Molecular Cloning and Expression Analysis of TH Gene in the Ant *Polyrhachis vicina* (Hymenoptera: Formicidae)

by

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ABSTRACT

Tyrosine hydroxylase (TH) plays an important role in the synthesis of L-DOPA. In this report, a TH homologue was isolated from *Polyrhachis vicina* Roger (Hymenoptera: Formicidae). The full –length cDNA of PvTH is 2429bp, which contains an open reading frame of 1713 bp. The 5'-and 3'- UTRs are 357 and 359 bp long, respectively. The open reading frame encodes a deduced 570 amino-acid peptide with a calculated molecular mass of 65.09 kilodaltons and an isoelectric point of 5.26. To compare PvTH mRNA expression during *P. vicina* development and the heads of different castes, real-time quantitative reverse transcription polymerase chain reaction was used. The results show that PvTH mRNA is differentially expressed in the ant development, in whole bodies and in the heads of different castes. During the development, the highest expression level is in pupae. The levels also vary among castes, the highest level is in workers. The investigation revel that PvTH is related with the developmental and caste-specifically at the level of transcription.

Key words: tyrosine hydroxylase (TH), molecular cloning, *Polyrhachis vicina* Roger, real-time quantitative PCR

INTRODUCTION

As a highly conserved enzyme, tyrosine hydroxylase (TH) a pterin-dependent monooxygenase, has been found in vertebrates and invertebrates,, and contains ferrous iron at the active site (Ulrich *et al*. 2007). As a first and rate limiting enzyme in the synthesis of catecholamine neurotransmitters (Nagatsu *et al*. 1964), TH is primarily expressed in the brain retina secreted area, sympathetic ganglia sympathetic noradrenergic neurons, sympathetic adrenal medulla norepinephrine epinephrine and norepinephrine cells

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(Peng 2002). In the presence of tetrahydrobiopterin (BH4) as a cofactor, TH catalyzes the oxidation of L-tyrosine to L-dopa, H_2O and quinoid dihydrobiopterin (Haavik *et al*. 1987). In dopa decarboxylase activation, L-dopa is decarboxylated to dopamine (Hopkins *et al*. 1992).TH also catalyzes activity of other aromatic amino acids, such as hydroxylation of L-phenylalanine and L-tryptophan (Meyer *et al*. 1992).

In vertebrates, generally, the TH gene encodes a single form. However, there are two isoforms in monkey (Ichikawa *et al*. 1990) and four in human (Kaneda *et al*. 1987). In insects, TH gene also generally encodes a single form except *Drosophila melanogaster* and *Pseudaletia separate* which possesses two TH isoforms encoded by a signle gene and generated by RNA splicing (Birman *et al*. 1994). TH plays an important role in the stress response of insects (Stathakis *et al*. 1999). The insect immune system is dependent on both humoral and cellular responses. Adaptive immunity of insects is usually expressed by humoral response. Melanin and the precursors which are necessary for many physiological processes such as wound healing, pathogen encapsulation, phagocytosis are cytotoxic and antimicrobial activity (Gotz *et al*. 1985; Söderhäll, *et al*. 1998; Kazuhiko *et al*. 2008). Studies have demonstrated that TH participates in tanning and immune response, and is expressed in the epithelial cells underlying the darkly pigmented cuticle (Futahashi *et al*. 2005; Ninomiya *et al*. 2007; Futahashi *et al*. 2009). TH might be involved in neural activity in the larvae (Wang. 2010). TH and dopamine immunoreactivity appear to localize to the same neurons (Budnik *et al*. 1988; Nässel *et al*. 1992; Lundell *et al*. 1994). In *Drosophila*, dopamine plays a role in many complex neuronal processes such as sleep and arousal, stress response, learning, visual attention, and sexual behavior (Andretic *et al*. 2005; Ganguly-Fitzgerald *et al*. 2006; Kume *et al*. 2005; Ye *et al*. 2004; Neckameyer *et al*. 2005; Schwaerzel *et al*. 2003; Chang *et al*. 2006). So in the larval and adult central nervous system, TH-immunoreactive neurons are commonly referred to as dopaminergic neurons (Matthias *et al*. 2008).

Polyrhachis vicina Roger is a typical social insect with extensive polymorphism and sophisticated behavior. We cloned the full-length cDNA of TH from the ant *P. vicina* for the first time and named PvTH. The expression patterns of PvTH mRNA at distinct development stages and at the heads of different castes were studied by using real-time quantitative reverse

transcription-polymerase chain reaction (RT-PCR). The results indicate that PvTH mRNA possesses a function to regulate the ant caste-specificity and development at the level of transcription.

