# Molecular Cloning and Expression Analysis of the Gene Encoding DDC in the Ant *Polyrhachis vicina* Roger (Hymenoptera: Formicidae)

by

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## ABSTRACT

L-3, 4-Dihydroxyphenylalanine decarboxylase (DDC) is an important enzyme which catalyses L-dopa decarboxylation to dopamine in the synthesis of catecholamine neurotransmitters and 5-hydroxytryptophan (5-HTP) to 5-hydroxytryptamine (5-HT). In this paper, a DDC homologue was isolated from *Polyrhachis vicinna* Roger (Hymenoptera: Formicidae). The full length cDNA of PvDDC is 1893 base pairs (bp) and contains a 5'-untranslated region of 71 nucleotides and a 3'-UTR 379 bp long. The open reading frame of PvDDC encodes a deduced 480-amino acid peptide. Real-time quantitative reverse transcription polymerase chain reaction was used to compare PvDDC mRNA expression during *P. vicina* development in different castes. The results show that PvDDC mRNA is differentially expressed in different stages of ant development, in whole bodies and 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 males. These investigations indicate that PvDDC mRNA possesses a function to regulate ant caste-specificity and development at the level of transcription.

Key words: L-3, 4-Dihydroxyphenylalanine decarboxylase (DDC), molecular cloning, *Polyrhachis vicina* Roger, real-time quantitative PCR

## INTRODUCTION

L-3, 4-Dihydroxyphenylalanine decarboxylase (DDC) is a homodimer, binding one subunit of pyridoxal-5'-phophate (PLP). Its molecular weight is approximately 50 kD. As a PLP requiring enzyme, it catalyses the decarboxylation of L-dopa to dopamine in the synthesis of catecholamine neurotransmitters, and 5-hydroxytryptophan (5-HTP) to 5-hydroxytryptamine

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(5-HT) (Sourkes 1966; Hodgetts *et al.* 2006). Due to a broad selectivity of dopa decarboxylase for substrate, most of the aromatic amino acids and their α-methyl derivatives can be decarboxylased by DDC. DDC is also referred to as aromatic L-amino acid decarboxylase (AADC) (Neckameyer *et al.* 2002; Nassel. 1996). DDC also catalyzes L-phenylalanine, histidine, and L-tryptophan to phenylethylamine, histamine, and tryptamine, respectively. These products may have some function as central neurotransmitter endogenous regulatory factors in the brain. In mammals, especially in humans, DDC plays an important role in Parkinson's disease pathogenesis research and treatment (Lopez-Real *et al.* 2003). Supplying DDC for Parkinson's patients may raise the efficiency of conversion of l-dopa to DA, thereby improving the clinical symptoms (Imaoka *et al.* 1998).

In insects, there are sequences similar to DDC which are involved in insect melanization. A DDC-deficient *Drosophila* mutant was the first demonstration that the dopamine-oxidation pathway plays a role in immune responses that involve melanization (Nappi *et al.* 1993). The synthesis of DDC is under the control of both ecdysone and juvenile hormone, the activity of DDC is regulated by several factors such as $\alpha_2$-adrenergic receptor antagonists, DA receptor agonists, and PK-A and PK-C mediated pathways (Poulikakos *et al.* 2001; Rosseti *et al.* 1989; Zhu *et al.* 1993; Young *et al.* 1993; Young *et al.* 1994). In *Drosophila* larvae, silencing of DDC neurons increases the response to light throughout larval development, including the later stages of the 3rd instar characterized by photo-neutral response (Verónica *et al.* 2009). Melanization limits the ability of some mosquito species to transmit pathogens (Adak *et al.* 2006; Christensen *et al.* 2005; Infanger *et al.* 2004). Knockdown of the DDC enzyme resulted in a significant reduction of melanization in *Anopheles gamibae*, which may provide new control strategies for these parasites (Paskewitz *et al.* 2008).

In this study, a DDC homologue from the social ant *Polyrhachis vicina* Roger was cloned and named PvDDC. PvDDC mRNA differentially expressed during *P. vicina* development in whole bodies as well as the heads of different castes were studied by using real-time quantitative RT-PCR. The results may suggest PvDDC mRNA is relevant to regulating functions in social communities.

