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Molecular Characterization and Gene Expression of Trehalase in the Bumblebee, *Bombus lantschouensis* (Hymenoptera: Apidae)

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Abstract

Trehalose provides the main energy source for the physiological activities of insects, especially in adverse conditions. Trehalase is the only enzyme that hydrolyzes trehalose, therefore it is important to clarify the distribution and expression of trehalase under adverse conditions such as high temperatures and starvation. Here, we have cloned the trehalase genes and investigated their expression in different tissues, at multiple development stages, and with the treatments of high temperature and starvation in *Bombus lantschouensis*, which is considered to be one of the most commercially viable native species in China. The results suggest that the membrane-bound (*BlTre-2*) cDNA has an open reading frame of 1986 nucleotides, which encodes a protein of 662 amino acids, and two putative transmembrane domains. qRT-PCR analysis indicated that *BlTre-2* was expressed in 10 tissues and at nine development stages, with the highest expression in general in 30-day-old workers, and in ovarian tissue in particular. The expression of *BlTre-1* for 15-day-old workers which were exposed to a pre-treatment of 45°C increased over the first 5 h and subsequently decreased over time. In contrast the expression of *BlTre-2* consistently decreased over time. The highest expression levels of *BlTre-1* and *BlTre-2* were observed the newly emerged adult workers when starved for 16-20 h. These results indicate that *BlTre-2* may be part of the carbohydrate metabolism of the bumblebee, and that *BlTre-1* is a key gene regulating energy metabolism and providing trehalose when exposed to a high temperature. Both *BlTre-1* and *BlTre-2* may balance trehalose and provide energy when *B. lantschouensis* is starved.

Introduction

Trehalose is a non-reducing disaccharide with two glycosidically linked glucose units present in all forms of life except mammals, including plants, bacteria, fungi, yeast, nematodes, and insects (Elbein et al., 2003; Thompson, 2003; Argüelles, 2014; Nardelli et al., 2019). In these organisms, trehalose plays an important role in protecting proteins and cellular membranes during unfavorable environmental conditions such as heat, desiccation, dehydration, freezing,

hyperosmosis, anhydrobiosis, and oxidation (Wyatt, 1967; Crowe et al., 1992; Thompson, 2003; Mitsumasu et al., 2010). As a major blood sugar of insects, trehalose is present in the hemolymph of larvae, pupae, and adults (Alumot et al., 1969; Becker et al., 1996; Thompson, 2003). Trehalose is the main metabolic source for physiological activity, such as growth, development (Łopieńska-Biernat et al., 2018), energy metabolism (Thompson, 2003), anhydrobiosis (Mitsumasu et al., 2010), feeding behavior (Tang et al., 2014; Yasugi et al., 2017), chitin synthesis (Tang et al., 2012, 2016, 2017, 2018; Shen et al., 2017; Zhang et al., 2017), and diapause (Kamei et al., 2011; Yang et al., 2013; Wang et al., 2018). On the other hand, it plays key roles in adverse circumstances, such as starvation (Tang et al., 2014; Shukla et al., 2014; Liu et al., 2016).

In insects, trehalase (α-glucoside-1-glucohydrolase, EC 3.2.1.28) is the only key enzyme that catalyzes the conversion of one molecule of trehalose to two molecules of glucose utilized through glycolysis (Clegg & Evans, 1961; Tatun et al., 2008). Two types of trehalase (soluble and membrane-bound) and corresponding genes (*Tre-1* and *Tre-2*) have been cloned in a variety of insects (Qin et al., 2015; Liu et al., 2016; Shukla et al., 2016; Zhao et al., 2016). Both forms contain signature motifs (PGGRFEFYYWDSY and QWDYPNAWPP) and a highly conserved glycine-rich region (GGGGEY). TRE-1 and TRE-2 break down the intracellular and extracellular trehalose, respectively (Shukla et al., 2014; Liu et al., 2016). The *Tre-1* gene, which was first cloned from *Tenebrio molitor* in 1992, is mainly found in the digestive and circulatory systems (Takiguchi et al., 1992). *Tre-2* contains a putative transmembrane domain (Takiguchi et al., 1992; Tang et al., 2008; Gu et al., 2009; Forcella et al., 2010) and is mostly identified in the muscle (Mitsumasu et al., 2005).

Recent studies have indicated that only *Tre-1* and *Tre-2* are found in the majority of insects, such as *Apolygus lucorum* (Tan et al., 2014), *Helicoverpa armigera* (Ai et al., 2018), *Spodoptera exigua* (Tang et al., 2008), *Omphisa fuscidentalis* (Tatun et al., 2008), *Drosophila melanogaster* (Shukla et al., 2016), *Cnaphalocrocis medinalis* (Tian et al., 2016), and *Bemisia tabaci* (Wang et al., 2014). Multiple *Tre-1* have been cloned in insects, including *Harmonia axyridis* (Tang et al., 2014; Shi et al., 2016), *Locusta migratoria* (Liu et al., 2016), *Nilaparvata lugens* (Zhao et al., 2016), *Tribolium castaneum* (Tang et al., 2016), and *Leptinotarsa decemlineata* (Shi et al., 2016). In addition, trehalase gene is expressed in various tissues inducing the diapause of silkworm eggs and improving the cold resistance (Kamei et al., 2011; Tan et al., 2014). Generally, two types of trehalase, TRE-1 and TRE-2 are involved in various physiological functions such as long-distance flight metabolism, chitin synthesis during molting, energy metabolism, muscular movement, reproduction, and cold tolerance (Chen et al., 2010; Tang et al., 2012; Zhang et al., 2012; Shi et al., 2019).

Bumblebees (Apidae, *Bombus* Latreille), important pollinators of many endangered alpine plants and agricultural crops, play a major role in maintaining biodiversity of ecosystems and agricultural production (Williams & Osborne, 2009; Gunnarsson & Federsel, 2014). Many species of bumblebees (such as *Bombus terrestris*, *B. impatiens*, *B. occidentalis*, and *B. ignitus*) have been used for commercial crop pollination (Velthuis & van Doorn, 2006). *B. lantschouensis* (Hymenoptera: Apidae), once mistaken for *B. hypocrita*, is widely distributed in the North China and is a native bumblebee species used in commercial applications – it is recognized in Chinese agriculture to be an excellent pollinator (An et al., 2014). There is not much information is available on the function, structure, tissue distribution, and expression patterns of the trehalase gene in *B. lantschouensis* even though trehalase has been studied in honeybees (Alumot et al., 1969; Brandt & Huber, 1979; Lee et al., 2007; Łopieńska-Biernat et al., 2018). Understanding the fundamental molecular characteristics and function of trehalase will enable us to improve the population and health of *B. lantschouensis*.

In the present study, we cloned and characterized the *Tre-2* gene from *B. lantschouensis* and measured patterns of expression in tissues at different chronological ages. In addition, we investigated the expression patterns of both *Tre-1* and *Tre-2* in *B. lantschouensis* at 45°C and under starvation conditions.

Materials and Methods

Bumblebees

Newly mated queens of the bumblebee *B. lantschouensis* were collected from the Hebei Province of China. These queens were reared under controlled climatic conditions at a temperature of 29 ± 0.5°C and 60±5% relative humidity in continuous darkness. Queens and their newly emerging workers were fed a 50% sucrose solution and pollen (from *Brassica campestris* L. and *Prunus armenica* L.). To ensure the adult bumblebees were the same age, all new workers were marked on the thorax with a white mark within 1 h. The workers used in this study were 15 days old but particular exceptions, which are noted later on. All the samples were taken from three colonies, and each colony was treated as an independent replicate.

