Differential expression patterns of two delta-9-acyl-CoA desaturases in *Thitarodes pui* (Lepidoptera: Hepialidae) during different seasons and cold exposure

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**Abstract**
*Thitarodes pui* larvae have a limited distribution in the Tibetan Plateau and are the host of a parasitic fungus, *Ophiocordyceps sinensis*. Low temperature is a main environmental stress. However, understanding of *T. pui* cold adaptation mechanisms is insufficient. Delta-9-acyl-CoA desaturase (D9D) is closely correlated with cold adaptation for many organisms. To further understand the cold adaptation processes in *T. pui* larvae, two D9Ds, TpdesatA and TpdesatB were sequenced, and expression patterns were investigated during different seasons and cold exposure (under 0°C) in the laboratory. The full lengths of two cDNAs are 1,290 bp and 1,603 bp, and the ORFs encode a polypeptide of 348 and 359 amino acids, respectively. Four transmembrane domains, three conserved histidine residues and five hydrophobic regions exist in these two sequences. The expression level of TpdesatA is up-regulated in the long-term cold exposure and negatively correlated with temperature in seasonal patterns. TpdesatB responds to cold temperature in short-term cold exposure and positively corresponds temporally in seasonal expression. Two D9Ds may have different substrate specificities, TpdesatA tends to use C16:0 and C18:0 as substrate while TpdesatB prefers C18:0. In conclusion, TpdesatA may play a very important role in *T. pui* cold tolerance and TpdesatB regulates function in short-term cold exposure and content change of fatty acids in the body.

**KEYWORDS**
acclimation, delta-9-acyl-CoA desaturases, experimental evolution, thermal adaptation, *Thitarodes pui*

**INTRODUCTION**

For overwintering insects, low temperature is one of the most serious environmental stresses affecting their survival. In fact, overwintering insects survive low temperature due to a variety of physiological and biochemical adaptations (Baust & Rojas, 1985; Clark et al., 2009). Until recently, cold hardiness in insects was most often discussed in terms of cryoprotectants, membrane lipids, and heat-shock proteins (Storey & Storey, 2012; Teets & Denlinger, 2013; Yocum, 2001). The transition of cell membrane lipids from a liquid crystalline phase to a gel phase is an important cause of cold injuries under nonfreezing conditions (Michaud & Denlinger, 2006). Further investigations revealed that accumulation of unsaturated fatty acids (UFAs) contributed to the fluidity of cell membranes which are susceptible to cold (Khani, Moharamipour, & Barzegar, 2007; Koštá, Berkova, & Šimek, 2003; Michaud & Denlinger, 2006). In most cases, more UFAs and less saturated fatty acids (SFAs)
were detected in response to cold exposure (Los & Murata, 2004; Yi, Guo, Zou, & Zhang, 2015). Studies on the composition of cell membrane lipids in many species of microorganisms, plants, and animals under different temperatures have revealed the universal occurrence of remodeling of cell membrane lipids in response to changes in ambient temperature, a phenomenon known as homeoviscous adaptation (Hazel, 1995; Teets & Denlinger, 2013). The increasing UFAs are considered to play a role in maintaining the liquid crystalline phase at low temperatures (Kayukawa, Chen, Hoshizaki, & Ishikawa, 2007).

Delta-9-acyl-CoA desaturase (D9D) is an important enzyme that introduces a double bond into SFAs, and has been shown to play an essential role in cold hardiness by increasing the ratio of UFAs to SFAs in cell membranes (Hsieh & Kuo, 2005; Tiku, Gracey, Macartney, Beynon, & Cossins, 1996). Certain groups of reports demonstrated that up-regulation of the D9D gene occurs during cold exposure. Tiku et al. (1996) indicated that transcription of the D9D gene increased tenfold in the liver of cold-exposed carp. Similar findings were also proved in Oreochromis niloticus (Zerai, Fitzsimmons, & Collier, 2010), as well as Chanos chanos and Ctenopharyngodon idella (Hsieh & Kuo, 2005). For insects, the D9D gene was firstly proved to participate in cold adaptation mechanisms in Delia antiqua. In that study, twofold to tenfold up-regulation of the D9D gene was induced in brain tissues, malpighian tubules, and the midgut when D. antiqua was exposed to cold (Kayukawa et al., 2007). This same result occurred in Sarcophaga crassipalpis (Rinehart, Robich, & Denlinger, 2010), Folsomia candida (Waagner, Holmstrup, Bayley, & Jesper, 2013), and Aedes albopictus (Reynolds et al. 2012). Meanwhile, another kind of D9D, which was associated with dietary alterations, was found in Cyprinus carpio and Acheta domesticus (Batcabe, Howell, Blomquist, & Borgeson, 2000; Polley et al., 2003).