MATERIAL AND METHODS

Insects

Polyrhachis vicina colonies were purchased from Hongfa Edible Ant Research Center in Guangxi Province, China. The ants were raised in a chamber supplied with fruit, fish food and honeydew under standard laboratory conditions at 28°C, 40% relative humidity and natural light-dark periods. Embryos, larvae, pupae, workers, male and female ants were collected from the colonies, and immersed immediately in liquid nitrogen and stored at -80°C (Lu *et al*. 2008; Ouyang *et al*. 2009).

RNA Preparation and cDNA Synthesis

Total RNA was extracted from pooled samples of 15 frozen workers selected randomly with RNAiso Plus (Takara Bio Inc., Shiga, Japan; http://www.takara-bio.com/), and then immediately reverse-transcribed for generating cDNA using the First-Strand cDNA Synthesis Kit with oligo(dT) primer (Fermentas Life Sciences, Burlington Ontario; http://www.fermentas.com/). All the processes followed the manufacturer's instructions.

The full length cDNA of TH was cloned according to the scheme shown in Fig. 1. A partial cDNA fragment of TH was obtained using degenerated primers (TS1 TX1) (Table 1) that were designed on the basis of the conserved motifs of published TH from other insect species (*Samia cynthia ricini*, *Papilio xuthus*, *Manduca sexta*, *Drosophila melanogaster*, *Nasonia vitripennis*,

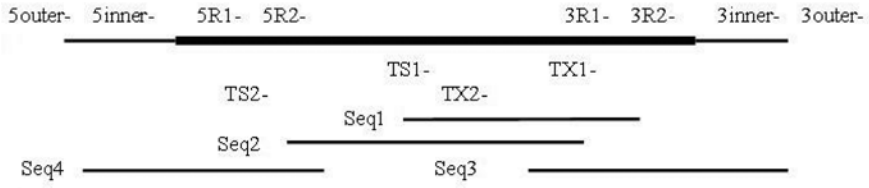


Fig.1. Cloning strategy and map of the primers for amplifying the full sequence of the PvTH gene; TS1 and TX1 for Seq 1 of 739bp; TS2 and TX2 for Seq 2 of 860bp; 3R1 and 3R2 for Seq 3 of 710bp; 5R1 and 5R2 for Seq 4 of 676bp.

Table 1. Oligonucleotide primers used for cDNA cloning and real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR).

Target	Name	Primer Sequence 5'-3'	Expected Size (bp)	Tm (°C)
TH fragment	TS1	CAACCAYCTBATGACBAART	739	56
	TX1	ACCARCGDCKGAAYTTBTC		
	TS2	GCYATCAAGAAATCYTACAG	860	48
	TX2	GTCCAGCACGGTGTTGAGA		
3'RACE	3R1	TCAATTCTCACAAGAAATCGGTCGGTCTG	710	55
	3R2	GCTTATGGTGCTGGTTTGTTGTC		
5'RACE	5R1	CTTCCGCTTTCGCTTCTTCTTTC	676	62
	5R2	CTACCCAAAGAACCAATACCCTC		
Real Time	RTS	AGCTGATTAGGAACCTGCGACAG	81	55
	RTX	GATCTTTGATGCTAACGGAATTG		
β-Actin	Y1	CCCTCTTCCAGCCATCGTTC	250	55
	Y2	CCACCGATCCAGACGGAGTA		

"Y" is C or T; "B" is C or G or T; "R" is A or G; "D" is A or G or T; "K" is G or T.

Apis mellifera). Based on the above fragment, primers TS2 and TX2 (Table 1) were designed and used to obtain the Seq2 fragment. PCR products were purified from agarose gel using a Gel Extraction Kit (Qiagen, Germany http:// www1.qiagen.com/) and subcloned into the pMD19-T simple vector (Takara, Japan). PCR positive clones were obtained for sequencing.

3'-and 5'- Rapid Amplification of cDNA Ends

The full length cDNA PvTH was amplified by specific primers used in nested PCR of 3'- and 5'- rapid amplification of cDNA ends (RACE) (3R1, 3R2 for 3'-RACE and 5R1,5R2 for 5'-RACE) (Table 1) using 3'Full RACE Core Set and 5'Full RACE Core Set (Takara Bio Inc.), following the manufacturer's instructions. The PCR products of 3'-RACE and 5'-RACE were cloned into pMD19-T simple vector (Takara) for sequencing.