RNA Extraction, cDNA Synthesis, and PCR

The total RNA was extracted from the sample with Trizol (Invitrogen, Carlsbad, CA, USA). First-strand cDNA synthesis was synthesized from 1 μg of RNA using a Transcriptor First Strand cDNA Synthesis Kit (Takara, Dalian, China), and the undiluted first-strand cDNA was used as the template for the polymerase chain reaction (PCR).

Conserved District Fragment Amplification

To obtain a conservative fragment of *BlTre-2* from *B. lantschouensis*, a pair of degenerate primers, Tre-2-F and Tre-2-R (Table 1), were designed based on the conserved

amino acid sequences of the known *Tre-2* in *B. terrestris* and *B. impatiens* (GenBank accession nos. XP_003393687 and XP_003490073). The PCR amplification reaction system contained 2 μL cDNA template, 1 μL of each primer (10 μmol/L) and 12.5 μL of the PCR mix, and finally was topped to 25 μL volume with nuclease-free water. PCR conditions were as follows: 94°C for 5 min, 35 amplification cycles of 94°C for 40 s, 50°C for 30 s, and 72°C for 1 min, and then an extension at 72°C for 7 min. The PCR products were electrophoresed for 10 min under 200 volts and then excised from agarose gels (1%) and purified with DNA Purification Kits (Version 2.0, Takara). Purified PCR products (4.2 μL) were ligated into 0.8 μL pMD-19T vectors using 5 μL Solution I at 16°C. The ligated constructs were transformed into Tran1-T1 competent cells and cultured for 1 h at 37°C. We selected an ampicillin-resistant clone and sub-cultured in 600 μL LB liquid medium to obtain the optimum amount of the expression vector, which was sequenced using M13-forward and M13-reverse.

To complete the 5' and 3' ends, a Transcriptor First Strand cDNA Synthesis Kit (Takara) was used to synthesize 5'-RACE and 3'-RACE first-strand cDNA according to the manufacturer's instruction. Specific primers 5'-OGSP and 5'-OP, 5'-IGSP, and 5'-IP for 5'-RACE, and 3'-OGSP and 3'-OP, 3'-IGSP, and 3'-IP for 3'-RACE (Table 1) were synthesized based on the cDNA sequence of the PCR fragment. The first PCR amplification reaction system contained 2.5 μL cDNA template, 1 μL of each primer (10 μmol/L) (5'-OGSP and 5'-OP/3'-OGSP and 3'-OP), 1 μL dNTP Mix, 2.5 μL 10×EX Buffer, 0.4 μL EX Taq HS, and was finally topped to 25 μL volume with nuclease-free water. PCR was performed under the following conditions: 94°C for 2 min, 35 amplification cycles of 94°C for 15 s, 60°C for 30 s, and 72°C for 40 s, and an extension at 72°C for 10 min. The products of the first PCR were diluted one-fold and then used as a template (2.5 μL) for the second PCR. The second PCR had the same reagent and content as the first PCR except for the primers (5'-IGSP and 5'-IP/3'-IGSP and 3'-IP) and was performed under the following conditions: 2 min at 94°C followed by 30 cycles of 15 s at 94°C, 30 s at 60°C, and 40 s at 72°C, and then 10 min at 72°C.

Protein and cDNA Sequence Analyses

The cDNA sequence of *BlTre-2* was analyzed to determine its similarity with Tre-2 genes of *B. terrestris* (XP_003393687) and *B. impatiens* (XP_003490073) deposited in GenBank by using BioEdit 7.0.9. The *BlTre-2* cDNA sequence was deposited in the NCBI GenBank (accession number MZ292465). The signal peptides, molecular mass and theoretical isoelectric point (pI), N-glycosylation sites and transmembrane helices of the deduced amino acid sequences of *BlTre-2* were predicted by using the SignalP 4.1 Server, the ExPASy Compute pI/Mw tool (https://web.expasy.org/compute_pi/), the NetNGlyc 4.0 Server, and the TMHMM Server v. 2.0, respectively. The deduced amino acid sequences of *BlTre-2* were compared with trehalase genes of other species available in GenBank. Multiple alignments of trehalase genes were conducted using the software Clustal X2. A phylogenetic tree was constructed based on the amino acid sequences by using the neighbor-joining (NJ) algorithm in MEGA 6.06. The reliability of the branching was tested using the bootstrap method (1,000 replications).

Table 1. Primers used in this study.

Primer uses	Primer name	Primer sequence (5'-3')
The part fragment	Tre-2-F	GGACTGGAAAAGAGCGGTCA
	Tre-2-R	GGTTTCAGATCGTCCTGGCACGATT
3'-RACE	3'-OGSP	GTTCGACGATTTGGAACGTC
	3'-OP	TACCGTCGTTCCACTAGTGATTT
	3'-IGSP	ATACGCGGAACGTAATTGGC
	3'-IP	CGCGGATCCTCCACTAGTGATTTCACTATAGG
5'-RACE	5'-OGSP	GGTCGAACGTGTTGTTGATG
	5'-OP	CATGGCTACATGCTGACAGCCTA
	5'-IGSP	GATCTGGTTCCTGGTCGGTG
	5'-IP	CGCGGATCCACAGCCTACTGATGATCAGTCGATG
qRT-PCR	Tre-1-F1	AGTGAGTTTGCCTTCTGG
	Tre-1-R1	GAGGTCTCGTGCTGTTGA
	Tre-2-F1	TCCTCGTTCGTTTGTGACGA
	Tre-2-R1	TTTGGCCAATTACGTTCCGC
Reference gene	Actin-88-F	GCGCGACATTAAGGAGAAAC
	Actin-88-R	CCATACCCAGGAAGGAAGGT

BlTre-2 Expression in Different Tissues and Different Chronological Ages

To clarify the distribution of *BlTre-2* in *B. lantschouensis,* we dissected the workers in the ice-cold lysis buffer on ice. Ten tissues (antennae, head, muscle, leg, wing, integument, midgut, Malpighian tubule, fat body, and ovary) were collected from workers aged 15 days and placed separately into PCR tubes for RNA extraction. To get enough samples for RNA extraction, 3 to 15 workers were dissected (Table 2).

Larvae, pupae, and adult workers aged 0 (new workers), 5, 10, 15, 20, 25, and 30 days old were dissected to investigate the relative expression of *BlTre-2* at different chronological ages. A sample of workers of the same age from each of three different colonies was dissected to extract RNA from the whole body, and this triplicated.

To determine the absolute copy of the target transcript, the cDNA template was diluted (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}) to gradient concentrations and then used to generate a standard curve. The qRT-PCR amplification reaction system contained 2 μL cDNA template, 0.8μL of each primer (10 μmol/L), 10 μL SYBR® Premix Ex TaqTM II, and 0.4 μL ROX reference dye and was finally topped to 20 μL volume with ddH_2O. The amplification conditions were as follows: 94°C for 30 s followed by 40 cycles of 94°C for 5 s and 60°C for 30 s. Each sample was replicated three times. *Actin-88* gene (Table 1) served as an endogenous reference gene for the determination of targeted mRNA for its continuously expressed in bumblebee (Li et al., 2010).

Table 2. The information of the number of individuals in each biological repetition for different tissues and chronological ages of *B. lantschouensis*.