Thitarodes pui (Zhang et al.) (Lepidoptera: Hepialidae) (Figure 1) was first reported as Heptalpus pui (Zhang, Gu, & Liu, 2007) in China but was later moved to the genus Thitarodes (Zou, 2009; Zou, Liu, & Zhang, 2010). Larvae of Thitarodes in Southeast Asia are the host of the fungus Ophiocordyceps sinensis (Berkeley) Saccardo (Dong Chong Xia Cao in Chinese) (Winkler, 2009), which is one of the most valuable resources for traditional Chinese medicine (Buenz, Bauer, Osmundson, & Motley, 2005; Yue, Ye, Lin, & Zhou, 2013; Zhu, Halpern, & Jones, 1998). T. pui has a limited distribution of 4,100–5,000 m surrounding Mount Segrila in Tibet (Zhang et al., 2007). In this region, the average annual temperature is below 5°C and the soil is periodically frozen and thawed, and low temperature is considered a main environmental stress for T. pui (Yi, Guo, et al., 2015). Nevertheless, Thitarodes larvae can endure extreme temperatures at −12 and −20°C, but the mechanism for this cold tolerance is unclear (Yang, Li, Shu, & Yang, 1996).

Our previous work indicated that proteins, total sugar, and total fat in the hemolymph of T. pui larvae showed negative correlation with soil temperature (Yi, Zhang, Guo, Min, & Zou, 2015). In addition, HSP90 of T. pui, rather than HSP70, responds to temperature changes and potentially plays a key role in cold tolerance (Zou, Sun, Li, & Zhang, 2011). In addition, trehalose-6-phosphate synthase is involved in the complicated cold adaptation process in T. pui (Min et al., 2016). To obtain a further understanding of cold adaptation, two D9D genes (TpdesatA and TpdesatB) were sequenced in T. pui larvae and their expression patterns were investigated by real-time PCR during different seasons and cold exposure under 0°C in the laboratory. The results might serve to build a framework for comprehensively understanding the biology and molecular mechanisms of T. pui adaptation to thermal stress.

2 | MATERIALS AND METHODS

2.1 | Temperature measurement of soil with T. pui larvae

Temperature of soil at 20 cm below the surface was measured with Hobo Pro temperature and RH data logger (Model H08-032-08, Eco-tech Co. LTD, USA). The data logger was set to record the temperature every 30 min, and the data were downloaded every 30 days with BoxCar Pro software (version 4.3, Onset Computer Corporation, USA) (Zou et al., 2011).
2.2 | Insect collection and cold exposure regime

The investigation of seasonal expression patterns was processed from July 2008 to June 2009. In the middle of every month, six individuals of the sixth instar T. pui larvae were collected from Mt. Segrià (4,156 m, 29°37’N, 94°37’E) in the Tibetan Plateau, and these samples were used to transcription level analysis. Experiments under cold exposure were processed from July to August in 2013. More than 100 individuals of sixth instar T. pui larvae were collected in July 2013 at the same area, then fed in soil at laboratory, and the environment-controlled at 10°C; after fifteen days, they were used to cold exposure experience. Ten individuals were used as a control group. The other were reared at 0°C (Thermo Scientific Precision, USA) and collected at different times, including short term (1, 3, 6, 12 hr), midterm (24, 48, 72 hr, 5 days), and long term (7, 10, and 15 days).

2.3 | Collection of fat body

All samples were dissected to obtain fat bodies. The fat bodies isolated from two larvae were mixed and then stored in RNA protect solution (TaKaRa, Japan) at −80°C.

