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Identification and Characterization of the Detoxification Genes Based on the Transcriptome of *Tomicus yunnanensis*

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Abstract: Bark beetle, as a trunk borer, has caused a large number of tree deaths and seriously damaged the mountain forest ecosystem. Bark beetles oxidize the secondary metabolites of plants, degrade them, and excrete them from the body or convert them into components needed by the body. This process is completed by the cooperation of CYPs, GSTs, and CCEs and occurs in different tissues of the insects, including the gut (i.e., the part where beetle pheromone is produced and accumulated) and antennae (i.e., the olfactory organ used to sense defensive monoterpenes and other plant-related compounds and pheromones in the air). In this study, we identified and characterized three gene superfamilies of CYPs, GSTs, and CCEs involved in the detoxification of endobiotics (e.g., hormones and steroids) and xenobiotics (e.g., insecticides, sex pheromones, and plant allelochemicals) through a combination approach of bioinformatics, phylogenetics, and expression profiles. Transcriptome analyses led to the identification of 113 transcripts encoding 51 P450s, 33 GSTs, and 29 CCEs from *Tomicus yunnanensis* Kirkendall and Faccoli, 2008 (Coleoptera, Scolytinae). The P450s of *T. yunnanensis* were phylogenetically classified into four clades, representing the majority of the genes in the CYP3 clan. The CCEs from *T. yunnanensis* were separately grouped into five clades, and the GST superfamily was assigned to five clades. Expression profiles revealed that the detoxification genes were broadly expressed in various tissues as an implication of functional diversities. Our current study has complemented the resources for the detoxification genes in the family Coleoptera and allows for functional experiments to identify candidate molecular targets involved in degrading plants' secondary metabolites, providing a theoretical basis for insect resistance in mixed forests.

Keywords: cytochrome P450; glutathione S-transferase; carboxylesterases; bark beetles; gene expression

Citation: Li, W.; Yang, B.; Liu, N.; Zhu, J.; Li, Z.; Ze, S.; Yu, J.; Zhao, N. Identification and Characterization of the Detoxification Genes Based on the Transcriptome of *Tomicus yunnanensis*. *Diversity* **2022**, *14*, 23. https://doi.org/10.3390/d14010023

Academic Editor: Luc Legal

Received: 27 November 2021
Accepted: 29 December 2021
Published: 31 December 2021

1. Introduction

To withstand the ingestion of insects, plants have developed a defense mechanism against insects through various methods such as morphology, biochemistry, and molecular regulation in the long-term evolution process [1]. The production of secondary metabolites in plants is a way of defending themselves against insects. Various secondary metabolites in plants can affect the feeding and food utilization of insects, and toxic secondary metabolites can lead directly to insect death or stunted growth [2,3].

However, in the long-term evolutionary process, insects are constantly adapting to plant defense mechanisms. Genetic variation in morphology, including polymorphic wing growth, and differentiation of different types of mouthparts, such as chewing, stabbing, and sucking [4]. Insects can also use the enzymatic detoxification system, which mainly includes cytochrome P450 monooxygenases (CYPs), glutathione S-transferases (GSTs), and

carboxylesterases (CCEs), to improve the ability of insects to metabolize secondary substances of plants, and cause insects to adapt to the defense mechanism of the host plant [5].

CYPs are a very large and diverse group of enzymes found in all living things. They constitute a very important system involving endogenous compounds and xenobiotics such as drugs, pesticides, secondary plant metabolites, mutagens, and hormones [6,7]. In addition, CYPs can reflect different levels of phylogenetic information such as subtypes, population, and species. It is a good molecular marker to explore the phylogenetic relationship between populations and population genetic diversity [8,9]. According to the similarity of amino acids, the CYPs of insects can be divided into four clades: CYP2, CYP3, CYP4, and mitochondrial CYP clades [10,11]. When exposed to toxic plant compounds, insects will induce reactions to other exogenous substances. For example, CYP6AE14 in *Helicoverpa armigera* Hübner, 1809 (Lepidoptera, Noctuidae) is involved in the metabolism of gossypol (plant toxins) [12]. GST is a dimeric protein, which plays an important role in intracellular metabolism, chemical cycle, and body defense [13]. Insect GST can be divided into six categories (delta, epsilon, omega, sigma, theta, and zeta). Among them, the delta and epsilon subtypes are unique to insects, and some GSTs play an important role in plant antitoxin [14,15]. In research on *Dendroctonus armandi* Tsai and Li, 1959 (Coleoptera, Scolytinae), it is assumed that DaGSTe1 catalyzes the combination of glutathione with terpenes and phenolic substances from *D. armandi*, so that toxic substances are transported out of the cell, thereby reducing the damage of exogenous toxins from the host to *D. armandi* [16]. In addition to CYPs and GSTs, another detoxification enzyme is CCE. Insect CCEs belong to the carboxyl/cholinesterase family, a branch of the α/β-hydrolase fold superfamily in which enzymes hydrolyze ester bonds, which are present in many plant volatiles, insect pheromones, and hormones, as well as pesticides [17].

