,the specific PCR products and the absence of primer-dimers were confirmed by viewing the single peak in the melting curve of the tested genes.

One of the cDNA samples was used to construct standard curves for AmERR and β-actin after serial dilution and the slopes of the curves were obtained. The relative quantification of AmERR was determined using the formula $F=2^{-\Delta\Delta CT}$. In order to analyze the expression of AmERR at different developmental stages, we chose the cDNA of first instar as the calibrator, while analyzing AmERR expression in the three castes, the drone was selected as the calibrator. AmERR relative expression levels were analyzed using one-way analysis of variance (ANOVA) with Dunnett's multiple comparison (SPSS Inc. 2004). Details of the procedures for measurement were same as those described previously.

RESULTS

Cloning and characterization of AmERR cDNA

The full-length cDNA of AmERR was amplified by RT-PCR and RACE. The full-length sequence is 1779 bp, contains a 1305 bp ORF which encodes a 434 amino- acid protein (Fig. 2). The length of the 5'- UTR is 197 bp ,and the 3'-UTR is 277 bp. A putative polyadenylation signal is found upstream from the 19-nucleotide poly (A) tail, which coincides with the fact that the polyadenylation signal is most often present 11-30 nucleotides upstream from the poly (A) tail (Fitzgerald & Shenk 1981). The calculated molecular mass of deduced AmERR protein is 49.131kDa,and the isoelectric point is 7.43.