2.4 | Cloning the full-length cDNA of two D9D genes

Total RNA was extracted from the fat body of one individual using Trizol Reagent Kit (Invitrogen, USA) according to the manufacturer’s instructions, then dissolved by 30 μl diethylpyrocarbonate (DEPC) water and stored at −80°C. The RNA was quantitated by NanoDrop 2000 (BioSpec-mini, Shimadzu) and transferred to cDNA by using AMV reverse transcriptase (TaKaRa, Dalian, China) under the manufacturer’s protocol. Degenerate primers were designed based on the conserved amino acid sequences of known D9D genes of other Lepidoptera insects (Table 1) and then used to amplify the initial segments of two D9D genes. The PCR was conducted with 30 cycles under condition of 30 s at 94°C for denaturation, 30 s at 45°C for annealing, 1 min at 72°C for extension. Specific primers for 5′-RACE (Rapid amplification of cDNA ends) and 3′-RACE (Table 1) were synthesized based on the initial segments of two D9D genes. The 5′ and 3′ RACE were processed using the SMART RACE cDNA Amplification Kit (Clontech, CA, USA). The PCR was placed in 50 μl volume with 30 cycles under the condition of 30 s at 94°C, 30 s at 55°C, 2 min at 72°C.

2.5 | Sequence analysis

The obtained fragments of two D9D genes were assembled by DNASTAR and the ORFs were identified through ORF Finder (Thompson, Higgins, & Gibson, 1994), respectively. Amino acid sequences were deduced from the corresponding cDNA sequences by using the translation tool on the ExPASy Proteomics Web site, and analogs were searched by BLASTP at the NCBI (Zou et al., 2011). The molecular weight (MW) and isoelectric point (pI) of the deduced amino acid sequences were predicted from the ExPASy Proteomics Web site. Analysis of the transmembrane domains and hydrophobic regions were performed using Kyte-Doolittle hydropathy plots in DNASTAR. Multiple alignments were performed among TpdesatA, TpdesatB as well as the analogous amino acid sequences by DNAMAN. Finally, base on the amino acid sequences of known D9D genes in Lepidoptera and Dipteran from GenBank (Kayukawa et al., 2007), phylogenetic tree was constructed using the neighbor-joining method in MEGA software with 1,000 bootstrap replications.

2.6 | Quantitative analysis of two D9D genes

The seasonal expression and cold adaptation changes of two D9D genes were investigated through RT-PCR in CFX96™ Real-Time System. Two pairs of primers were designed for the quantitative analysis of two genes as well as a pair of primers for the control (β-actin) (Table 1). The reaction was performed following the manufacturer’s instructions of SYBR® Premix Ex Taq™ (TaKaRa, Dalian, China) with the conditions as followed: 3 min at 95°C followed by 40 cycles of 95°C for 15 s, 30 s at 60°C and 30 s at 72°C. The remaining curve analysis at the end of program was used to test the specificity of primers. Experimental operation was repeated three times for each group. 2−ΔΔCt method was used to determine the

### Table 1 | Primers used for cloning and expression analysis of two D9D in Thitarodes pui

| Fragment | Primer | Primer sequence (F/R) 5′→ 3′ |
|----------|--------|-----------------------------|
| TpdesatA | TdAF1  | TTTTCTCTCATATGGGCTGTCG |
| TdAF2   | TCCGTCAGCCTGTTACCCCT |
| TdAR1   | GGCTACGAAAAAAGCCAG |
| TdAR2   | CTTCTGAAATGTAACGATGGGT |
| TdAR3   | ATAAAGCCAGCCCATATAGAGAA |
| QdesatAF | CGTCAGCCTGTTACCTCCG |
| QdesatAR | GCCGGTCTGTATGATCCTCTTC |
| TpdesatB | TdBF1  | TGATACAGACGCCGACCCG |
| TdBF2   | TGCCGGCTGTCCTCATT |
| TdBF3   | CGACCCCTATCCTAGCTTTCA |
| TdBR1   | GGATAGGGCTGTTCTC |
| TdBR2   | TGGTCCGGCTGCGGTCTGATTC |
| TdBR3   | CTATCACAGTCTCCTGAAATG |
| QdesatBF | TGATACAGACGCCGACCCG |
| QdesatBR | GGCAAAAATGACGACGACGAC |
| β-Actin | QActinF | TAACCCCCAAGCCGAACAGA |
| QActinR | GCCAACGTCGACGGAGAAG |

ΔΔCt method was used to determine the
expression profiles of TpdesatA and TpdesatB. The relative mRNA levels of TpdesatA and TpdesatB in July and 0 hr were set as 1, respectively.