Tomicus yunnanensis Kirkendall and Faccoli, 2008 (Coleoptera, Scolytinae) is one of the main pests of *Pinus yunnanensis*, which is widely distributed in Yunnan, Sichuan, and Guizhou. The whole life cycle of *T. yunnanensis* is almost entirely on *P. yunnanensis* [18]. The damage to *P. yunnanensis* by *T. yunnanensis* can be divided into two periods: feeding on treetops and trunks. When feeding on the treetops, the adult worms feed on the pith tissues of the branches and supplement nutrients to complete their sexual development; when feeding on the trunk, the mature adult worms transfer from the treetop to the trunk and feed on phloem, lay eggs, and form mother tunnel and sub tunnel. Then, larvae develop under the bark, pupate, and emerge, and adults will burrow out of the phloem and return to the treetops to feed [19,20]. This concealed lifestyle brings many inconveniences to the study of its habits, prevention, and treatment. *T. yunnanensis* first broke out in central Yunnan Province in the 1980s, and then spread to 65 counties in 15 regions of Yunnan Province, infesting more than 200,000 ha of Yunnan pine forests and blighting more than 93,000 ha of *P. yunnanensis*, and seriously damaged the mountain forest ecosystem [21]. To mitigate the damage of this insect, some common measures can play a certain role for a short time, such as biological control, chemical control and tending, and mixed forest [22]. Among them, the mixed forest has achieved remarkable results in insect resistance and contributed to the protection of mountain forest ecosystem, but its anti-insect mechanism still needs further research. The identification of the detoxification genes of *T. yunnanensis* enriches the detoxification gene family in Coleoptera and provides data support for future research on the function of detoxification genes. We hope that the study can provide a theoretical basis for insect resistance in mixed forests.

2. Materials and Methods

2.1. Insect and Tissue Collection

The adults of *T. yunnanensis* used in this experiment were originally collected from Jiulong Mountain Forest Farm in Zhanyi County, Qujing City, Yunnan Province in June 2018. The antennae, heads, legs, and carcasses (excluding antennae, heads, and legs) of 200 pairs of male and female adults of *T. yunnanensis* were collected separately (three groups of

biological replicates). We quickly froze the collected samples with liquid nitrogen and stored them in a refrigerator at −80 °C. qPCR samples were treated the same way.

2.2. Total RNA Extraction and cDNA Synthesis

Total RNA samples of tissues were isolated using TRIzol Reagent according to the manufacturer's protocol (Ambion, Life Technologies, Carlsbad, CA, USA). The quality of RNA was confirmed using a NanoVue UV–vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and RNA integrity was verified using a standard 1% agarose gel electrophoresis. Genomic DNA was digested by treatment with DNase I (Fermentas, Thermo Fisher Scientific, USA). First-strand cDNA was synthesized with a first strand cDNA synthesis kit (TaKaRa, Dalian, Liaoning, China). The synthesized cDNA templates were stored at −20 °C.