# MATERIAL AND METHODS

## Insects

*Polyrhachis vicinna* colonies were purchased from Hongfa Edible Ant Research Center in Guangxi Province, China. They were raised in a chamber supplied with fruit, fish 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 until used (Lu *et al.* 2008; Ouyang *et al.* 2009).

## RNA Preparation and cDNA Synthesis

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

A partial cDNA fragment of DDC was amplified by PCR using degenerated primers (DS1, DX1) (Table 1) based on the conserved motifs of published DDC from other insect species (*Apis mellifera, Drosophila simulans, Tribolium castaneum, Aedes aegypti, Papilio xuthus*). Based on the above fragment, another sequence was amplified using primers (DS2, DX2). The full length cDNA PvDDC was amplified by 5'and 3'rapid amplification of cDNA ends (RACE) using 5' Full RACE Core Set and 3' Full RACE Core Set (Takara Bio Inc.) by specific primers used in nested PCR, following the manufacturer's instructions (5R1,5R2 for 5'-RACE and 3R1,3R2 for 3'-RACE, Table 1, Fig. 1). The PCR products were purified from agarose gel using a Gel Extraction Kit (Axygen: http//www.axygen.com) and cloned into pMD 19-T simple vector (Takara) for sequencing.

## Structural and phylogenetic analyses of PvDDC

The open reading frame (ORF) of PvDDC was searched by the National Center for Biotechnology Information ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Using the SingalP program (Centre for Biological Sequence Analysis; http://www.cbs.dtu.dk/services/Signalp/; Bendtsen

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

| Target | Name | Primer Sequence 5'-3' | Expected Size (bp) | Tm (°C) |
|---|---|---|---|---|
| DDC fragment | DS1 | CACGCVTAYTTYCCMACVGC | 807 | 65 |
| | DX1 | CCVARYGGGATYTGCCAGTG | | |
| | DS2 | ATGCCGCTTACGCAGGTTCT | 511 | 57 |
| | DX2 | ATBGCSAWVCGYAGGAAGTA | | |
| 5'RACE | 5R1 | TTGACAGGAACAGTATGCGACTA | 600 | 58 |
| | 5R2 | TCCAATCAGGATGTTGCTCT | | |
| 3'RACE | 3R1 | CCTGAAACTTTGGTTCGTGCTA | 530 | 62 |
| | 3R2 | TTCACCTCGTCCCATCAAAG | | |
| Real Time | RTS | GGTAGCGTTACTTGGAGCGAAGG | 112 | 55 |
| | RTX | TTGACAGGAACAGTATGCGACTA | | |
| β- Actin | BS | CCCTCTTCCAGCCATCGTTC | 250 | 55 |
| | BX | CCACCGATCCAGACGGAGTA | | |

"V" is A or C or G; "Y" is C or T; "M" is A or C; "R" is A or G; "B" is C or G or T; "S" is C or G; "W" is A or T.

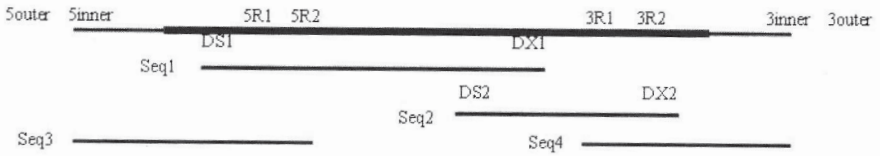


**Fig. 1.** Cloning strategy and map of the primers for amplifying the full sequence of the PvDDC gene; DS1 and DX1 for Seq 1 of 847bp; DS2 and DX2 for Seq 2 of 532 bp; 5R1 and 5R2 for Seq 3 of 594bp; 3R1 and 3R2 for Seq 4 of 531bp.