2.7 | Statistical analysis

Means and variances of treatments were analyzed using SPSS program (version 19.0, IBM Inc., USA), and the relative mRNA levels of D9D in July or control group was set as 1. All data were shown as mean ± SD. The means were compared with variance (ANOVA) and Tukey’s studentized range test with the level of significant difference at p < .05 and highly significant difference at p < .01.

3 | RESULTS

3.1 | Sequence identification and characterization of two D9D genes

The full length of two D9D genes, TpdesatA and TpdesatB, were obtained through overlapping PCR and RACE. They are 1,290 bp and 1,603 bp, and their nucleotide sequences and deduced amino acid sequences are shown in Figure 2a,b, respectively. Their nucleotide sequences have been deposited in NCBI GenBank with accession numbers GU126468 and GU205814, respectively.

The full length of TpdesatA cDNA contains 65 bp in the 5′-untranslated region (UTR), 1,041 bp in the open reading frame (ORF) and 184 bp in 3′-UTR. The ORF encodes a polypeptide of 348 amino acids. The inferred molecular mass of the mature protein is 107.5 kDa with an estimated PI of 5.02 (Figure 2a). The TpdesatB includes 103 bp in 5′-UTR, 420 bp in 3′-UTR and 1,080 bp in an ORF encoding a polypeptide of 359 amino acids with MWs of 133.4 kDa and PI of 4.96 (Figure 2b). Otherwise, three histidine clusters can be found in two D9D (HXXH, HXXHH, and EXXHXXHH) (Figure 2a,b). The presence of five hydrophobic regions and four transmembrane domains in TpdesatA and TpdesatB was revealed with Kyte–Doolittle hydropathy analysis. The amino acids of TpdesatA and TpdesatB were aligned to those D9D genes of other 12 species, and analysis results showed that TpdesatA is similar to Manduca sexta (65.6%), Bombyx mori (65.6%), Lampronia capitella (65.3%); TpdesatB showed a similarity with L. capitella (59.4%) and Dendrolimus punctatus (59.4%), followed by Ephippus postvittana (59.1%). Otherwise, the similarity of TpdesatA and TpdesatB is 45.5%. Same feature sequences were also existed in others species D9D sequence (Figure 3).

A phylogenetic tree was constructed by the neighbor-joining method, based on amino acid sequences of 20 known D9D in Lepidopteran and Dipteran. Four denatures groups with different substrate specificities were clearly identified as follows (substrate preferences are indicated in parentheses): △9 (16 > 18), △9 (16 = 18), △9 (18 > 16) and △9 (14–26), respectively. It was shown that TpdesatA gene belongs to the △9 (16 > 18) group and TpdesatB gene belongs to the △9 (18 > 16) (Figure 4).

3.2 | Seasonal expression patterns of two D9D genes

Quantitative analysis was performed to indicate the seasonal expression patterns of two D9D genes through RT-PCR. The soil temperature kept low level in whole year (Figure 5a). As shown in Figure 5b, the expression of TpdesatA exhibited a negative correlation with temperature (y = 4.286 − 0.227x) (r = −.388, p = .390). The transcription of TpdesatA reached the highest level in December while the temperature remained at low level. In spite of the lowest level in March and July, the expression of TpdesatA remained stable in January, May, August as well as October. Expression of TpdesatB showed a positive correlation with soil temperature (y = 0.656 + 0.035x) (r = .437, p = .326). During the investigation, the expression of TpdesatB sustained in high level in July, August, and October while it dropped and remained at low level from December to May (Figure 5c).