2.3. Library Construction, Sequencing, and Functional Annotation

Oligosaccharide (DT)-containing magnetic beads were used to enrich the mRNAs in the total RNA of antennae, head, foot, and residue. Then, according to the protocol in NEBNext® Ultra™ RNA Library Prep Kit for llumina® (NEB, Ipswich, MA, USA), we cut them into short fragments with a fragment buffer. The first-strand cDNA was synthesized using this fragment as a template, and then the second-strand cDNA was synthesized by DNA polymerase I and RNaseH. After purification with AMPure XP beads, the second-strand cDNA was repaired, α-end ligated, and ligated with indexed adapters. The products of suitable size were selected, amplified by PCR, and purified with AMPure XP beads to establish a digital gene expression (DEG) library. Raw reads were processed through a rigorous filtering process to eliminate low-quality reads (base calling). The clean reads were then assembled using Trinity (v2.4.0) and then grouped using Corset (v1.0.5) to eliminate redundant data in the assembled transcripts. [23]. Databases used for annotation included the non-redundant nucleotide (Nt), non-redundant database (Nr), Swiss-Prot, Protein family (Pfam), Kyoto Encyclopedia of Genes and Genomes (KEGG) and Clusters of Orthologous Groups (COG/KOG). Gene ontology (GO) analysis was performed using blast2go (b2g4pipe_v2.5) software. Nr, Swiss-Prot, and COG/KOG were analyzed by diamond v0.8.22 software, and Nt was analyzed by NCBI blast v2.2.28+; KEGG was analyzed with KAAS r140224 and Pfam was analyzed with hmmscan HMMER 3. For the DEG analysis, the resulting clean reads were then mapped to the transcriptomic unigenes using RSEM software with default parameters [24]. The differential expression of different conditions/groups (genes and samples) was analyzed by DESeq2 software. The number of read counts normalized by TMM for each mapped gene was used to calculate gene expression levels following the FPKM (fragments per kilobase of transcript sequence per millions base) method [25,26].

2.4. Gene Identification and Sequence Analysis

To identify candidate detoxification genes from *T. yunnanensis*, detoxification gene families from other coleopteran species were selected as queries to search the new stand-alone transcriptome of this beetle. *Anoplophora glabripennis* Motschulsky, 1854 (Coleoptera, Lamiinae), *Lepeophtheirus salmonis* Kroyer, 1838 (Copepoda, Caligoida), *Dendroctonus ponderosne* Hopkins, 1902 (Coleoptera, Scolytidae), and *Tribolium castaneum* Herbst, 1797 (Coleoptera, Tenebrionidae) were used for CYPs; *D. ponderosae*, *A. glabripennis*, *Rhynchophorus ferrugineus* Oliver, 1790 (Coleoptera: Curculionidae), *D. armandi* and *T. castaneum* for GSTs, and *D. ponderosae*, *D. armandi*, and *T. castaneum* for CCEs (https://www.ncbi.nlm.nih.gov/ (accessed on 7 June 2021)). TBLASTN was used to search and identify candidate detoxification genes against the *T. yunnanensis* transcriptome, with an E-value cutoff of 1×10^{-5}. Further, these identified genes were verified using TBLASTX against the NCBI non-redundant protein sequences database. Open reading frames (ORFs) were identified using the ORF Finder in NCBI (https://www.ncbi.nlm.nih.gov/orffinder/ (accessed on 29 June 2021)). CYP names use the CYP prefix, followed by an Arabic numeral, designates the family (all

members nominally >40% identical), a capital letter designates the subfamily (all members nominally >55% identical), and an Arabic numeral designates the individual gene or message and protein [27]. In the set of trees, a multiple sequence alignment was performed using the Muscle method in MEGA7.0 [28]. An ML tree of candidate detoxification genes was constructed by Evoliview [29]. Accession numbers of all protein sequences from other Coleoptera species used in the phylogenetic analysis are listed in Supplementary Materials.

2.5. Quantitative Real-Time PCR

We used qRT-PCR to verify the expression of randomly selected candidate *T. yunnanensis* detoxification genes in different tissues. The cDNA was synthesized with an input of 1 μg of total RNA using the Prime ScriptRT Reagent Kit with gDNA Eraser to remove gDNA (AG, Changsha, China). For quantitative real-time PCR (qPCR), various tissues including antennae, heads (without antennae), legs, and carcasses from female and male adults were collected and immediately immersed in liquid nitrogen. qPCR was performed using SYBR Premix EX Taq™ (AG, Changsha, China) with three technical replicates of each template from three independent biological pools. For the qPCR analysis, the primers (Table S1) were designed by Primer Premier 5.0 [30]. Each reaction contained a total volume of 20 μL, consisting of 10 μL of SYBR Green PCR Master Mix, 0.8 μL of each primer (10 μM), 2 μL (20 ng) of cDNA template, and 6.4 μL of nuclease-free water. The β-actin gene was used as an endogenous control. qPCR cycling parameters were: 94 °C for 4 min followed by 40 cycles at 94 °C for 20 s and 60 °C for 30 s. Relative gene expression level was calculated using the Q-GENE statistical analysis package [31].