Tissue name	Number of individuals	Chronological Age	Number of individuals
Antennae	15	Larva	3
Head	5	Pupa	3
Muscle	3	Day 0 worker	3
Leg	5	Day 5 worker	3
Wing	15	Day 10 worker	3
Integument	5	Day 15 worker	3
Midgut	15	Day 20 worker	3
Malpighian tubule	15	Day 25 worker	3
Fat body	15	Day 30 worker	3
Ovary	15		

BlTre-1 and *BlTre-2* Expression at 45°C and During Starvation

In this study, 15-days old workers were exposed to 0, 1, 2, 3, 4, and 5 h at 45°C, representing the temperature treatments for our experiments. For the starvation treatments, the newly emerged (0 day) adult workers were starved for 0, 4, 8, 12, 16, 20, and 24 h. When the treatments were completed, RNA was extracted from three workers exposed to the same treatment. To investigate the relative expression of the *BlTre-1* and *BlTre-2* genes, we designed two pairs of specific primers: Tre-1-F1 and Tre-1-R1; Tre-2-F1 and Tre-2-R1 (Table 1) based on the conserved amino acid sequences of the two known forms of trehalase gene in *B. lantschouensis* (*BlTre-1*: GenBank accession nos. KJ025078; *BlTre-2*: GenBank accession nos. MZ292465), and the primers of the reference gene Actin-88-F and Actin-88-R (Li et al., 2010; Qin et al., 2015). The qRT-PCR reactions were conducted using an Mx3000 qPCR system (Agilent, USA) with buffers at 94°C for 30 s in 1 cycle; 94°C for 5 s and 60°C for 20 s in 40 cycles with a melt curve over a temperature ranging from 55 to 90°C. In each reaction, 25 μL of final volume was produced containing 10 μL of the SYBR® Premix Ex TaqTM II, 2 μL of cDNA sample, 0.8 μL of primer (10 μmol/L), 0.4 μL of ROX reference dye, and 11 μL of RNase-free and DNAase-free H_2O, according to the manufacturer's instructions of SYBR® Premix Ex TaqTM II Kit (Takara, Dalian, China). All samples were run in triplicate. The qRT-PCR values of the focal genes were normalized using the *Actin-88* gene.

Statistical Analyses

Transcript quantifications were calculated using the $2^{-\Delta\Delta Ct}$ (Livak & Schmittgen, 2001). The lowest expression levels in different tissues and at different chronological ages were stated as 1. All data were analyzed using LSD tests of one-way ANOVA in SPSS 13.0.

Results

Cloning and Characterization Analyses of *BlTre-2*

The *BlTre-2* is 4,051 bp long, including an open reading frame (1,989 bp), a 5'-untranslated region (UTR) (1,307 bp), and a 3'-UTR (1,684 bp). The *BlTre-2* transcript encoded a protein of 662 amino acids (about 76.81 kDa and an estimated pI of 5.94). The amino acids contained two signature motifs, PGGRFREFYYWDSY (residues 180-193) and QWDYPNAWPP (481-490), a highly conserved glycine-rich region, GGGGEY (545-550), a signal peptide of 30 amino acids and a cleavage site (CYA-ST) between 30 and 31 (Fig 1), and five putative N-glycosylation sites (amino acids 79, 276, 352, 386, and 527) suspected to be a glycoprotein. Residues 13-31 and 598-620 comprised two putative transmembrane domains, MLLSAAFLALLVVAPCYAS QVMTGILALVISLAAGFIGMVVY (Fig 1).

The deduced amino acid sequence of trehalase from *B. lantschouensis* was aligned with the corresponding sequences of another insect trehalases (Fig 2). *BlTre-2* is most similar to the other Hymenopterans, such as *BtTre-2* (*B. terrestris)*, *BiTre-2* (*B. impatiens)*, *AmTre-2 (A. mellifera)* and *AfTre-2 (A. florea)* (Table 3). It is also similar to *Tre-1* and *Tre-2* from *Harpegnathos saltator*, *S. exigua*, *Bombyx mori*, *O. fuscidentalis*, *N. lugens*, *B. tabaci*, and *A. lucorum* (Table 3).

The alignment of multiple sequences indicated that the insect *Tre-2* gene is highly conserved, particularly in the middle of the putative catalytic domain (Fig 2). In addition, we used the amino acid sequences of selected trehalase genes to construct a phylogenetic tree, which shows that *BlTre-2* has a higher identity with other *Tre-2* genes in insects. The entire *Tre-2* gene clustered together as a subgroup, and the *Tre-1* gene clustered into another subgroup (Fig 3).

Table 3. The similarity comparison between amino acid sequences of trehalase genes from *B. lantschouensis* and other insects.

Species names	Gene names	GenBank No.	Similarity to *BlTre-1*	Similarity to *BlTre-2*
Bombus lantschouensis	*BlTre-1*	KJ025078	–	55.7 %
	BlTre-2	MZ292465	55.7 %	–
Bombus terrestris	*BtTre-1*	XP_003400853	99.5 %	55.2 %
	BtTre-2	XP_003393687	56.0 %	98.9 %
Bombus impatiens	*BiTre-1*	XP_003491166	99.0 %	54.9 %
	BiTre-2	XP_003490073	55.1 %	97.5 %
Apis mellifera	*AmTre-1*	XP_393963	88.3 %	54.5 %
	AmTre-2	BAF81545	59.6 %	84.2 %
Apis florea	*AfTre-1*	XP_003695047	87.4 %	53.9 %
	AfTre-2	XP_003696950	56.1 %	91.8 %
Harpegnathos saltator	*HsTre-1*	EFN81352	72.1 %	53.5 %
	HsTre-2	EFN85130	56.2 %	85.8 %
Spodoptera exigua	*SeTre-1*	ABY8628	58.5 %	54.1 %
	SeTre-2	ABU95354	56.2 %	69.4 %
Bombyx mori	*BmTre-1*	NP_001037458	60.4 %	51.7 %
	BmTre-2	NP_001036910	57.3 %	68.6 %
Omphisa fuscidentalis	*OfTre-1*	ABO20846	59.9 %	53.8 %
	OfTre-2	ABO20845	56.0 %	67.5 %
Nilaparvata lugens	*NlTre-1*	ACN85420	60.9 %	54.4 %
	NlTre-2	ACN85421	54.2 %	72.9 %
Bemisia tabaci	*BtTre-1*	AFV79626	57.1 %	50.4 %
	BtTre-2	AFV79627	57.7 %	70.4 %
Apolygus lucorum	*AlTre-1*	AGK89789	58.3 %	65.0 %
	AlTre-2	AGL34007	58.0 %	71.2 %

BlTre-2 Expression in Different Tissues and at Different Chronological Ages

The qRT-PCR results detected the expression of *BlTre-2* in 10 tissues and at various chronological ages of *B. lantschouensis*. The *BlTre-2* had the highest expression levels in the ovary, followed by the midgut, antennae, muscle, Malpighian tubule, integument, wings, head, and fat bodies, with the lowest expression level in legs (Fig 4A). The *BlTre-2* gene expression in the larval stage is higher than that in the pupal stage (P = 0.011). In addition, analysis of different chronological ages revealed that *BlTre-2* had the lowest expression in 0-day-old workers, and highest expression in 30-day-old workers. From day 0 to 15, the gene expression increased gradually (Fig 4B).

BlTre-1 and *BlTre-2* Expression at 45°C and During Starvation

The expression of *BlTre-1* increased as temperature treatment time increased, reaching the highest level at 3 h, and then declined progressively (Fig 5A). *BlTre-2* expression declined with treatment time in the first 3 h, and then it increased at the 4 h time point (Fig 5B). Overall, the results showed that in the 45°C treatments, the change of expression patterns differ between *BlTre-1* and *BlTre-2*. In the starvation experiment, both *BlTre-1* and *BlTre-2* had the highest expression levels in adult bees starved for 16 and 20 h, as compared to other time points (Fig 5C, D). The expression levels of *BlTre-1* in bees starved for 4 to 12 h and 24 h did not differ significantly from that of bees starved for 0 h (Fig 5C). However, the expression level of *BlTre-2* in starved adult *B. lantschouensis* was higher than that in individuals starved for 0 h, and its expression increased with starvation time.