3.3 | Expression patterns of two D9D genes during cold exposure under 0°C

0°C was set to explore the cold adaptation mechanism under stable cold exposure in laboratory. Significant change was detected in the expression level of TpdesatA during the cold stress (F_{11, 35} = 40.777, p < .001). In the short-term and midterm cold exposure, TpdesatA was stable, and remained at a low level from 1 hr to 5 days with no substantial change detected (Figure 6a). In the long-term cold exposure, the expression of TpdesatA increased from 5 days to the highest level at 10 days (2.43-fold) and slightly declined to 1.88-fold at 15 days. Expression of TpdesatB was significantly affected by cold exposure (F_{11, 35} = 109.469, p < .001), the expression of TpdesatB was up-regulated from 6 hr (2.55-fold) to 5 days (2.97-fold) with a highest level appeared at 24 hr (3.59-fold), and TpdesatB was down-regulated before 3 hr and after 7 days.

4 | DISCUSSION

As a plateau insect with high cold tolerance, a series of physiological and biochemical mechanisms are evolved in T. pui. The proteins, total sugar, and total fat in the hemolymph as well as the fatty acid in whole body had the negative correlation with soil temperature (Yi, Guo, et al., 2015; Yi, Zhang, et al., 2015). Moreover, trehalose-6-phosphate synthase, HSP90 of T. pui, rather than HSP70, responds to temperature changes (Min et al., 2016; Zou et al., 2011). In this paper, TpdesatA and TpdesatB were found to have the relation with cold tolerance of T. pui.

FIGURE 2 Nucleotide and deduced amino acid sequences of TpdesatA (a) and TpdesatB (b). The start and stop codons were showed as bold. Four transmembrane domains and three conserved histidine residues were boxed and underlined, separately. Five hydrophobic regions were leaned and underlined.
Multiple alignment of Tpdesat amino acids in insects Four transmembrane domains and three conserved histidine residues were boxed and underlined, separately. Five hydrophobic regions were leaned and underlined.
In T. pui larvae, the lipid content changes in response to soil temperature. In phospholipids, C18:1 and C18:2, showed significant negative correlation with soil temperature. However, the fluctuation soil temperature did not cause any significant changes in any of the individual's triacylglycerols fatty acids (Yi, Guo, et al., 2015), to which the molecular mechanism remains unknown. D9D plays an essential role in cold hardiness, by increasing the ratio of unsaturated and saturated fatty acids (UFA/SFA) (Rinehart et al., 2010; Tiku et al., 1996). In this study, two D9D genes were isolated for the first time in Thitarodes insects, and their expression patterns were investigated during different seasons and cold exposure under 0°C.

The two D9D genes separately encoded 346 AA and 359 AA amino acids, which correspond to the size range in other insects from NCBI. Alignment of two D9D genes and isoforms of thirteen other insects revealed D9D genes exist in several highly conserved regions. Four transmembrane domains existed in D9D, suggesting that the sequence spans the lipid bilayer of membranes four times (Kayukawa et al., 2007; Los & Murata, 1998). Three histidine residues in these D9D genes were the highly conserved regions which are catalytically essential in desaturases (Shanklin, Whittle, & Fox, 1994). These histidine residues are suggested to combine with iron atoms at the catalytic center (Los & Murata, 1998). According to the N-J tree that was constructed in current study, two D9D genes in T. pui occurred in two independent clades, indicating that two D9D has different substrate specificities.

D9D desaturase is a key enzyme in synthetic pathway of UFAs, contributing to the formation of C16:1, C18:1, and C18:2. And these three UFAs are crucial to sustaining the fluidity of membranes under cold conditions (Kayukawa et al., 2007; Khani et al., 2007; Miyazaki, Kayukawa, Chen, Nomura, & Ishikawa, 2006). Previous investigations had proved that D9D gene was critical for cold adaptation in fish (Tiku et al., 1996), bacteria (Sakamoto & Bryant, 1997), and plants (Vega, Del Rio, Bamberg, & Palta, 2004). In this study, the expression level of TpdesatA was up-regulated in the long-term cold exposure and remained at a low level at short term and midterm; this indicates that TpdesatA contributes to long-term cold hardness.