Structural and phylogenetic analyses of PvTH

The open reading frame (ORF) of PvTH was searched using the National Center for Biotechnology Information ORF Finder (http:// www.ncbi.nlm.nih.gov/gorf/gorf.html). The secondary structure of the PvTH protein was predicted by SOPM (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopm.html), and the tertiary structure were predicted by Swiss model First Approach Mode (http://swissmodel.expasy.org/). Signal peptide prediction was performed using the SingalP program

(Centre for Biological Sequence Analysis; http://www.cbs.dtu.dk/services/Signalp/; Bendtsen *et al*. 2004). Sequence alignments based on the amino acid sequences of known THs were performed with Clustal X1.81 (Jeanmougin *et al*. 1998) followed by manual inspection. Phylogenetic tree were generated using MEGA version 4.0 from these alignments (Tamura *et al*. 2007) based on the neighbor-joining method with bootstrap test calculated with 2000 replicates and a Poisson correction model.

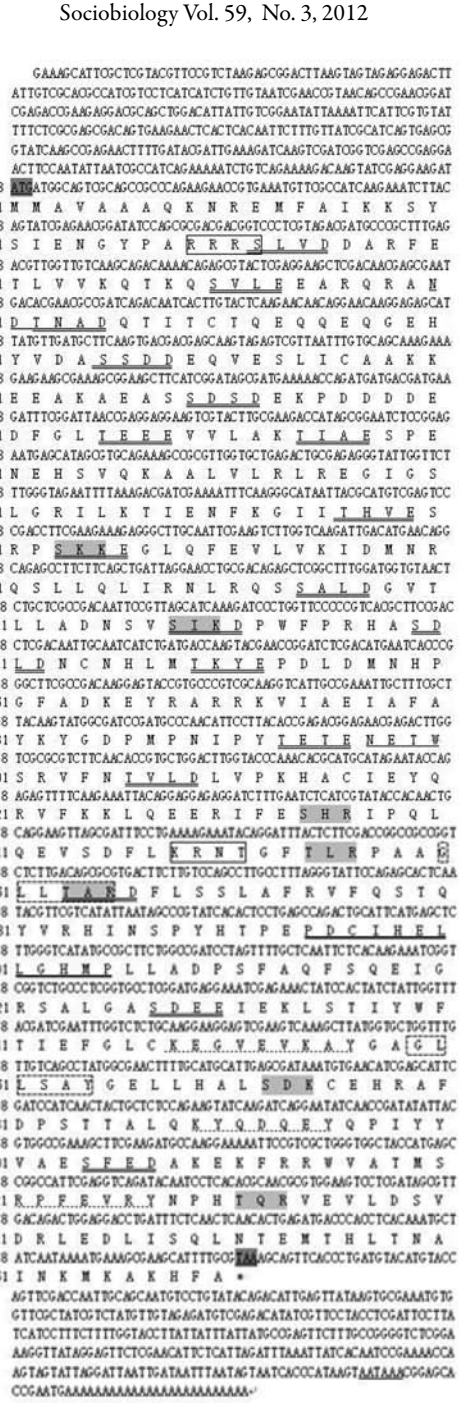
Real-time Quantitative PCR

The mRNA expression levels of PvTH at different development stages and from the heads of ants of different castes was quantified by Quantitative RT-PCR. Reactions were performed using a iQ5® apparatus (Bio-Rad Laboratories, Inc.) with a SYBR Premix Ex Taq Kit (Takara Bio Inc.). The β-actin gene was used as the endogenous control. Primers (RTS, RTX) of PvTH and primers (Y1, Y2) of β-actin were used in real-time RT-PCR (Table 1). The detailed protocol was as follows: 95°C for 1 min, 40 cycles of 95°C for 10 s and 55°C for 30 s, 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. Expression levels were determined basing on the formula $F=10^{\Delta Ct,t/At-\Delta Ct,r/Ar}$ (Zhang *et al*. 2005). The cDNA of the forth instar was selected as the calibrator in analyzing PvTH expression at different development stages in this study and the heads of workers was selected as the calibrator in analyzing PvTH expression in the heads of the three castes. To analyze the relative expression levels, all the counted *F* values were put into SPSS 13.0 software using a one-way analysis of variance (ANOVA) with Dunnett's multiple comparison (SPSS Inc. 2004).