3. Results

3.1. Transcriptome Assembly

An Illumina HiSeq platform was used to sequence antennae, head, legs, and carcasses of adult female and male *T. yunnanensis* transcriptomes. We obtained 61.51 million (MA-1), 47.40 million (MA-2), and 52.92 million (MA-3) raw reads from the antennae of a male adult, 58.15 million (MH-1), 50.67 million (MH-2), and 61.09 million (MH-3) raw-reads from the head of a male adult, and there are raw readings from other organizations in Table 1. Filtering resulted in 58.28 million (MA-1), 46.26 million (MA-2), 51.60 million (MA-3), 55.80 million (MH-1), 49.30 million (MH-2), 59.09 million (MH-3), 55.93 million (ML-1), 53.53 million (ML-2), and 56.48 million (ML-3) clean reads (Table 1). The percentages of reads with Q20 and Q30 values for each library were approximately 98% and 93%, respectively. The GC content ranged from 38.55% to 52.60% (Table 1). The final transcript dataset contained 100,455 unigenes with a mean length of 877 bp and an N50 length of 1153 bp (Table 2), indicating the high quality of our assembly.

3.2. Functional Annotation of the Unigenes in T. yunnanensis

A total of 100,455 unigenes were annotated by searching against six databases using BLAST. Specifically, 60,067 (59.79%) unigenes were matched in the non-redundant (NR) database, which accounted for the largest match. Further, 54,782 (54.53%) were annotated in the nucleotide (NT) database, 50,102 (49.87%) were annotated by blasting against the Swiss Port database. Additionally, the euKaryotic Ortholog Groups (KOG) database had the lowest number of annotated unigenes, with 19,808 (19.71%) unigenes (Table 3). Species with the highest proportion of similar genes were *Nephila clavipes* (21.00%), followed by *Dendroctonus ponderosae* (19.2%), *Hyalella azteca* (8.0%), *Toxocara canis* (4.2%) and *Lucilia cuprina* (2.1%) (Figure 1).

Table 1. Summary of the transcriptome sequencing data from *T. yunnanensis*.

Sample	Raw Reads	Clean Reads	Q20 (%)	Q30 (%)	GC (%)
MA_1	615,114,60	582,887,10	97.95	94.15	47.57
MA_2	474,005,30	462,665,14	97.90	93.91	42.76
MA_3	529,246,96	516,082,96	98.06	94.27	42.81
MH_1	581,529,36	558,021,48	97.75	93.46	38.56
MH_2	506,735,06	493,021,74	97.96	94.00	38.74
MH_3	610,987,72	590,914,14	98.04	94.16	40.09
ML_1	582,201,60	559,355,36	98.04	94.29	45.54
ML_2	561,873,52	535,354,48	98.05	94.30	43.28
ML_3	587,793,32	564,878,02	97.88	93.93	42.65
MC_1	640,881,58	628,616,66	97.63	93.21	38.55
MC_2	651,352,06	633,407,70	97.87	93.79	39.15
MC_3	591,512,94	566,883,04	97.10	92.16	41.95
FA_1	523,288,02	510,649,40	98.11	94.44	46.17
FA_2	568,975,58	533,953,70	98.00	94.23	46.38
FA_3	535,300,88	500,120,04	98.00	94.24	46.14
FH_1	513,240,72	502,592,66	97.95	93.94	39.67
FH_2	558,425,54	547,795,96	97.81	93.65	39.48
FH_3	640,915,78	629,613,86	97.62	93.18	39.24
FL_1	586,049,54	572,336,12	97.83	93.72	43.91
FL_2	503,729,98	491,791,08	97.82	93.72	42.40
FL_3	435,955,04	420,544,90	97.94	94.19	52.60
FC_1	572,708,34	560,824,28	97.67	93.30	40.48
FC_2	533,416,24	517,611,84	97.87	93.78	41.24
FC_3	505,727,10	495,473,28	97.77	93.55	39.88

MA: male antennae, MH: male head, ML: male leg, MC: male carcasses, FA: female antennae, FH: female head, FL: female leg, FC: female carcasses.

Table 2. Summary of transcriptome splicing length distribution data from *T. yunnanensis*.