It was shown that seasonal expression patterns of TpdesatA exhibited a negative correlation with soil temperature \( r = -.388, p = .390 \). Kayukawa et al. (2007) proved that the expression of D9D increased to enhance the cold hardiness in Delia antiqua through up-regulating the abundance of C16:1 and C18:1. So far, the same results were seen in S. crassipalpis (Rinehart et al., 2010), A. albopictus (Reynolds, Poolchau, Rahman, Armbruster, & Denlinger, 2012) and F. candida (Waagner et al., 2013). At the same time, seasonal cold-hardening is defined as cold-hardening that requires at least days to weeks for induction (Teets & Denlinger, 2013). Therefore, we suggest that TpdesatA has contributed to seasonal cold-hardening. In phospholipids of T. pui larvae, C18:1 was the most abundant UFAs and exhibited a weak negative correlation with soil temperature. C18:2, the second abundant UFA, was highly accumulated at early days of overwintering and fluctuated in lower levels during warmer seasons. Prewinter accumulation was also detected in C18:3 (Yi, Guo, et al., 2015). These three UFAs content change in phospholipids may associate with the regulation of TpdesatA. Moreover, C16:0 was the second abundant in triacylglycerols, with a significant negative correlation with soil temperature (Yi, Guo, et al., 2015). At the same time, TpdesatA gene was clustered in the \( \Delta 9 \ (16 = 18) \) group in phylogenetic tree. It indicates that TpdesatA works in seasonal cold-hardening, and C16:0 and C18:0 served as main substrate in triacylglycerols and phospholipids, respectively.

During cold exposure at 0°C, TpdesatB up-regulated from 6 hr to 5 days and down-regulated after 5 days; this indicates TpdesatB may responds to cold temperature in short-term cold exposure. The same results were obtained in the winter diapause pupae of D. antiqua (Hao et al., 2012). In the seasonal expression pattern of TpdesatB, it remained at high levels in summer and dropped in winter. It is obvious that high temperature contributed to the transcription of TpdesatB and low temperature suppressed the process \( r = .437, p = .326 \). Polley et al. (2003) indicated that one D9D gene (Cds 1) in carp was well expressed at 30°C and repressed at 15°C, and the regulated pattern was associated with dietary. Down-regulation in two D9Ds was induced in Drosophila montana (desat 1) and Drosophila virilis (desat 2) by cold acclimatization (Vesala, Salminen, Laiho, Hoikkala, & Kankare, 2012), and there is also evidence that desaturases function in stress resistance (Greenberg, Moran, Coyne, & Wu, 2003). Based on the results showed above, TpdesatB seems to be not responsible for seasonal cold-hardening. Many researchers believed that the UFAs accompanied by
FIGURE 5 Relative mRNA levels of TpdesatA (b) and TpdesatB (c) of Thitarodes pui in different seasons with different soil temperature (a). Data represent means ± SD from three replicate experiments (p < .05), actin gene was used as reference one.

FIGURE 6 Relative mRNA levels of TpdesatA (a) and TpdesatB (b) of Thitarodes pui under different cold exposure at 0°C Data represent means ± SD from three replicate experiments. Different letters indicate significant differences (p < .05), actin gene was used as the reference one.
triaclylglycerols increased and functioned as energy resources to help organism overcome cold (Joanisse & Storey, 1996; Teets & Denlinger, 2013). Therefore, TpdesatB may act as important short-term regulate substance in cold exposure and cause the change of the proportion of fatty acids in the body. Meanwhile, TpdesatB rather than TpdesatA was clustered closer to the 9 (16 > 16) group in phylogenetic tree. It indicated that C18 served as the substrate of TpdesatB prior to C16 in the short-term cold exposure.

Our results suggest that, during cold exposure at 0°C, TpdesatA and TpdesatB contributed to the cold tolerance in T. puui larvae. TpdesatA has contributed to cold hardiness in long term and TpdesatB in short term during cold exposure at 0°C. The seasonal expression pattern of TpdesatA exhibited a negative correlation with temperature. While TpdesatB showed another expression pattern compared to TpdesatA, it is contributed to short-term cold hardiness and positively corresponds to temperature in seasonal expression pattern. These results indicated that TpdesatA played a very important role in seasonal cold-hardening and TpdesatB acted as an important regulating substance in short-term cold exposure and the proportion change of fatty acids in larvae. Different D9D have diverse substrate specificities, TpdesatA tends to use C16:0 and C18:0 as substrate, and TpdesatB prefers to C18:0.

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CONFLICT OF INTEREST

None declared.

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