Fig 1. Nucleotide and deduced amino acid sequences of *BlTre-2*. The numbers on the left are the positions of nucleotides and amino acids in the sequences; underlined amino acid residues represent the signal peptide; the cleavage site is indicated by an arrow; the N-glycosylation sites are indicated by a box; the highly conserved glycine-rich region is shaded gray and the trehalase signature motif is shaded red; transmembrane domains are gray and boxed.

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1 CATCGTTATCTTGCGAATTCGAAGCAAAAGAGGAACTTGTTAAAAGAAGGGAAGTAA
58 AGGAGACAAGAGAGAAGAAGAGGGATTGTGTGAAAGGGTCGACCGGTGGAGAAAAAA
115 atggcttggagctgcacgcgctgcggttcgacgaatatgctgctgagtgctgcgttcctc
1  M  A  W  S  C  T  R  C  G  S  T  N  M  L  L  S  A  A  F  L
175 gcgcttctcgtcgttgctccgtgttacgctagcacagagaaggcaagctacgtgaaaccg
21  A  L  L  V  V  A  P  C  Y  A  S  T  E  K  A  S  Y  V  K  P
235 cctccgtgtcagagcgatatttactgccatggcgagctgctgcacacgatacagatggcc
41  P  P  C  Q  S  D  I  Y  C  H  G  E  L  L  H  T  I  Q  M  A
295 tcgatctacaaggactcgaagacgttcgtcgacatgaagatgaaattctcgccgaacgag
61  S  I  Y  K  D  S  K  T  F  V  D  M  K  M  K  F  S  P  N  E
355 acgctgctcctatttcgcgaattcatggaaagcgtgaatcaaacaccgaccaggaaccag
81  T  L  L  L  F  R  E  F  M  E  S  V  N  Q  T  P  T  R  N  Q
415 atcgaacaattcatcaacaacacgttcgaccaagaaggatccgagttcgaggaatggaac
101 I  E  Q  F  I  N  N  T  F  D  Q  E  G  S  E  F  E  E  W  N
475 ccagtggactggaccagccaaccgaagtttcttaacaaaatccacgatcacgatcttcgc
121 P  V  D  W  T  S  Q  P  K  F  L  N  K  I  H  D  H  D  L  R
535 aaatttgcctctgatttgaaccaaatttggaaaatgttgggacgaaagatgaaagacgac
141 K  F  A  S  D  L  N  Q  I  W  K  M  L  G  R  K  M  K  D  D
595 gtgcgggtcaacgaggatcgatattccatcatctacgtgccgaatccggtgatcgtgccc
161 V  R  V  N  E  D  R  Y  S  I  I  Y  V  P  N  P  V  I  V  P
655 ggcggccgattccgcgagttctactactgggactcgtactggatcgtgaaagggctgctg
181 G  G  R  F  R  E  F  Y  Y  W  D  S  Y  W  I  V  K  G  L  L
715 ctttcggagatgtacaccaccgtcaaaggaatgttaaccaatttcgtctctctggtggac
201 L  S  E  M  Y  T  T  V  K  G  M  L  T  N  F  V  S  L  V  D
775 aagatcggtttcatcccgaacggaggcagaatctactacgctaggagatctcagcctccc
221 K  I  G  F  I  P  N  G  G  R  I  Y  Y  A  R  R  S  Q  P  P
835 atgttgattcctatggtcgaagagtatctgaaggtgaccatcgactacaaatgcctggag
241 M  L  I  P  M  V  E  E  Y  L  K  V  T  I  D  Y  K  C  L  E
895 gataaccttcaccttctagagaaggagtttgaattttggatgaccaataggacggtggac
261 D  N  L  H  L  L  E  K  E  F  E  F  W  M  T  N  R  T  V  D
955 gttgaagtggatggagtgaagtacactttagccagattcttcgaggagtcttcgggacct
281 V  E  V  D  G  V  K  Y  T  L  A  R  F  F  E  E  S  S  G  P
1015 cgaccagaatcctacaaagaggattacctgaccagccaaagttttcgcacgaacgaagag
301  R  P  E  S  Y  K  E  D  Y  L  T  S  Q  S  F  R  T  N  E  E
1075 aaggacaactattacgcggaattgaagaccgcggccgagtccggctgggacttttctagt
321  K  D  N  Y  Y  A  E  L  K  T  A  A  E  S  G  W  D  F  S  S
1135 cgatggttcatactagacggcacgaacaaaggtaacctgacgaacttgaaaacgagatac
341  R  W  F  I  L  D  G  T  N  K  G  N  L  T  N  L  K  T  R  Y
1195 attgtccccgtggacttgaattcgataatatatcgaaacgcgcagctgctagaacagtac
361  I  V  P  V  D  L  N  S  I  I  Y  R  N  A  Q  L  L  E  Q  Y
1255 aatcaaaggatgggcaacgagaccaaggccgcgtattaccggaaaagagcggaggactgg
381  N  Q  R  M  G  N  E  T  K  A  A  Y  Y  R  K  R  A  E  D  W
1315 aaaagagcggtcacggccgtactgtggcacgatgaagtcggtgcttggctcgactacgat
401  K  R  A  V  T  A  V  L  W  H  D  E  V  G  A  W  L  D  Y  D
1375 ttactgaacgacatcaaaagagattatttttatccgacgaacgttctgccgctttggacc
421  L  L  N  D  I  K  R  D  Y  F  Y  P  T  N  V  L  P  L  W  T
1435 gattgttacgacatcgcaaagagagaggaatacatagcgaaggtgctcaagtatctagag
441  D  C  Y  D  I  A  K  R  E  E  Y  I  A  K  V  L  K  Y  L  E
1495 aaaaatcaaataatgttaaatttgggcggtataccgaccaccctcgaacactctggtgaa
461  K  N  Q  I  M  L  N  L  G  G  I  P  T  T  L  E  H  S  G  E
```

Fig 1. Nucleotide and deduced amino acid sequences of *BlTre-2*. (Continuation)

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1555 caatgggattacccgaatgcctggccgcccttgcaatactttgtcatcatgtcgttgaat
481  Q  W  D  Y  P  N  A  W  P  F  L  Q  Y  F  V  I  M  S  L  N
1615 aacaccggagacccgtgggcgcagaggctcgcctacgagatcagccaacgatgggttcgc
501  N  T  G  D  P  W  A  Q  R  L  A  Y  E  I  S  Q  R  W  V  R
1675 agcaactggaaggcgttcaacgagacgcacagcatgttcgagaagtatgacgccacggta
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1735 tcaggcggtcacggaggtggcggtgagtacgaggtgcaactaggtttcggttggagcaac
541  S  G  G  H  G  G  G  G  E  Y  E  V  Q  L  G  F  G  W  S  N
1795 gggatcatcatggacttgctgaacaagtacggagatagactgacagccgaaattttcctc
561  G  I  I  M  D  L  L  N  K  Y  G  D  R  L  T  A  E  I  F  L
1855 gccatagtgcagagcttggcccctccagccgtcgtcgtttcgaccgccggtcaagtgatg
581  A  I  V  Q  S  L  A  P  P  A  V  V  V  S  T  A  G  Q  V  M
1915 accggtattctcgccctcgtaatatcgttggccgcgggattcatcggaatggtggtttac
601  T  G  I  L  A  L  V  I  S  L  A  A  G  F  I  G  M  V  V  Y
1975 aaaaggcgacactactatgttcctggaccatcgacgatgccaaacaagagaaaagtgatc
621  K  R  R  H  Y  Y  V  P  G  P  S  T  M  P  N  K  R  K  V  I
2035 tcaccgaccggaaacgtttatcgaaagaggatcgcctacactgaattgaaggacatgaac
641  S  P  T  G  N  V  Y  R  K  R  I  A  Y  T  E  L  K  D  M  N
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661  N  D  *
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3715 TGCATCTTCCGCTAATCGCGATCGATCGCCGGCCGACGAGCATCGACCAACGAGCACCGC
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3895 CGAGTGAACGAAAAATCGGTCGAGAGCCGACGAAGAATGATCGCGGTTGATCCTATGAGG
3955 AAATTTACCGCATCACGAAGGATGACGACGCGGTCGGATGGACTCGATCGTTAGCTGCGA
4015 CTAAAAAAAAAAAAAAAAAACCTATAGTGAAATCACT
```

Fig 2. Comparison of the amino acid sequences of *Tre* in *B. lantschouensis* with those in other orthologs. Letters on the left and numbers on the right are the gene names and positions of amino acids on the sequences, respectively. The conserved and similar amino acid residues are labeled in black and gray backgrounds, respectively. The highly conserved glycine-rich regions of trehalase are indicated by a double underline.