RESULTS

Cloning and characterization of PvTH cDNA

The full–length cDNA of PvTH, 2429bp, was obtained using RT-PCR and RACE. It contains a ORF of 1713 bp that encodes a 570-amino-acid protein. The full-length cDNA sequences are shown in Fig.2. The 5'-and 3'-UTRSs are 357 and 359 bp long, respectively. A putative polyadenylation signal AATAAA is found 14 bp upstream form the 25-nucleotide poly (A) tail, which coincided with the fact that the polyadenylation signal is most



often present 11-30 nucleotides upstream from the poly (A) tail (Fitzgerald *et al*. 1981). The deduced PvTH protein consists of 570 amino-acid residues with a calculated molecular mass of 65.09 kilodaltons and an isoelectric point (pI) of 5.26. The GenBank accession number is JF499654. Analysis with the SignalP program showed that there was no N-terminal signal sequence in this protein. It may suggest that PvTH may be an unglycosylated protein. Using PROSITE protein, a series of predicted function motifs were found in the PvTH protein (Table 2).

Alignment analysis and phylogenetic-tree construction

The predicted secondary structure of the PvTH shows that alpha helix is 55.44%, extended strand is 10.53%, beta turn is 6.32% and random coil is 27.22% (Fig.3). The result of the tertiary structure prediction from the Swiss model First Apprpach shows that the protein model is 2xsnD (Human tyrosine hydroxylase); catalytic domain; moedlled residue range, 231-568; sequence identify, 57.69%; X-ray resolution, 2.68; E value, 0.00e-1. A multiple alignment of the deduced animo acid sequence of PvTH with other known TH homologues was performed with ClustalX 1.81 (Fig. 5). MegAlign analysis of the PvTH protein sequence with other TH protein sequences revealed that the PvTH protein shares 99% identity with *A.mellifera*. A phylogenetic tree was constructed using MEGA based on the neighbor-joining method (Tamura *et al*. 2007; Fig. 6). Result show that PvTH clustered with the THs of other Hymenoptera and is most closely related to that of *A.mellifera*.

Fig.2 (facing page). The nucleotide and deduced amino acid sequence of PvTH. The sequence is 2429bp long and encodes a protein of 570 amino acid residues. The initiating codon ATG and stop codon TAA are underlined and shaded; the olyadenylation signal AATAAA is underlined. A series of important functional motifs in the PvTH protein sequence are marked. Two N-glycosylation sites at 60-63 (NDTN), 297-300 (NETW); two cAMP- and cGMP-dependent protein kinase phosphorylation sites at 29-32 (RRRS), 348-351 (KRNT); seven protein kinase C phosphorylation sites at 163-165 (SKK), 228-230 (SIK), 334-336 (SHR), 354-356 (TLR), 363-365 (TAR), 472-474 (SDK), and 531-533 (TQR); eighteen casein kinase II phosphorylation sites at 32-35 (SLVD), 50-53 (SVLE), 62-65 (TNAD), 85-88 (SSDD), 109-112 (SDSD), 125-128 (TEEE), 134-137 (TIAE), 176-179 (THVE), 183-186 (SKKE), 214-217 (SALD), 228-231 (SIKD), 239-242 (SDLD), 249-252 (TKYE), 293-296 (TETE), 306-309 (TVLD), 363-366 (TARD), 427-430 (SDEE), and 504-507 (SFED); three tyrosine kinase phosphorylation sites at 448-456 (KEGVEVKAY), 489-495 (KYQDQEY), and 521-527 (RPFEVRY); two N-myristoylation sites at 360-365 (GLLTAR), 459-464 (GLLSAY); one biopterin-dependent aromatic amino acid hydroxylases signature at 394-405 (PDCIHELLGHMP).

Table 2. Predicted function motifs in the PvTH protein of *Polyrhachis vicina*.