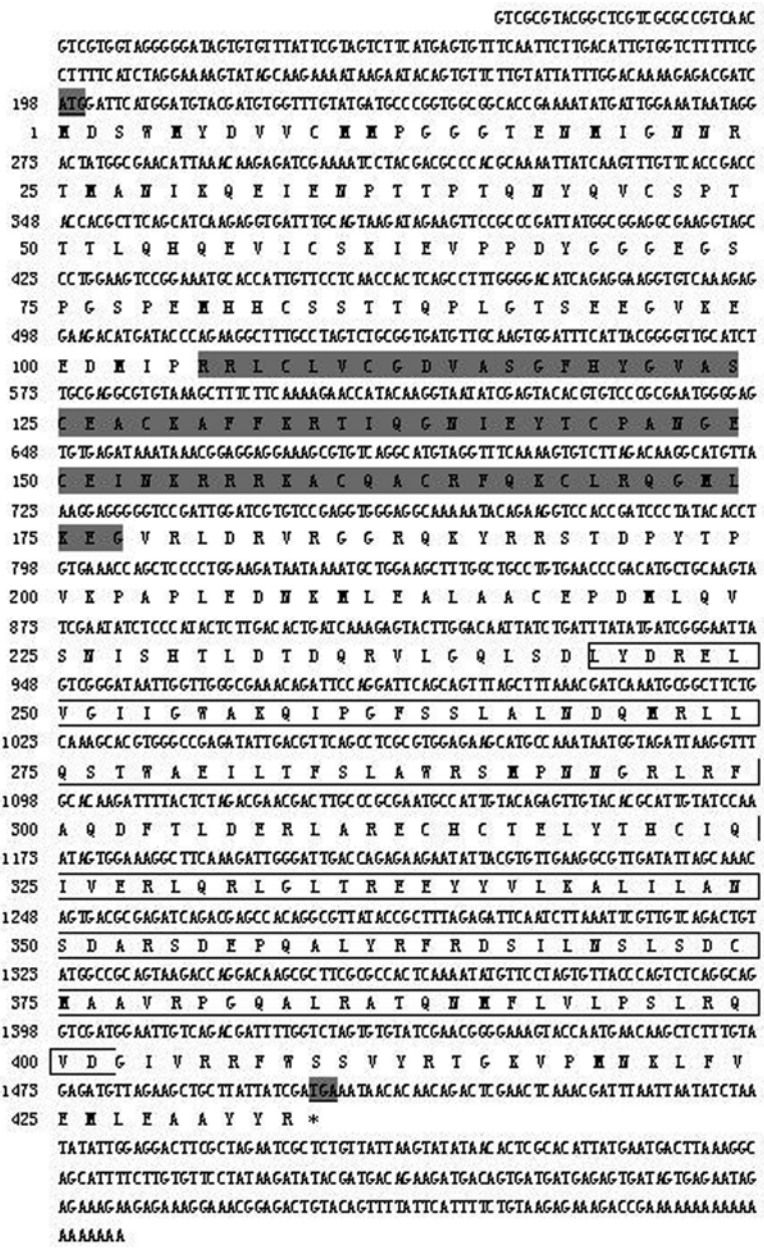

Fig.2. ThecDNA and deduced amino acid sequence of AmERR. The sequence is1779bp long encoding a protein of 424 amino acid residues. The initiating codon ATG and the stop codon TGA are underlined and in shadow, and a DBD domain is in shadow, a LBD domain is boxed.

The GenBank accession number of the AmERR cDNA is EF506198.

Alignment analysis and phylogenetic-tree construction in insects

A multiple alignment of the deduced animo acid sequence of AmERR with other known ERR homologues was performed with Clustal X 1.81 (Fig.3) and modified by BOXSHADE (European Molecular Biology Network; http://www.ch.embnet.org/software/BOX_form.html). Species and GenBank accession numbers mentioned are as follows:*Apis mellifera* (Accession No.XP_392385); *Nasonia vitripennis*(Accession No.XP_1604033); *Drosophila melanogaster*(Accession No.NP_729340); *Aedes aegypti* (Accession No.EAT34188);*Culexquin quefasciatus* (Accession No.XP_1862743);*Polyrhachis vicina* (Accession No. EF474463);*Homo sapiens* L. (Accession No.NP_004443); *Teleogryllus emma* (Accession No. ACW84414); *Bombus terrestris* (Accession No. XP_003395673); *Tribolium castaneum* (Accession No.XP_001812322). The phylogenetic tree was constructed using the neighbor joining method with Poisson correction, with

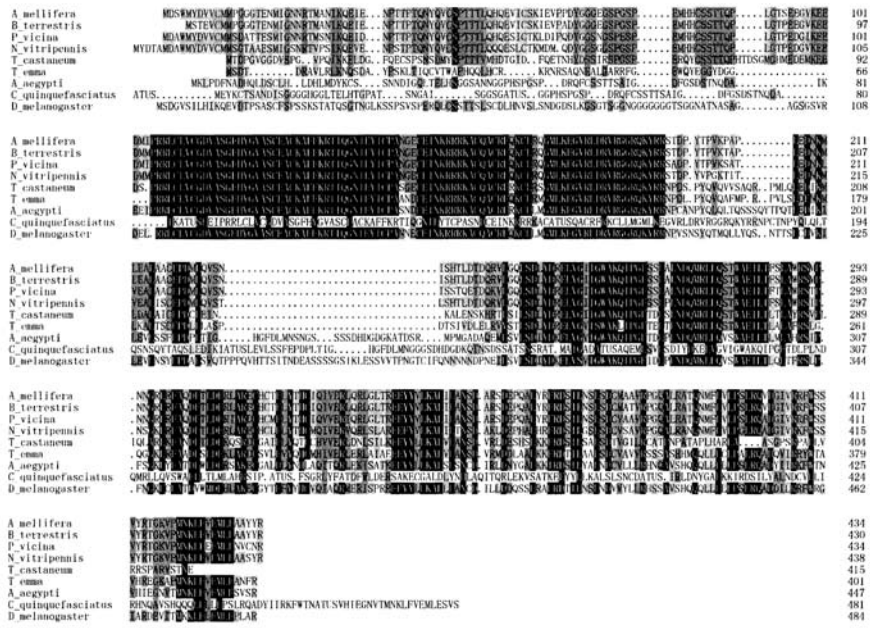

Fig.3. Amino acid sequence alignments of AmERR with other homologous sequences from other organisms. The highly conservative amino acid residues are highlighted in black and grey.

Homo sapiens L. used as an outgroup.

The results of phylogenetic analysis revealed that AmERR shares high identity with *Polyrhachis vicina* and *Bombus terrestris*, as expected (Fig.4). Also, the phylogenetic relationship of AmERR is closer to human ERR than to *Drosophila* ERR in the evolutionary pathway. The phylogenetic relationship of ERR in insects shows the protein is very highly conserved in the evolutionary pathway.