	Min Length	Mean Length	Max Length	N50	Total Nucleotides
Transcripts	154	1005	59,000	1465	184,171,785
Genes	301	877	59,000	1153	880,711,34

Table 3. Statistics of gene annotation success rate.

	Number of Genes	Percentage (%)
Annotated in NR	60,067	59.79
Annotated in NT	54,782	54.53
Annotated in SwissProt	50,102	49.87
Annotated in PFAM	53,205	52.96
Annotated in GO	53,205	52.96
Annotated in KOG	19,808	19.71
Annotated in all databases	1666	1.65
Annotated in at least one database	79,508	79.14
Total unigenes	100,455	100

Using a gene ontology (go) method, annotated genes were divided into three categories (a total of 56 functional groups): biological process, cell composition, and molecular function. Among the biological processes, the subcategories cellular process, metabolic process, and single-organism process contained the most unigenes. In the cellular component class, the subcategories cell and cell part contained the most unigenes. Binding and catalytic activity were the most numerous subcategories in the "molecular function" category (Figure 2a). For the euKaryotic Ortholog Groups (KOG) functional classification, we annotated about 19,808 unigenes and divided them into 25 molecular families (Figure 2b). Among them, the largest category was the general function prediction only, followed by amino acid transport and metabolism and energy production and conversion. Cell motility

and nuclear structure were the smallest groups (Figure 2b). A KEGG analysis was used to classify the annotated genes into different KEGG pathway functional categories (Figure 2c). The most representative pathways were amino acid metabolism, carbohydrate metabolism, overview, and signal transduction (Figure 2c).

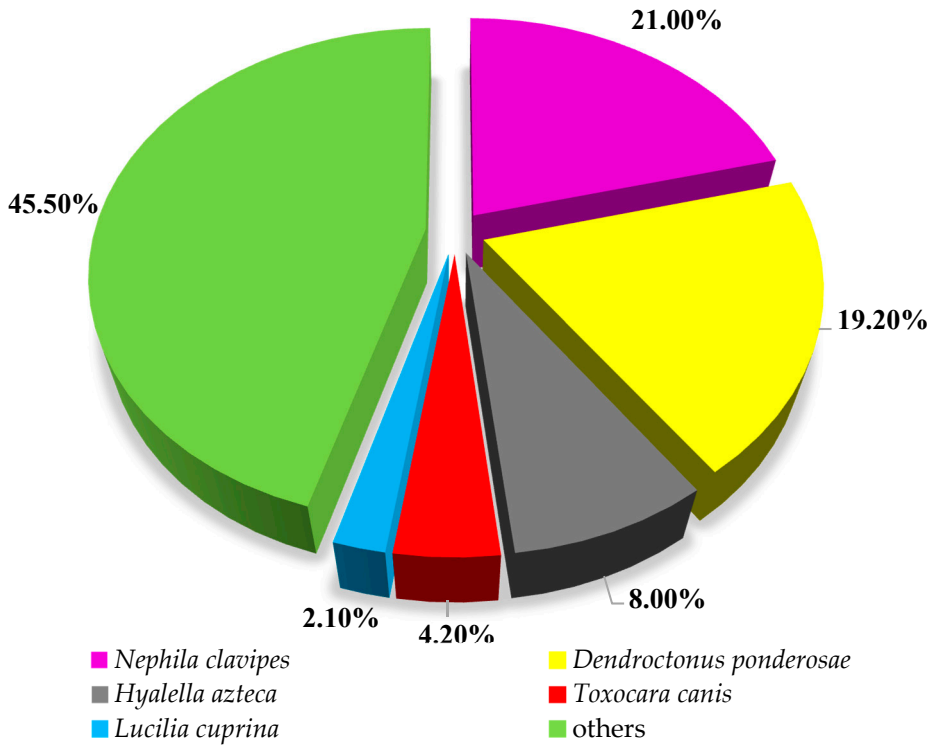

Figure 1. The unigene BLASTx searches against the Nr database for species distribution analysis.