Domain name	Canonical sequence	Position in sequence
N-glycosylation site	N[^P][ST][^P]	60-63(NDTN), 297-300(NETW)
cAMP- and cGMP-dependent protein kinase phosphorylation site	[RK]{2}.[ST]	29-32(RRRS), 348-351(KRNT)
protein kinase C phosphorylation site	[ST].[RK]	163-165(SKK), 228-230 (SIK), 334-336(SHR), 354-356(TLR), 363-365(TAR), 472-474(SDK), 531-533(TQR)
casein kinase II phosphorylation site	[ST].{2}[DE]	32-35(SLVD), 50-53(SVLE), 62-65(TNAD), 85-88(SSDD), 109-112(SDSD), 125-128(TEEE), 134-137(TIAE), 176-179(THVE), 183-186(SKKE), 214-217(SALD), 228-231(SIKD), 239-242(SDLD), 249-252(TKYE), 293-296(TETE), 306-309(TVLD), 363-366(TARD), 427-430(SDEE), 504-507(SFED)
tyrosine kinase phosphorylation site	[RK].{2,3}[DE].{2,3}Y	448-456(KEGVEVKAY), 489-495(KYQDQEY), 521-527(RPFEVRY)
N-myristoylation site	G[^EDRKHPFYW].{2}[STAGCN][^P	360-365(GLLTAR), 459-464(GLLSAY)

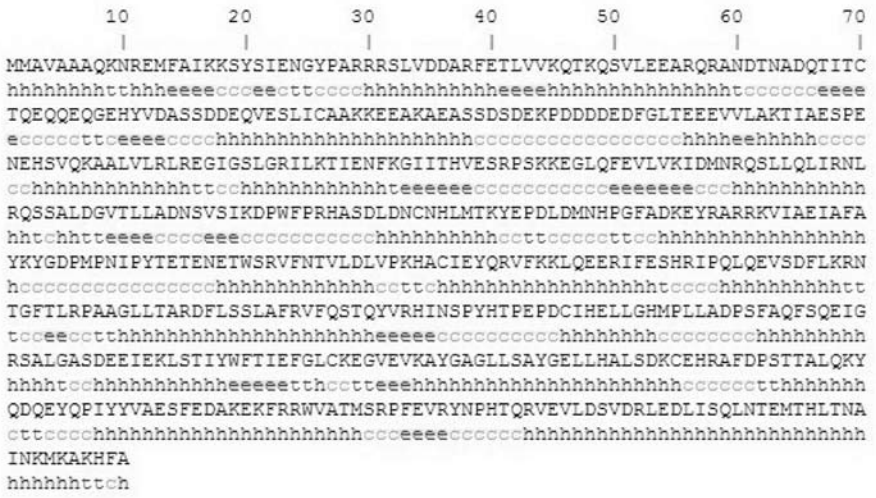


Fig.3. Predicted secondary structure of the PvTH protein, h is alpha helix; e is extended strand; t is beta turn; c is random coil.

Table 3. PvTH expression levels calculated for three replicates based on the formula $F=10^{\Delta Ct,t/At-\Delta Ct,r/Ar}$.

	TH C_t	β-actin C_t	ΔCt,t	ΔCt,r	$F=10^{\Delta Ct,t/At-\Delta Ct,r/Ar}$
Embryo	21.65	18.65	0	0	1
	21.48	18.44	0	0	1
	21.73	18.11	0	0	1
First instar	17.24	14.80	-4.38	-3.60	1.82
	16.69	14.49	-4.93	-3.91	2.16
	16.90	14.68	-4.72	-3.72	2.12
Second instar	22.45	20.07	0.83	1.67	1.74
	22.61	19.02	0.99	0.62	0.77
	22.15	19.82	0.53	1.42	1.81
Third instar	22.37	21.28	0.75	2.88	4.17
	22.86	20.73	1.24	2.33	2.05
	22.60	20.92	0.98	2.52	2.79
Fourth instar	19.52	14.65	-2.10	-3.75	0.34
	19.37	14.36	-2.25	-4.04	0.31
	19.73	14.22	-1.89	-4.18	0.22
Pupa	21.99	21.79	0.37	3.39	7.66
	21.89	21.73	0.27	3.33	7.88
	22.06	21.58	0.44	3.18	6.33
Worker	20.83	21.30	-0.79	2.90	10.02
	20.69	21.28	-0.93	2.88	13.53
	20.31	21.00	-1.31	2.60	17.36
Female	22.90	17.39	1.28	-1.01	0.21
	23.64	17.43	2.02	-0.97	0.13
	24.48	17.25	2.86	-1.15	0.06
Male	21.75	21.63	0.13	3.23	8.11
	22.35	21.88	0.73	3.48	6.34
	22.23	21.67	0.61	3.27	5.98
Head					
Worker	27.17	26.30	0	0	1
	26.68	26.26	0	0	1
	26.59	26.40	0	0	1
Female	29.07	31.02	2.26	4.70	4.85
	29.35	31.77	2.54	5.45	6.58
	29.03	31.58	2.22	5.26	7.19
Male	23.23	25.96	-3.58	-0.36	8.50
	23.28	25.91	-3.53	-0.41	7.96
	23.19	25.66	-3.62	-0.66	7.17