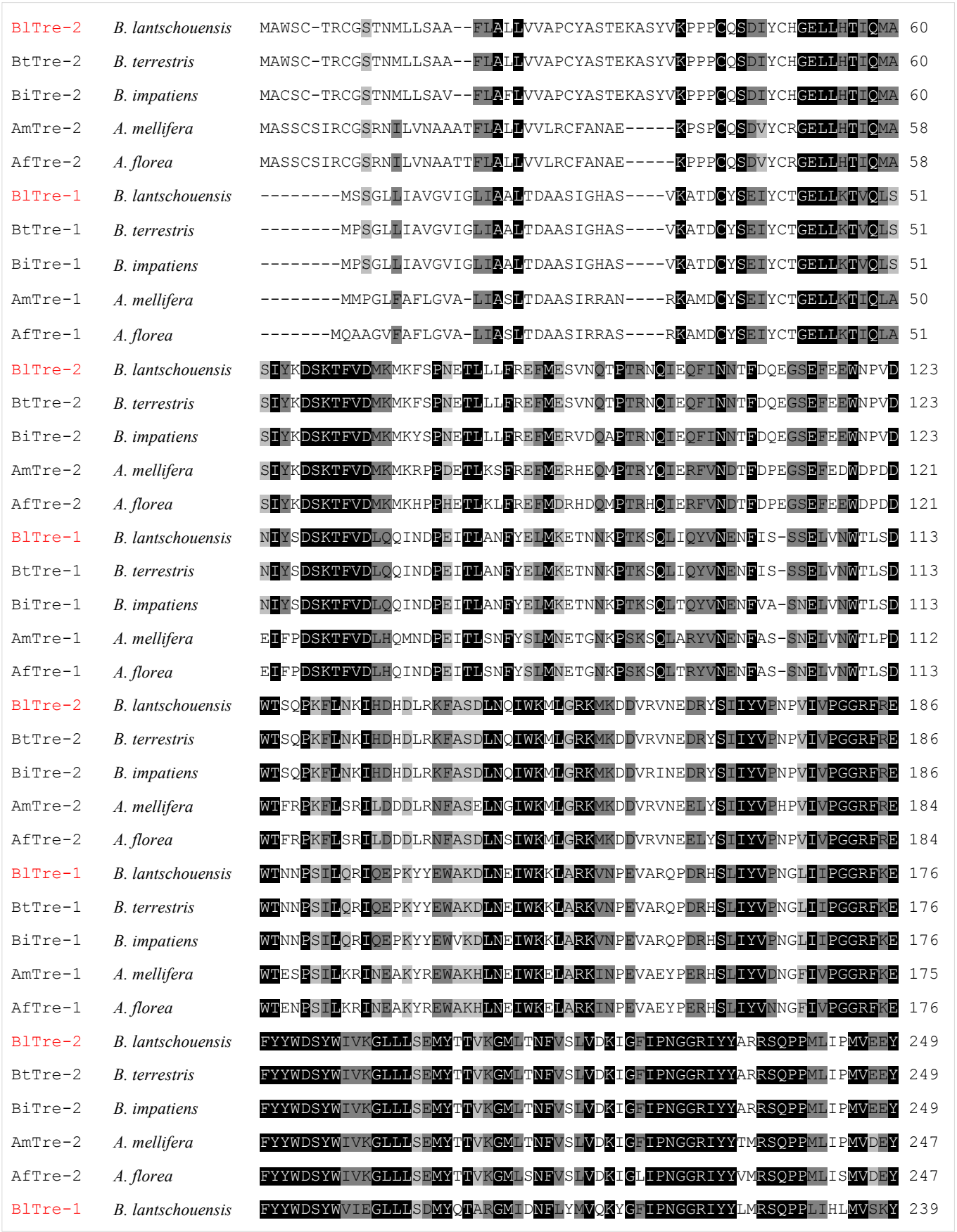


Fig 2. Comparison of the amino acid sequences of *Tre* in *B. lantschouensis* with those in other orthologs. (Continuation)