*et al.* 2004) signal peptide prediction was performed. Potential functional motifs of the protein sequence were analyzed using the PROSITE database (Expert Protein Analysis System. Swiss Institute of Bioinformatics, Basel; http//myhits.isb-sib.ch/cgi-bin/motif_scan). Followed by manual inspection, sequence alignments based on the amino acid sequences of known DDCs including homologues of some vertebrates and invertebrates obtained from NCBI were performed with Clustal X1.81 (Jeanmougin *et al.* 1998). The identities of PvDDC protein and above DDCs were analyzed using MegAlign. Phylogenetic trees were constructed using the Neighbor-Joining method with bootstrap test calculated with 2000 replicates and a Possion correction model implemented in MEGA (Tamura *et al.* 2007).

## Real-time quantitative RT-PCR

Quantitative RT-PCR was performed to quantify the mRNA expression levels of PvDDC at different development stages in the whole bodies and from the heads of ants of different castes. Reactions were performed using a iQ5® apparatus (Bio-Rad Laboratories, Inc.) with a SYBR Premix Ex Taq Kit (Takara Bio Inc.). The β-actin was used as the endogenous control. Primers (RTS, RTX) of PvDDC and primers (BS, BX) of β-actin were used in real-time RT-PCR (Table 1). One of the cDNA samples was used to construct standard curves for PvDDC and β-actin after serial dilution. The slopes of the curves were obtained from that. The formula $F=10^{\Delta Ct,t/At-\Delta Ct,r/Ar}$ was used for determining the relative quantification of PvDDC (Zhang *et al.* 2005). In this study, the cDNA of embryos was selected as the calibrator in analyzing PvDDC expression at different development stages and that of the heads of female ants was selected as the calibrator in analyzing PvDDC expression in the heads of the three castes. The relative expression levels were analyzed using one-way ANVOA with Dunnett's multiple comparison (SPSS Inc. 2004).

# RESULTS

## Cloning and characterization of PvDDC cDNA

The DDC cDNA fragments were isolated by RT-PCR using total RNA extracted from workers of *P. vicina*. Degenerate primers were designed for PCR in the conserved motifs of other insects' DDCs. The full-length cDNA was amplified by 5'RACE and 3'RACE. The full-length cDNA of PvDDC is 1893 bp. It contains a ORF of 1443 bp that encodes a 480-amino-acid protein. The 5'-untranslated region is 71 nucleotides followed by an initiating ATG codon. Stop codon TGA is at positions 1512-1514. The 3'-UTR is 379 bp long. A putative polyadenylation signal AATAA is found 19 bp upstream form the 17-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 cDNA sequence has been submitted to GenBank. The accession number is JN979785. The full-length cDNA sequences are shown in Fig.2. The deduced DDC protein with a calculated molecular mass of 54.60 kilodaltons (kDa) and an isoelectric point of 6.38 is shown in Fig.2. Using SignalP program showed that there was no N-terminal signal sequence in PvDDC, which suggests that PvDDC