Analysis of PvTH mRNA expression

Levels of expression of PvTH mRNA at different developmental stages and in different castes were detected by means of real-time quantitative RT-PCR. PvTH relative expression levels were calculated using the formula $F=10^{\Delta Ct,t/At-\Delta Ct,r/Ar}$ for each replicate (Table 3). PvTH was expressed in all templates in-

vestigated at different levels (Figs.7, 8). During development, the level of expression of PvTH transcripts was low in embryo and each instar, especially in fourth instar larvae. However, the level of expression greatly increased in pupae. In adults, the gene expression was highest in workers and lowest in females. In the heads of different castes, the expression was highest in the heads of males and lowest in the heads of workers.

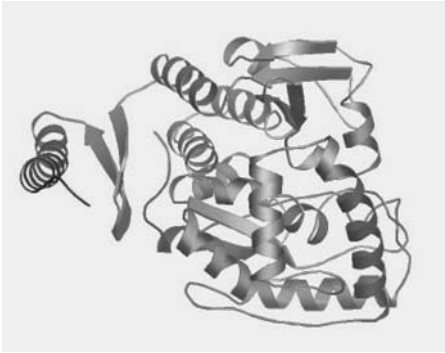


Fig.4. Predicted tertiary structure of catalytic domain in the PvTH proteins by SwissModel First Approach.

Note: The three relicate samples yielded three C_t values. ΔCt,t is the difference in C_t values for PvTH between the samples and the calibrator. ΔCt,r is in C_t values for β-actin. In the analysis of development, the F value for embryos is designated 1 and the slope of the PvTH curve and the β-actin curve was -3.325 and -3.406. In the analysis of the head, the F value for worker heads is designed 1 and the slope of the PvTH curve and theβ-actin curve was -3.47 and -3.515

DISCUSSION

A full-length cDNA sequence of TH from *P.vicina* was isolated and characterized in this study. The results of multiple aligments showed that PvTH reflects a high degree of homology with the TH genes identified from other animals. Phylogenetic tree which shows the evolutionary relationship of PvTH with other members of the TH family constructed on the basis of alignment of the amino acid sequences of TH homologues. Numbers at branch nodes indicate percent bootstrap confidence values derived from 2000 replications. Results showed that PvTH clustered with the THs of other species and is most closely related to that of *A.mellifera*. It is consistent with the species' evolution.

Real-time quantitative RT-PCR indicated that PvTH mRNA is expressed at all stages of *P.vicina* development and the heads of different castes, but at different levels. In embryos and larvae, the expression was very low, espe-