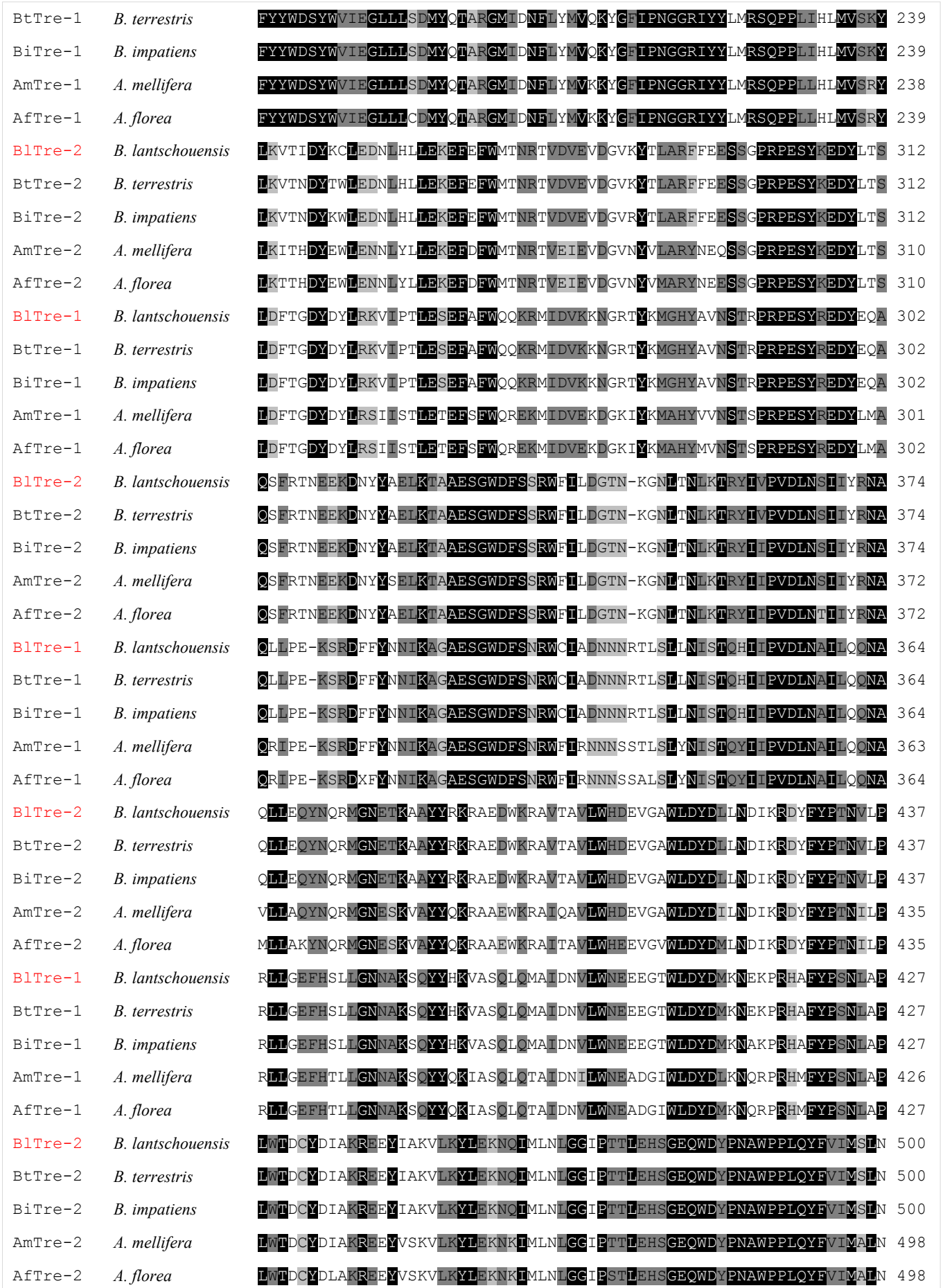
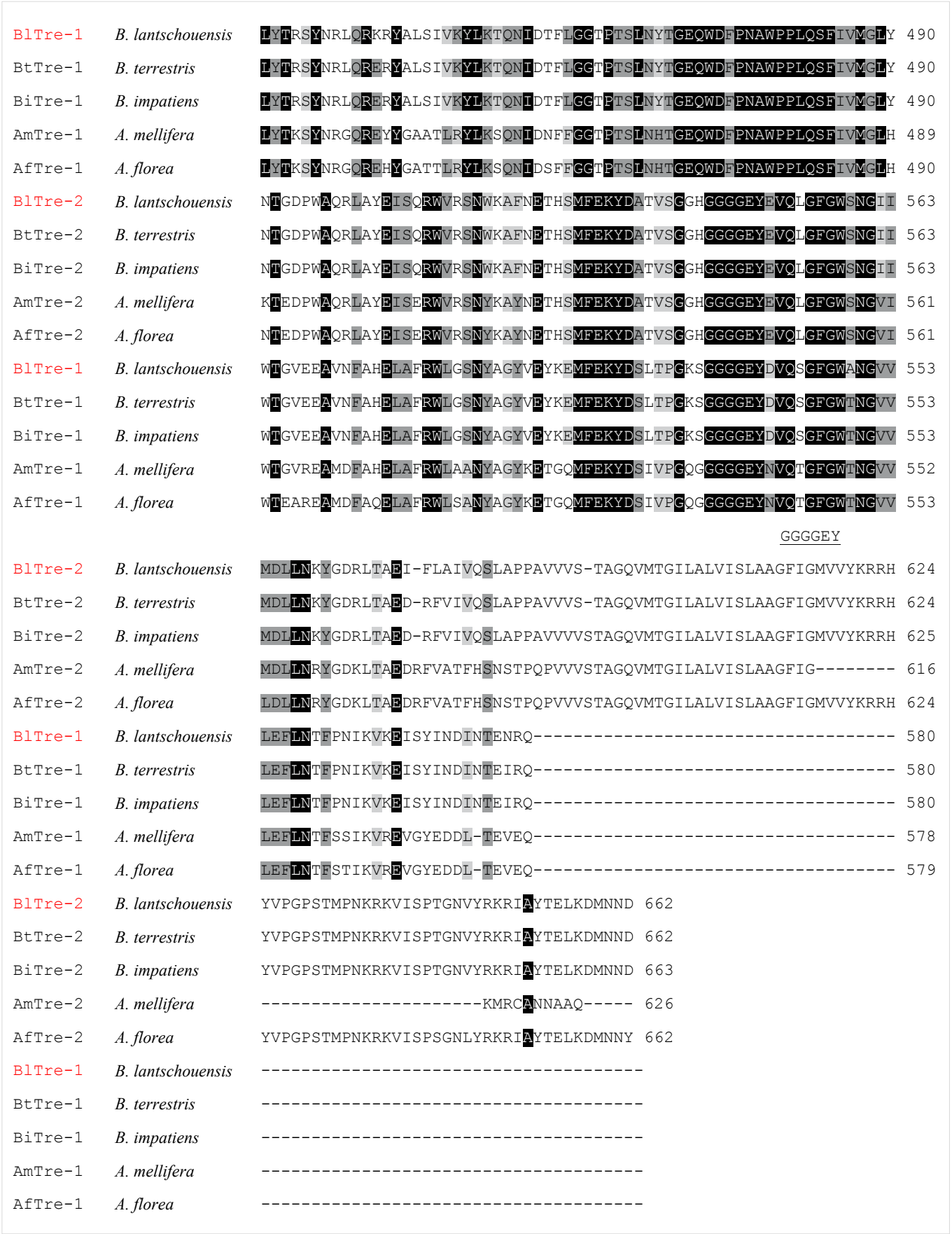


Fig 2. Comparison of the amino acid sequences of *Tre* in *B. lantschouensis* with those in other orthologs. (Continuation)



Discussion

In our study, we cloned the *BlTre-2* gene from *B. lantschouensis* using the homologous cloning and RACE techniques. The deduced amino acid sequence shared similarities with the *Tre-1* and *Tre-2* genes from various species, including a signal peptide leader, a glycine-rich region (GGGGEY), two signature motifs (PGGRFREFYYWDSY and QWDYPNAWPP), and putative N-glycosylation sites (Santos et al., 2012). Functions of the most conservative residues and regions remain unknown. Additionally, only one transmembrane domain has been found in most insects, including *B. mori* (Mitsumasu et al., 2005), *Nasonia vitripennis* (Tang et al., 2012), *N. lugens* (Gu et al., 2009), *O. fuscidentalis* (Tatun et al., 2008), *S. exigua* (Chen et al., 2010), *T. castaneum* (Tang et al., 2012), *L. migratoria* (Liu et al., 2016), and *C. medinalis* (Tian et al., 2016). However, the *BlTre-2* gene contained two putative transmembrane domains, MLLSAAFLALLVVAPCYAS and QVMTGILALVISLAAGFIGMVVY (Fig 1), which are like those of *A. mellifera* (Lee et al., 2007), *Laodelphax striatellus* (Zhang et al., 2010), *Spodoptera frugiperda* (Silva et al., 2009),

Fig 3. Phylogenetic analysis of trehalase amino acid sequences from various species. *B. lantschouensis* (*BlTre-1*: KJ025078); *B. terrestris* (*BtTre-1*: XP_003400853; *BtTre-2*: XP_003393687); *B. impatiens* (*BiTre-1*: XP_003491166; *BiTre-2*: XP_003490073); *A. florea* (*AfTre-1*: XP_003695047; *AfTre-2*: XP_003696950); *A. mellifera* (*AmTre-1*: XP_393963; *AmTre-2*: BAF81545); *Acyrthosiphon pisum* (*ApTre-1*: XP_001956264; *ApTre-2*: XP_001949459); *Laodelphax striatella* (*LsTre-1*: AFL03409; *LsTre-2*: AFL03410); *A. lucorum* (*AlTre-1*: AGK89789; *AlTre-2*: AGL34007); *B. mori* (*BmTre-1*: NP_001037458; *BmTre-2*: NP_001036910); *S. frugiperda* (*SfTre-1*: ABE27189; *SfTre-2*: ACF94698); *L. migratoria* (*LmTre-1*: ACP28173); *T. molitor* (*TmTre-1*: AGO32658); *Megachile rotundata* (*MrTre-1*: XP_003705482).