3.3. Identification of Candidate CYPs, GSTs, and CCEs

In this study, a total of 51 predicted CYPs transcripts were identified from the transcriptomes of different issues of *T. yunnanensis* using the BLASTx program. The sequence identities of these candidate CYPs with other Coleoptera insects ranged from 32.69% to 96.54% in the NCBI database (Table 4). According to the CYP nomenclature, we classified 51 CYP sequences into four families (CYP2, CYP3, CYP4, mitochondrial CYP). After removing 25 short sequences (aa < 390) [27], 26 sequences with complete open reading frames were used to construct a phylogenetic tree (Figure 3). The phylogenetic tree showed that TyunCYP had a high homology with DponCYP. The largest family was the CYP3 family, which included 13 members and the CYP3 family contained two subfamilies, CYP6 and CYP9. The results of the phylogenetic tree show that five CYPs (TyunCYP6BW2, TyunCYP6DE1, TyunCYP6DF1, TyunCYP6BX1, TyunCYP6DJ1) belong to the CYP6 subfamily. The second largest family is the CYP4 family; we identified eight genes (TyunCYP393A1, TyunCYP6BK1, TyunCYP411A1, TyunCYP349B2, TyunCYP4CV1, TyunCYP4BQ1, TyunCYP4G2, TyunCYP4BG1) belonging to the CYP4 family.