GAAAGCATTCGCTCGTACGTTCCGTCTAAGAGCGGACTTAAGTAGTAGAGGAGACTT
ATTGTCGCACGCCATCGTCCTCATCATCTGTTGTAATCGAACCGTAACAGCCGAACGGAT
CGAGACCGAAGAGGACGCAGCTGGACATTATTGTCGGAATATTAAAATTCATTCGTGTAT
TTTCTCGCGAGCGACAGTGAAGAACTCACTCACAATTCTTTGTTATCGCATCAGTGAGCG
GTATCAAGCCGAGAACTTTTGATACGATTGAAAGATCAAGTCGATCGGTCGAGCCGAGGA
ACTTCCAATATTAATCGCCATCAGAAAAATCTGTCAGAAAAGACAAGTATCGAGGAAGAT
358 ATGATGGCAGTCGCAGCCGCCCAGAAGAACCGTGAAATGTTCGCCATCAAGAAATCTTAC
1 M M A V A A A Q K N R E M F A I K K S Y
418 AGTATCGAGAACGGATATCCAGCGCGACGACGGTCCCTCGTAGACGATGCCCGCTTTGAG
21 S I E N G Y P A R R R S L V D D A R F E
478 ACGTTGGTTGTCAAGCAGACAAAACAGAGCGTACTCGAGGAAGCTCGACAACGAGCGAAT
41 T L V V K Q T K Q S V L E E A R Q R A N
538 GACACGAACGCCGATCAGACAATCACTTGTACTCAAGAACAACAGGAACAAGGAGAGCAT
61 D T N A D Q T I T C T Q E Q Q E Q G E H
598 TATGTTGATGCTTCAAGTGACGACGAGCAAGTAGAGTCGTTAATTTGTGCAGCAAAGAAA
81 Y V D A S S D D E Q V E S L I C A A K K
658 GAAGAAGCGAAAGCGGAAGCTTCATCGGATAGCGATGAAAAACCAGATGATGACGATGAA
101 E E A K A E A S S D S D E K P D D D D E
718 GATTTCGGATTAACCGAGGAGGAAGTCGTACTTGCGAAGACCATAGCGGAATCTCCGGAG
121 D F G L T E E E V V L A K T I A E S P E
778 AATGAGCATAGCGTGCAGAAAGCCGCGTTGGTGCTGAGACTGCGAGAGGGTATTGGTTCT
141 N E H S V Q K A A L V L R L R E G I G S
838 TTGGGTAGAATTTTAAAGACGATCGAAAATTTCAAGGGCATAATTACGCATGTCGAGTCC
161 L G R I L K T I E N F K G I I T H V E S
898 CGACCTTCGAAGAAAGAGGGCTTGCAATTCGAAGTCTTGGTCAAGATTGACATGAACAGG
181 R P S K K E G L Q F E V L V K I D M N R
958 CAGAGCCTTCTTCAGCTGATTAGGAACCTGCGACAGAGCTCGGCTTTGGATGGTGTAACT
201 Q S L L Q L I R N L R Q S S A L D G V T
1018 CTGCTCGCCGACAATTCCGTTAGCATCAAAGATCCCTGGTTCCCCCGTCACGCTTCCGAC
221 L L A D N S V S I K D P W F P R H A S D
1078 CTCGACAATTGCAATCATCTGATGACCAAGTACGAACCGGATCTCGACATGAATCACCCG
241 L D N C N H L M T K Y E P D L D M N H P
1138 GGCTTCGCCGACAAGGAGTACCGTGCCCGTCGCAAGGTCATTGCCGAAATTGCTTTCGCT
261 G F A D K E Y R A R R K V I A E I A F A
1198 TACAAGTATGGCGATCCGATGCCCAACATTCCTTACACCGAGACGGAGAACGAGACTTGG
281 Y K Y G D P M P N I P Y T E T E N E T W
1258 TCGCGCGTCTTCAACACCGTGCTGGACTTGGTACCCAAACACGCATGCATAGAATACCAG
301 S R V F N T V L D L V P K H A C I E Y Q
1318 AGAGTTTTCAAGAAATTACAGGAGGAGAGGATCTTTGAATCTCATCGTATACCACAACTG
321 R V F K K L Q E E R I F E S H R I P Q L
1378 CAGGAAGTTAGCGATTTCCTGAAAAGAAATACAGGATTTACTCTTCGACCGGCCGCCGGT
341 Q E V S D F L K R N T G F T L R P A A G