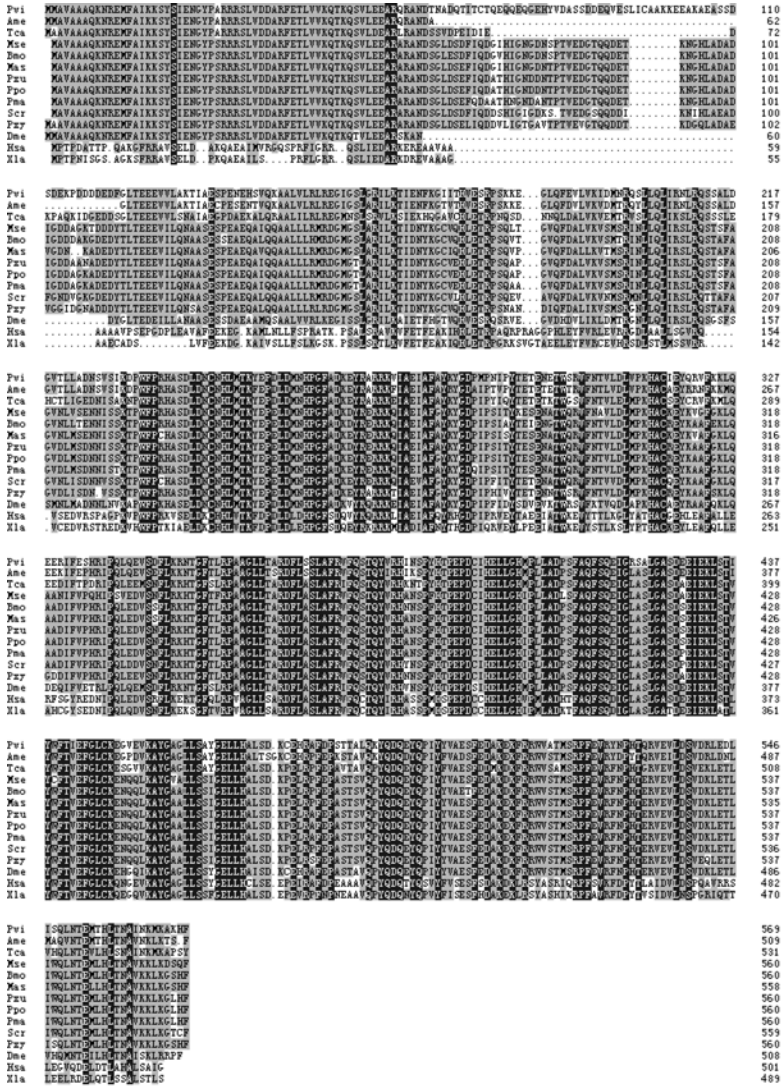


Fig.5. Amino acid sequence alignments of PvTH (*Polyrhachis vicinna* (Pvi)) with other species: *Apis mellifera* (Ame) NP_001011633; *Tribolium castaneum* (Tca) NP_001092299; *Mythimna separata* (Mse) BAF32573; *Bombyx mori* (Bmo) NP_001138794; *Manduca sexta*(Mas) ABQ95973; *Papilio xuthus* (Pxu) BAE43824; *Papilio polytes* (Ppo) BAJ07593; *Papilio machaon* (Pma) BAJ07587; *Samia cynthia ricini* (Scr) BAF64534; *Plutella xylostella* (Pxy) ADK12633; *Drosophila melanogaster* (Dme) NP_476897; *Homo sapiens*(Hsa) AAI43615; *Xenopus laevis*(Xla) NP_001091392. Conserved amino acids in all THs are shown in black and residues that are similar with respect to side chains in gray; gaps are introduced to optimize the alignments.

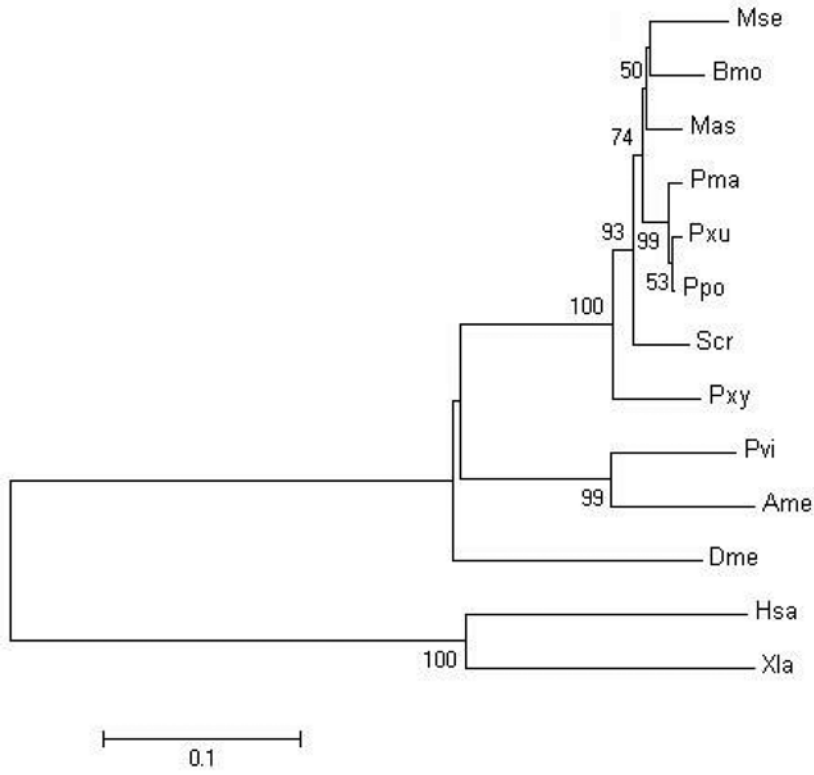


Fig.6. Phylogenetic tree which shows the evolutionary relationship of PvTH with other members of the TH family constructed on the basis of alignment of the animo acid sequences of TH homologues. Numbers at branch nodes indicate percent bootstrap confidence values derived from 2000 replications. See Figure 5 for the explanation of species abbreviations