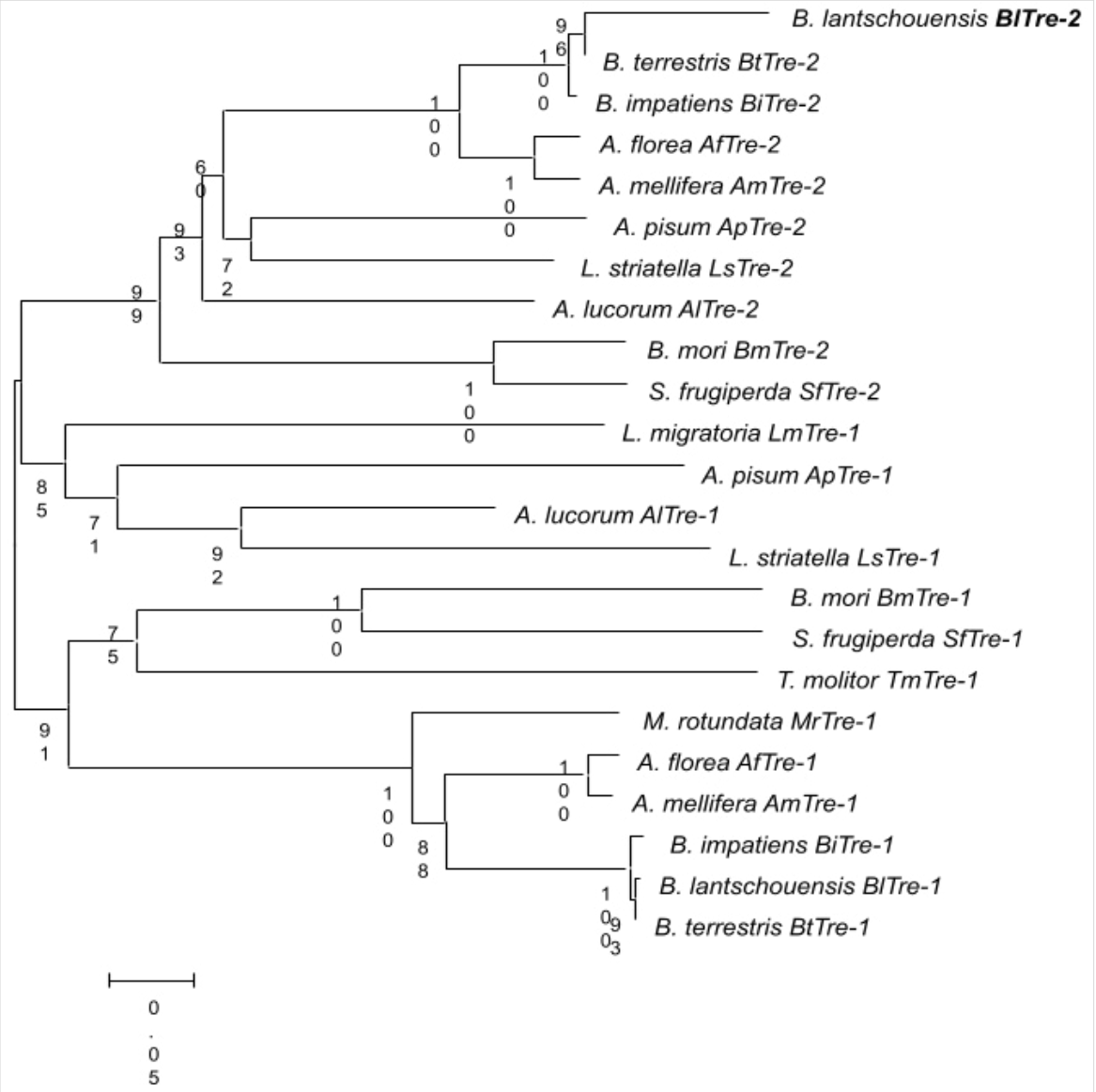
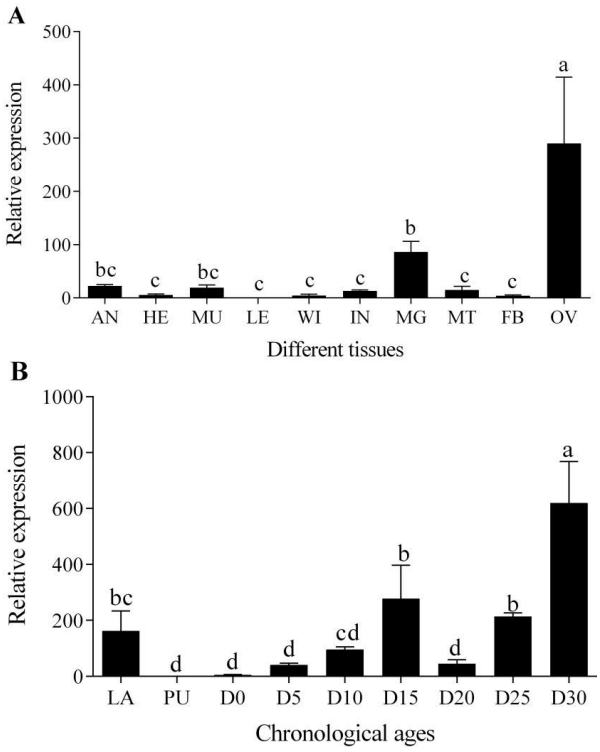


Fig 4. The relative expression levels of *BlTre-2* in different tissues (A) and chronological ages (B) of *B. lantschouensis*. AN: Antennae; HE: Head; MU: Muscles; LE: Legs; WI: Wings; IN: Integument; MG: Midgut; MT: Malpighian tubules; FB: Fat body; OV: Ovary; LA: Larva; PU: Pupa; D0-D30: Day 0 to day 30 worker. Each value represents mean ± S.D., and different letters above bars indicate a significant difference (p-0.05).