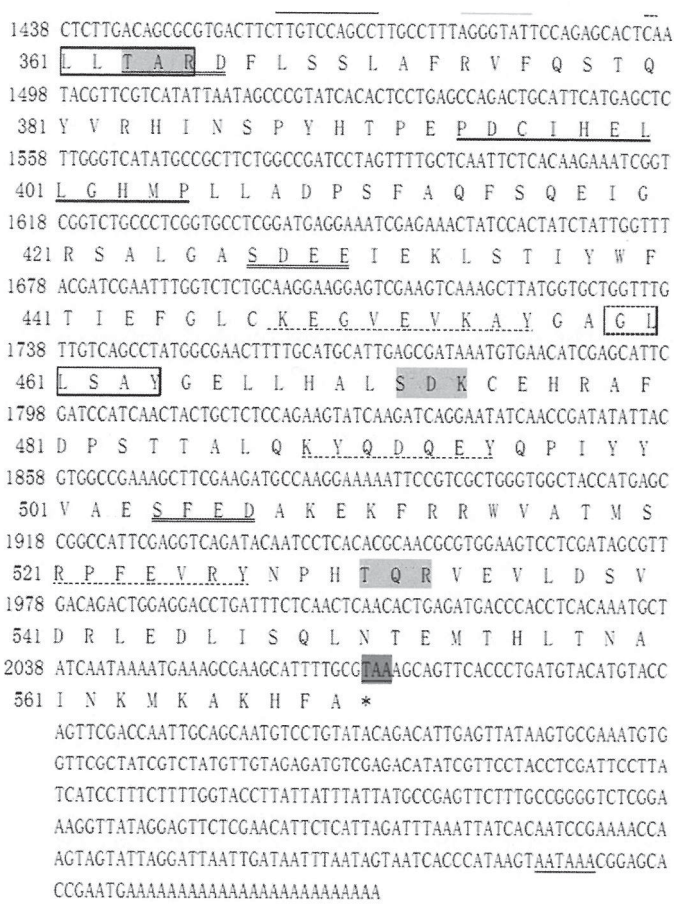


Fig.2. The nucleotide and deduced amino acid sequence of PvDDC. The sequence is 1893 bp long, contains a ORF of 1443 bp which encodes a protein of 480 amino acid residues. The initiating codon ATG and stop codon TGA are underlined and shaded; the olyadenylation signal AATAAA is underlined. Dopa deC like domin hit, which is dopa decarboxylase family is shaded from 76-466. The family belongs to pyridoxal phosphate (PLP)-dependent aspartate amino transferase superfamily. A series of function motifs in the PvDDC protein sequence are marked, including three protein kinase C phosphorylation sites at 5-7 (SFK), 73-75 (SPK), and 461-463 (SWK); eight casein kinase II phosphorylation sites at 5-8 (SFKD), 112-115 (TEIE), 147-150 (TASE), 175-178 (TENE), 193-196 (SSVE), 221-224 (TMAE), 340-343(SAPD), and 461-464 (SWKE); a tyrosine kinase phosphorylation site at 454-460 (KSKDIQY); six N-myristoylation sites at 96-101 (GAIACI), 137-142 (GGRGGG), 199-204 (GLLGGI), 244-249 (GTTVNC), 258-263 (GIVANR), and 290-295 (GIELAD); a amidation site at 351-354 (LGRR); a cell attachment sequence at 218-220 (RGD); a DDC / GAD / HDC / TyrDC pyridoxal-phosphate attachment site at 296-317 (SFNFNPHKWMLVDFDCSTMWLK).

may be an unglycosylated protein. By the PROSITE program, a series of predicted function motifs were found in the PvDDC protein, including three protein kinase C phosphorylation sites, [ST].[RK] at 5-7 (SFK), 73-75 (SPK), and 461-463 (SWK); eight casein kinase II phosphorylation sites, [ST].{2}[DE] at 5-8 (SFKD), 112-115 (TEIE), 147-150 (TASE), 175-178 (TENE), 193-196 (SSVE), 221-224 (TMAE), 340-343(SAPD), and 461-464 (SWKE); a tyrosine kinase phosphorylation site, [RK].{2,3}[DE].{2,3}Y at 454-460 (KSKDIQY); six N-myristoylation sites, G[^EDRKHPFYW].{2}[STAGCN][^P] at 96-101 (GAIACI), 137-142 (GGRGGG), 199-204 (GLLGGI), 244-249 (GTTVNC), 258-263 (GIVANR), and 290-295 (GIELAD); a amidation site, .G[RK][RK] at 351-354 (LGRR); a cell attachment sequence at 218-220 (RGD); and a DDC / GAD / HDC / TyrDC pyridoxalphosphate attachment site at 296-317 (SFNFNPHKWMLVDFDCSTMWLK)(Fig.2). The dopa decarboxyase domain is shown in Fig. 2.

## Alignment analysis and phylogenetic-tree construction

A multiple alignment of the deduced animo acid sequence of PvDDC with other known DDC homologues including mammals and insects was performed with Clustal X 1.81 (Fig. 3). MegAlign analysis of the PvDDC protein sequence with other DDC protein sequences showed that PvDDC has similarity to *Apis mellifera*. A phylogenetic tree was constructed using MEGA based on the neighbor-joining method (Tamura *et al.* 2007; Fig. 4). Results showed that PvDDC clustered with the DDCs of other Hymenoptera and is most closely related to that of *A. mellifera*.