Expression analysis of AmERR mRNA

The relative level of expression of the AmERR mRNA in different developmental periods and castes was analyzed by means of real-time quantitative RT-PCR. Previous studies clearly indicated that the analyzed β-actin mRNA levels remain fairly constant in tissues of insects regardless of their developmental or physiological condition (Claeys *et al*. 2003; Simonet *et al*. 2004). The results showed that AmERR is expressed in each tested sample. During the development stages of the three castes, the highest expression levels are all found in adults. During the development stages of worker bees (Fig.5), the lowest expression level is found in the third instar, then it increased from pupae to adults. During the development stages of queen bees (Fig.6), first, the expression level increased from the first instar to the second instar, then

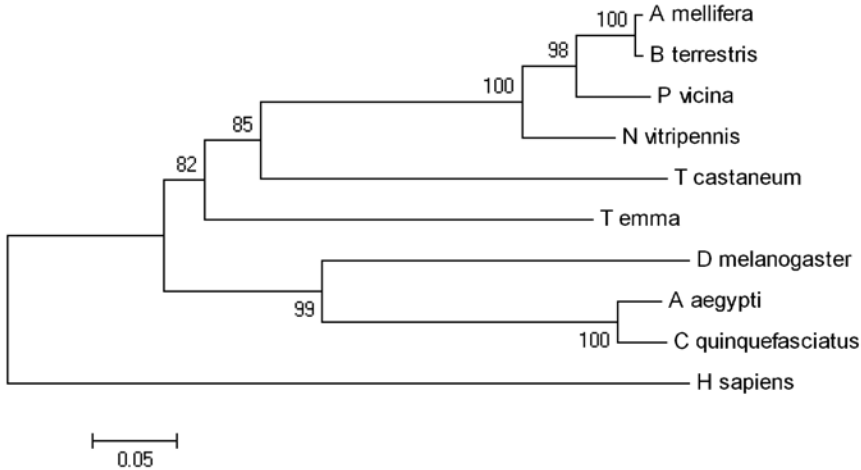

Fig.4. The phylogenetic tree based on NJ method, confidence values by 2000 repeats are shown in the nodes; the abbreviation of the species is shown in the text.

it declined significantly in the third instar, and finally, the expression of the pupa increased again. The expression level of AmERR in drones (Fig.7) is similar to that in queen bees. Among three caste adults (Fig.8), the highest expression of AmERR was in queen bees, the second-highest was in worker bees, and the lowest was in drones. Additionally, we analysed the AmERR mRNA expression among the developmental periods of the three castes (Fig.9), the results showed that the expression level increases gradually dur-

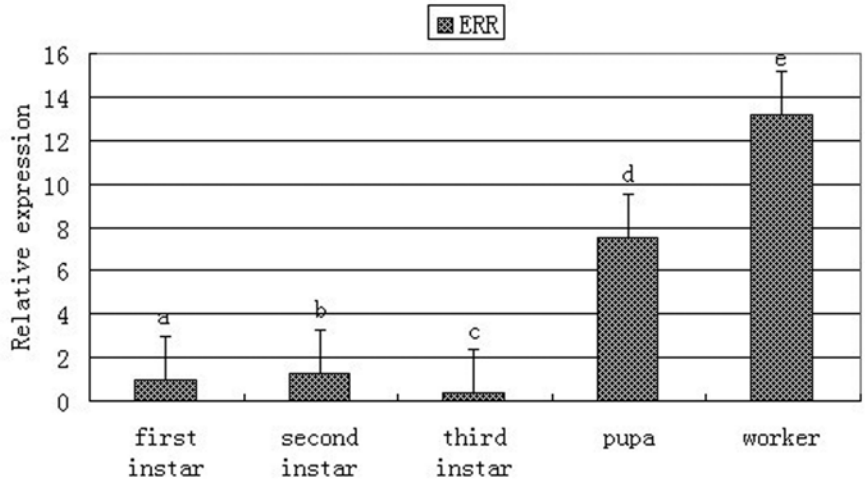

Fig.5. Relative expression of AmERR in different developmental stages of worker bees (bars with different letters indicate means are significantly different, t-test for LSD, $p<0.05$).