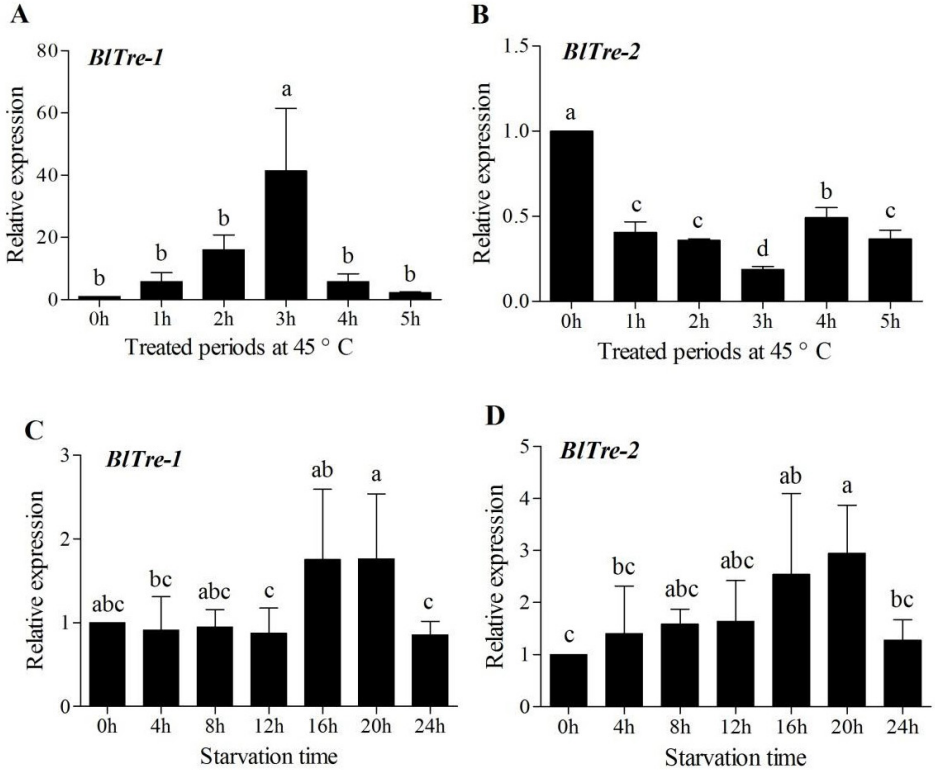


and *Aedes aegypti* (Tang et al., 2012). However, previous research suggested that *BlTre-1* gene have no transmembrane domain (Qin et al., 2015). In this study, although the proteins encoded by *BlTre-2* gene showed obvious similarity to *B. terrestris* (98.9%), *B. impatiens* (97.5%), and *A. mellifera* (84.2%), the protein encoded by *BlTre-2* gene only showed 55.7% similarity to those encoded by *BlTre-1* gene (Table 3).

In insects, *Tre-1* and *Tre-2* are involved in many physiological processes (Mitsumasu et al., 2005; Shi et al., 2019). Su et al. (1994) indicated that *Tre-2* can help transport sugars into oocytes of *B. mori*. Furthermore, it has been shown that trehalose, glycogen, and glucose can be stored in growing oocytes during the period of vitellogenesis of *Rhodnius prolixus*, (Santos et al., 2012). Similarly, in *B. tabaci* and *R. prolixus*, the expression levels of *Tre-2* were much higher in the ovary than in other tissues (Santos et al., 2012; Wang et al., 2014). In our study, the *BlTre-2* gene had the highest expression levels in the ovary and then in the midgut of *B. lantschouensis* (Fig 4A). These findings suggest that the *BlTre-2* gene may play an important role in providing materials and energy for oocyte development in *B. lantschouensis*.

The *Tre-2* gene is distributed differently in different tissues in insects, and this may be linked to its function. Previous studies have shown that the *Tre-2* gene has higher expression in the integument of *Spodoptera litura* (Zou et al., 2013), the wing bud of *N. lugens* (Zhang et al., 2017), and the gut of *S. exigua* (Tang et al., 2008) and *Bactrocera dorsalis* (Xie et al., 2013) as compared with other tissues.

Fig 5. The relative expression levels of two trehalase genes under adverse conditions. The *BlTre-1* and *BlTre-2* relative expression in workers which were exposed to 45°C ambient temperature for 0, 1, 2, 3, 4 and 5 h (A, B). The *BlTre-1* and *BlTre-2* relative expression in workers which were starved for 0, 4, 8, 12, 16, 20 and 24 h (C, D). Each value represents the mean ± S.D., and different letters above the bars indicate significant differences (p-0.05).



In *B. lantschouensis*, the *BlTre-2* gene may be involved in providing energy to the chitin synthesis process in the midgut or in supporting peristaltic movement of the midgut. These results suggest that the *BlTre-2* gene may perform specific functions in different tissues.

Our study found, for the first time, that *BlTre-2* has the highest expression level in 30-day-old worker bees (Fig 4B), so we can assume that this expression level of *BlTre-2* is associated with the biological behavior of *B. lantschouensis*. Older workers may depend mainly on *BlTre-2* to break down extracellular trehalose (mainly from food). Next, the expression of *BlTre-2* was significantly higher in the larvae than in the pupae. It is possible that *BlTre-2* expression is involved in the feeding and development of larvae. In *B. dorsalis*, *Tre-2* was found to be highly expressed in metabolic tissues at both the adult and larval stages (Xie et al., 2013). The results also revealed that expression of *BlTre-2* in 0- to 20-day-old workers first increased and then declined with age (Fig 4B). This result may be due to the social division of labor in *B. lantschouensis* colonies. Both younger and older workers are engaged in various activities, such as helping the queen secrete wax, nesting, and nursing, and strong workers take part in foraging and guarding.

Previous studies suggested that insect trehalase, including TRE-1 and TRE-2 play critical roles in energy supply, growth, metamorphosis, stress recovery, chitin synthesis, and flight by catalyzing the hydrolysis of trehalose to glucose in insects (Wyatt, 1967; Thompson, 2003; Shukla et al., 2014). Insects adapt to changes in environmental temperature and maintain their energy by regulating their own body temperature. A high temperature not only breaks the moisture balance of the internal environment and interferes with normal metabolism but it also causes body temperature to rise and affects enzyme activity and protein function in insects (Du et al., 2007). Previous studies showed that trehalase optimizes temperature in the range of 40–65°C (Zou et al., 2013; Shukla et al., 2014; Youngjin & Yonggyun, 2017). At a high temperature, the parasitic nematode *Aphelenchoides besseyi* improves its resistance by upregulating the trehalase gene (Chen et al., 2016). In our study, the expression levels of *BlTre-1* and *BlTre-2* were, respectively, higher and lower than at 0 h under the 45°C treatment conditions. We hold the opinion that the activity of soluble trehalase was high at 45°C because trehalose was gradually hydrolyzed into glucose and used for stress recovery. Bumblebees maintain vital activities by accumulating trehalose through soluble trehalase catalysis in such high-temperature conditions. By comparison, the expression level of *BlTre-2* was lower than that of *BlTre-1* for the 45°C treatment. This result suggests that *BlTre-1* may be involved in providing energy for physiological activity and that *BlTre-2* expression maybe constrained at a high temperature.

Trehalose is a feedback-regulating substance involved in the feeding behavior and nutrient intake of insects (Wyatt, 1967). Trehalose provides energy when insects are starving; thus, it has a role in the regulation of insect functions under starvation conditions (Tang et al., 2014; Youngjin & Yonggyun, 2017). In *H. axyridis* adults, the stored food reserves can provide energy to sustain vital activities for 8 h, but energy limitations have a direct impact on the desire to find food (Tang et al., 2014). Our results showed no significant difference in the expression levels of both *BlTre-1* and *BlTre-2* in *B. lantschouensis* adults starved for 4 to 12 h as compared with those starved for 0 h, which may be because the stored food reserves were able to provide energy to sustain vital activities for 12 h in adults. In addition, the expression levels of *BlTre-1* and *BlTre-2* increased in adults starved for 16 to 20 h, which suggests that trehalose stores were degraded by trehalase.

BlTre-1 and *BlTre-2* function to facilitate the uptake and utilization of trehalose from blood and food, respectively (Yaginuma et al., 1996). Result of the present study show that *BlTre-1* is a key gene involved in regulating energy metabolism and providing glucose at a high temperature. *BlTre-1* and *BlTre-2* might balance trehalose and provide energy during periods of starvation.

Our study sheds light on the molecular function and gene expression of trehalase in *B. lantschouensis*, which adds further important information about the characteristics of this gene in the physiology and development of bumble bees in China. Different native populations in different regions of China may display different types of adaptations to coldness, which is associated with trehalase expression. We provide new knowledge to assist future selection and breeding of *B. lantschouensis* in China. Furthermore, The *Tre-1* and *Tre-2* genes participate in energy metabolism during developmental and physiological activities in various insects, which provides a reference for the protection and utilization of pollination insects.

Acknowledgments

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Authors Contribution

JMQ: conceptualization, investigation, validation, methodology, writing.
FL: investigation, methodology.
JW: supervision, project administration.
SYH: supervision.
MI: review, resources.
WL: resources.
HML: writing.
SDL: conceptualization, funding acquisition, supervision, project administration, writing.

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