## Analysis of PvDDC mRNA expression

Levels of expression of PvDDC mRNA in the whole body and head of different castes were detected by means of quantitative RT-PCR. PvDDC relative expression levels were calculated using the formula $F=10^{\Delta Ct,t/At-\Delta Ct,r/Ar}$ for each replicate. PvDDC was expressed in all samples but at different levels (Figs.5, 6). During development, the expression level was lowest in embryos and increased in the third instar. The expression of PvDDC was highest in pupae. Among the three castes, PvDDC mRNA expression was highest in males and lowest in females. The expression of PvDDC in the heads of ants of different castes increased from workers, to females to males.

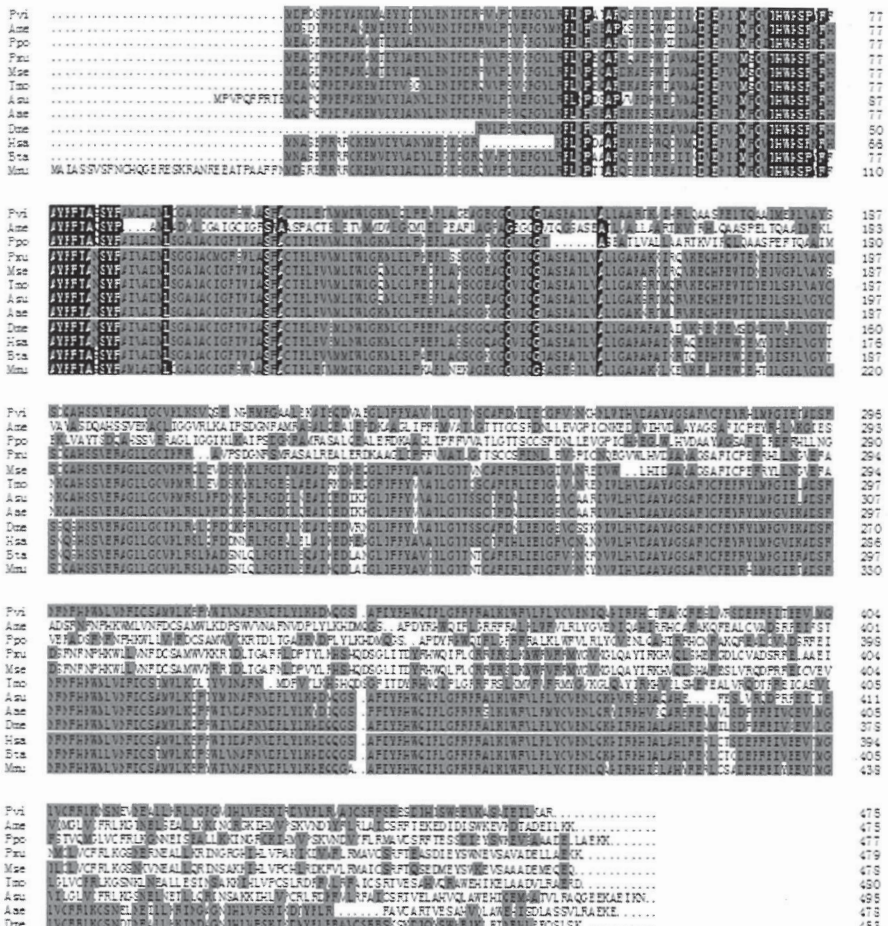

Fig.3. Amino acid sequence alignments of PvDDC(*Polyrhachis vicinna* (Pvi)) with other species: *Apis mellifera*(Ame) XP_394115; *Papilio polytes*(Ppo) BAJ07594; *Papilio xuthus*(Pxu) BAE43825; *Mythimna separate(*Mse) BAB68549; *Tenebrio molitor*(Tmp) BAA95568; *Armigeres subalbatus* (Asi) AAT75222; *Aedes aegypti*(Aae) XP_001648264; *Drosophila melanogaster*(Dme) AAC67582; *Homo sapiens*(Hsa) CAG33005; *Bos Taurus*(Bta) AAI42254; *Mus musculus*(Mmu) EDL40647. Conserved amino acids in all DDCs are shown in black and residues that are similar with respect to side chains are shown in gray; gaps are introduced to optimize the alignments.