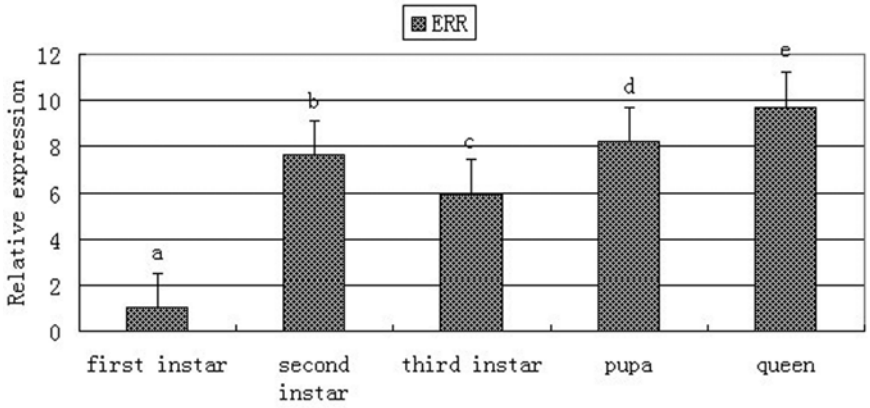

Fig.6. Relative expression of AmERR in different developmental stages of queen bees (bars with different letters indicate means are significantly different, t-test for LSD, $p<0.05$).

ing the development stages. A remarkable increase of the AmERR expression from pupa to adult may indicate the gene has important roles in regulating tissue differentiation. The differerent expression in castes and high mRNA levels in adults may suggest AmERR is related to various functions in social communities.

DISCUSSION

In this study, we have isolated and characterized a full-length cDNA sequence of an estrogen receptor-related receptor homolog from the honeybee species *Apis mellifera*. The cDNA sequence of AmERR and its deduced amino acid sequence reflect a high degree of homology with the ERR homologs identified from other animals, indicating that this newly isolated cDNA encodes the *Apis mellifera* estrogen receptor-related receptor protein. The results of multiple alignments showed that the DBD and the LBD of the AmERR proteins are highly conserved, suggesting that these regions might include the main functional domains.

Real-time quantitative RT-PCR analysis indicated that AmERR is expressed in each sample we tested, but at different levels. The highest expression level was found in adults. During the developmental stage, the expression level increased from the first instar to second instar, and declined during the third

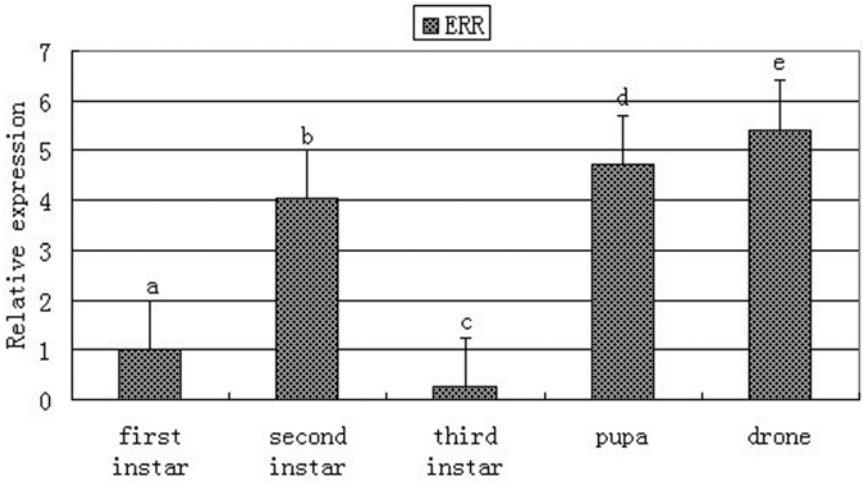

Fig.7. Relative expression of AmERR in different developmental stages of drones (bars with different letters indicate means are significantly different, t-test for LSD, $p<0.05$).

instar, then in pupae the expression increased again. The high mRNA levels in adults suggest the essential roles of ERR in honeybee development.