cially in fourth larval stages. In pupa, the expression reached very high levels. It is widely known that TH is the first, and rate limiting, enzyme in the synthesis of dopamine. Dopamine, which is necessary in development and melanin synthesis in insects, is an important neurotransmitter of the central nervous system and also a material to resist pressure (Goldstein *et al*. 1992; Kobayashi *et al*. 1995; Restifo *et al*. 1990; Monastirioti. 1999; Hopkins *et al*. 1992; Stathakis *et al*. 1999). Tissues, including nervous tissue, are gradually developed from the embryonic stage to the fourth instar stage. The low expression may reflect incomplete development of nervous tissue. In complete

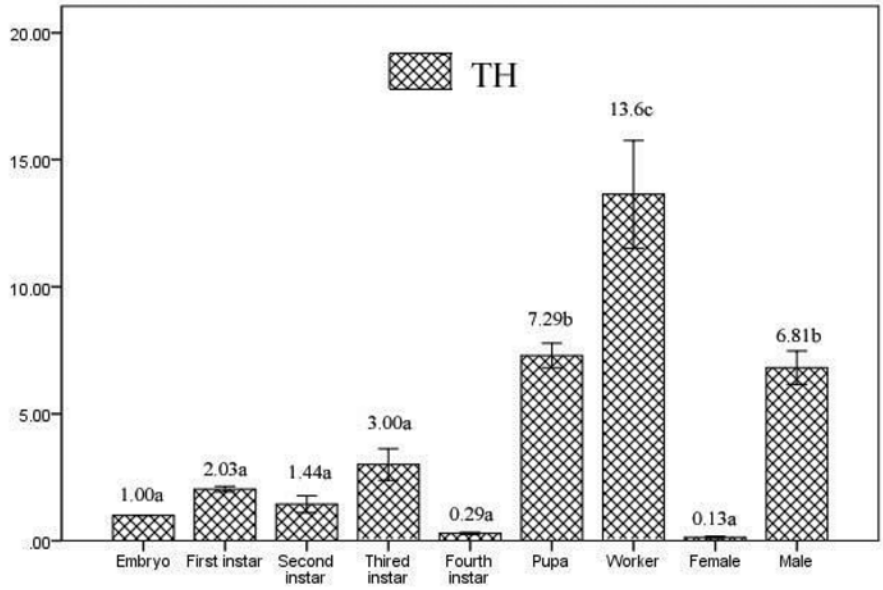


Fig.7. Relative expression profiles (± standard error of the mean; n=3) for PvTH in different developmental stages of P.vicina based on real-time PCR. All expression levels are shown relative to the expression level in embryos. Means followed by the same letter are not significantly different ($p<0.05$).

metamorphosis, the central nervous system is formed in the pupa period (Truman 1996). In adults, PvTH mRNA expression is high except in females. The obvious differences in different castes may reflect the different functions in social insects. As the females of the colony are important, they are more well-protected. In the brain, the lowest level was found in workers. The expression is high in females and males. It may play a role in sexual behavior (Chang *et al.* 2006). The highest level expression in bodies is different than in brains. The result may be related to the complex

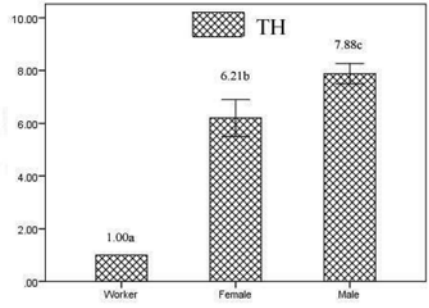


Fig.8. Relative expression profiles (± standard error of the mean; n=3) for PvTH in the heads of different castes. All expression levels are shown relative to the expression level in the heads of workers. Means followed by the same letter are not significantly different ($p<0.05$).

physiological function of dopamine in different body locations.

In summary, our study describes, for the first time, TH cloned from a common Chinese ant, *P. vicina*. The finding of differential expression of PvTH mRNA in heads of different castes and distinct development stages suggests PvTH may play an important role in ant development. The study made a preliminary discussion of TH physiological function.

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