## DISCUSSION

In our study, a full-length cDNA sequence of DDC from *P. vicina* was isolated and characterized. Multiple alignment and phylogenetic analyses showed that PvDDC cDNA and its deduced amino acid sequence share a high degree of homology with other animals. Among the insects in this study, the DDC proteins of the hymenopterans *P. vicina* and *A. mellifera* are more closely related to each other than to the DDC proteins of the other insects.

The high degree of conservation of DDC proteins may indicate a significant physiological role in organisms. The levels of PvDDC mRNA expression at different development stages in the whole bodies were measured by real-time

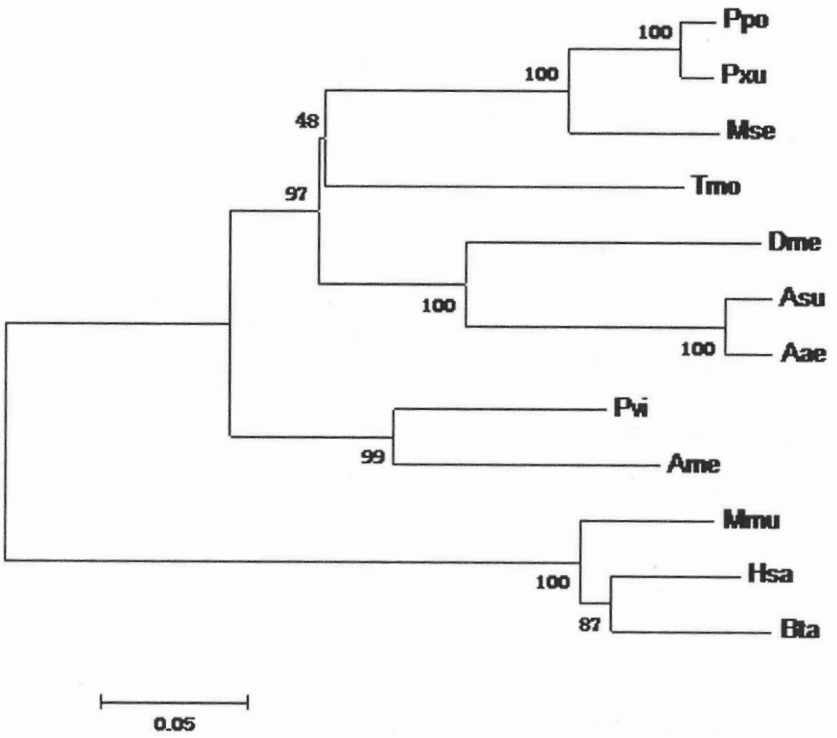


Fig.4. Phylogenetic tree which shows the evolutionary relationship of PvDDC with other members of the DDC family constructed on the basis of alignment of the animo acid sequences of DDC homologues. Numbers at branch nodes indicate percent bootstrap confidence values derived from 2000 replications. An explanation of species abbreviations is shown in Fig. 3.

quantitative RT-PCR in our study. It indicated that DDC mRNA was expressed at all stages of *P. vicina* development, but at different levels. The expression was very low in embryos and early larval stages then increased at the third larval stages and reached the highest level at the pupal stage. During the pupal stage, the tissues of adult insects began to form while the tissues for larvae began to disintegrate. For example, in ant larvae in the pupal stage reorganization of neural circuits begin to form while the body has also undergone a drastic change (Nemoto *et al.* 2007; Ishii *et al.* 2005). In complete metamorphosis, the central nervous system is formed in the pupa period (Truman. 1996). In this study, the levels of PvDDC mRNA expression in whole bodies and brains from adult of three castes were also measured. The highest level was found in males, the lowest level in females. The levels in female heads was higher than in workers'. DDC catalyses the decarboxylation of L-dopa to dopamine and 5-HTP to 5-HT (Sourkes 1966; Hodgetts *et al.* 2006). Dopamine is neces-