In three different castes of adults, the AmERR expression was highest in queen bees, second-highest in worker bees, and lowest in drones. The AmERR mRNA expression analysis among the development stages of the three castes

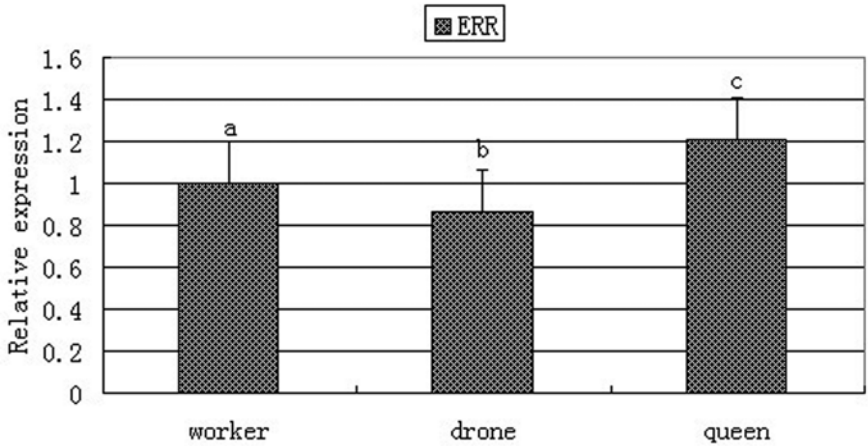

Fig.8. Relative expression of AmERR in different castes of *Apis melifera* (bars with different letters indicate means are significantly different, t-test for LSD, $p<0.05$).

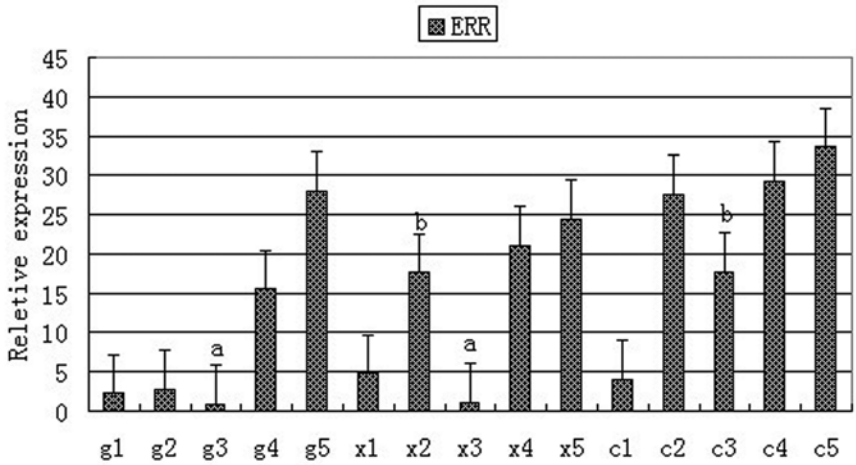

Fig.9. Relative expression of AmERR in different developmental stages of *Apis melifera* g1-g5,x1-x5,c1-c5 represent five developmental stages respectively of workers, drones and queens (bars with different letters indicate means are significantly different, t-test for LSD, $p<0.05$).

showed that, no matter which stage, the expression level of queen bees is always the highest. These results indicate that AmERR may have an essential role of regulating development in honeybee social communities. However, more research is needed to determine the precise role of ERR in the different castes of *Apis melifera*.

In summary, this study describes an ERR homolog gene cloned from *Apis melifera*, abbreviated as AmERR. The results indicated that the evolutionary pathways of ERR genes in animals, especially insect ERR homolog genes, are similar to each other. AmERR also is similar to mammalian ERRs in both sequence and structure, especially in the DBD and LBD, which implies that AmERR may be evolutionarily related to the mammalian ERRs. The findings that AmERR mRNA is differentially expressed at distinct developmental stages and different castes suggest that the AmERR protein may play a role in regulating honeybee development through its production. It will be interesting to extend the analysis on the precise role of ERR homolog gene in the different castes and anatomical parts of *Apis melifera*.

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