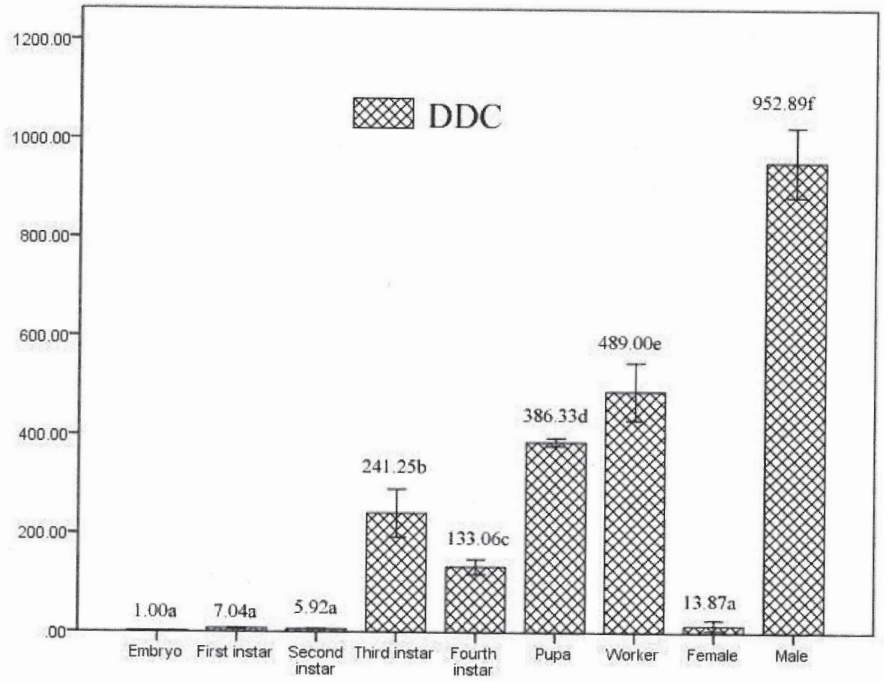


Fig. 5. Relative expression profiles (± standard error of the mean; n=3), determined by real-time PCR, for PvDDC mRNA during different development stages. All levels of expression are shown relative to the level in embryos. Means followed by the same letter are not significantly different.

sary in development and melanin synthesis in insects. It is an important neurotransmitter in 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). Dopamine also plays a role in sexual behavior (Chang *et al.* 2006). In insects, 5-HT is widely distributed in the central nervous system, involved in visual accuracy and reproduction (Nassel *et al.* 1988). Male ants' function is primarily sexual reproduction and their visual system is the most developed in the three castes (Yang *et al.* 2009). This may explain the highest level of expression that was found in male bodies and brains. It is interesting that the levels were found to be higher in whole bodies of workers than in reproductive females, but in brains the higher level was detected in females. This may reflect the important physiological function of PvDDC not only in neuronal activity but also in other systems of the body.

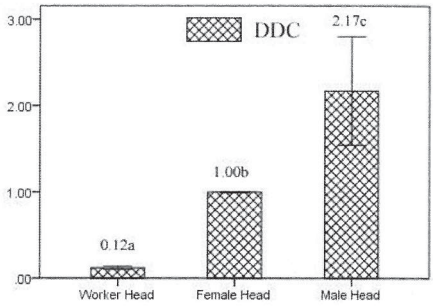


Fig.6. Relative expression profiles (± standard error of the mean; n=3), determined by real-time PCR, for PvDDC mRNA in the heads of different castes. All levels of expression are shown relative to the level in the heads of female ants. Means followed by the same letter are not significantly different.

In summary, DDC was cloned from a common Chinese weaver ant, *P. vicina* for the first time in our study. The finding that the PvDDC gene is differentially expressed at distinct developmental stages and in different head of castes may indicate the physiological importance of PvDDC in ant development. More evidence is needed to confirm the function of the PvDDC gene.

## ACKNOWLEDGEMENTS

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