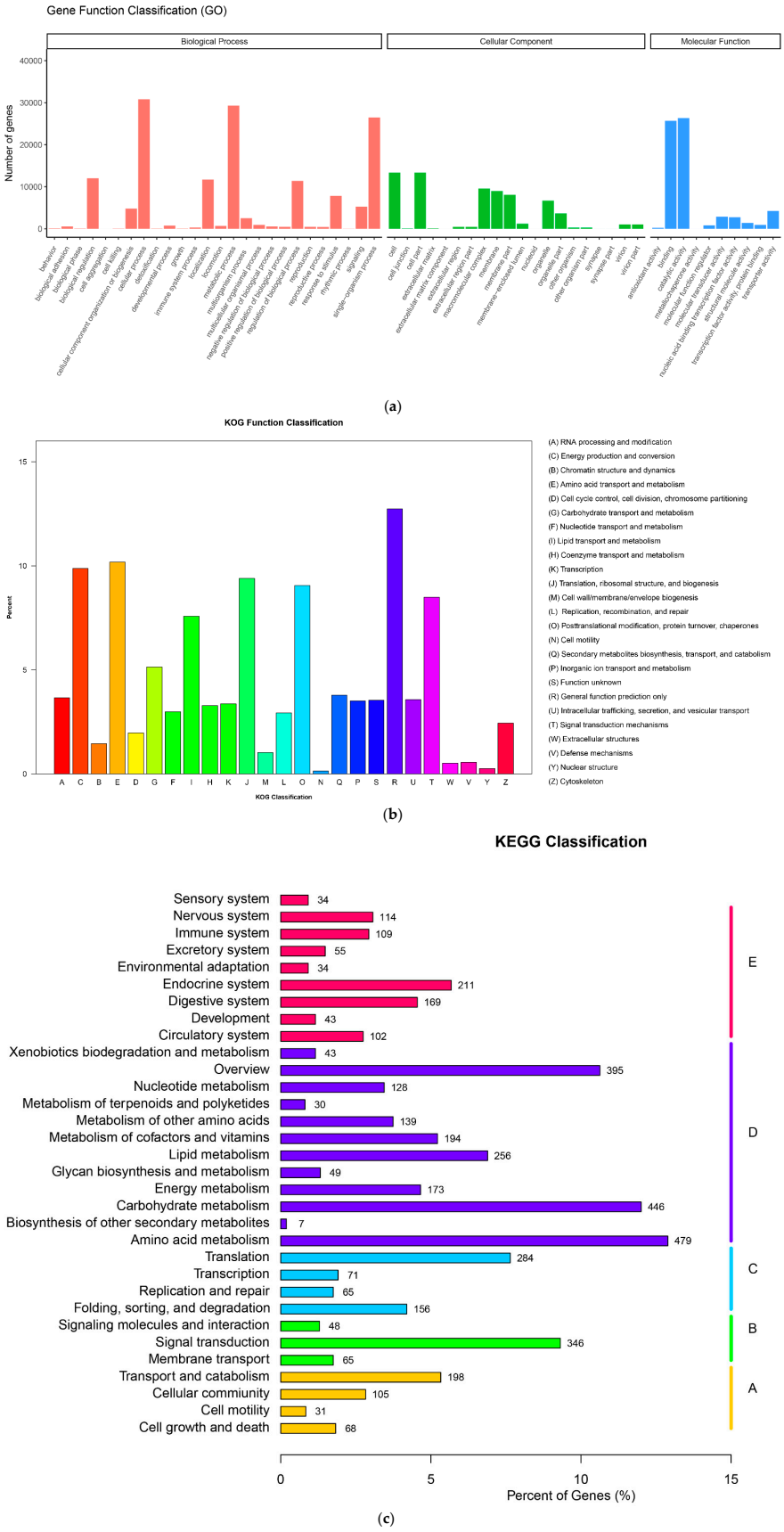

Figure 2. Results of BLASTx matches of *T. yunnanensis* transcriptome unigenes, gene ontology, KOG classification, and KEGG pathway annotation. (**a**) Gene ontology classifications of *T. yunnanensis* unigenes. (**b**) KOG classifications of *T. yunnanensis* unigenes. (**c**) KEGG classification of *T. yunnanensis* unigenes.

Table 4. Best BLASTX matches of *T. yunnanensis* CYPs.

Gene Name	Gene ID	aa	E-Value	Identity	Accession	Species Name
TyunCYP349A1	i2_LQ_TYUN_c39805/f1p0/2021	60	1×10^{-20}	65.00%	AEL88544.1	*Dendroctonus rhizophagus*
TyunCYP9Z1	i2_LQ_TYUN_c22584/f1p0/2717	145	5×10^{-82}	82.64%	AEL88550.1	*Dendroctonus rhizophagus*
TyunCYP314A1	i1_LQ_TYUN_c152368/f1p0/1050	147	6×10^{-78}	83.33%	AFI45004.1	*Dendroctonus ponderosae*
TyunCYP347B1	i4_LQ_TYUN_c2131/f1p0/4666	447	0	81.94%	AFI45010.1	*Dendroctonus ponderosae*
TyunCYP410C1	Cluster-27834.11928	456	1×10^{-107}	39.43%	AFI45013.1	*Dendroctonus ponderosae*
TyunCYP4CV1	Cluster-27834.3883	507	0	77.47%	AFI45020.1	*Dendroctonus ponderosae*
TyunCYP6BW1	i3_LQ_TYUN_c10935/f1p0/4006	283	9×10^{-180}	85.16%	AFI45024.1	*Dendroctonus ponderosae*
TyunCYP6BW2	i1_HQ_TYUN_c416/f4p1/1768	506	0	86.76%	AFI45026.1	*Dendroctonus ponderosae*
TyunCYP6BX1	Cluster-27834.4019	478	0	71.43%	AFI45028.1	*Dendroctonus ponderosae*
TyunCYP6DJ1	i1_LQ_TYUN_c33388/f1p0/1763	507	0	70.71%	AFI45041.1	*Dendroctonus ponderosae*
TyunCYP9Z4	i1_LQ_TYUN_c78340/f1p0/1911	526	0	84.22%	AFI45045.1	*Dendroctonus ponderosae*
TyunCYP6DJ2	i2_LQ_TYUN_c23405/f1p0/2443	66	6×10^{-7}	67.65%	AGF69211.1	*Dendroctonus valens*
TyunCYP4G1	i3_LQ_TYUN_c4999/f1p0/3248	242	5×10^{-158}	93.80%	ALD15896.1	*Dendroctonus armandi*
TyunCYP305F1	Cluster-27834.3827	491	0	87.53%	ALD15904.1	*Dendroctonus armandi*
TyunCYP352B1	i2_LQ_TYUN_c27719/f1p0/2254	225	4×10^{-74}	69.36%	ALD15909.1	*Dendroctonus armandi*
TyunCYP4BD4V	i1_LQ_TYUN_c15852/f1p0/1569	166	8×10^{-68}	67.31%	ALD15912.1	*Dendroctonus ponderosae*
TyunCYP6DF1	Cluster-27834.8537	505	0	72.31%	ALD15922.1	*Dendroctonus armandi*
TyunCYP9Z2	i2_LQ_TYUN_c15205/f1p0/2172	575	0	81.30%	ALD15924.1	*Dendroctonus armandi*
TyunCYP434A1	Cluster-27834.7270	371	7×10^{-147}	71.53%	XP_019754109.1	*Dendroctonus ponderosae*
TyunCYP4BQ1	Cluster-27834.7070	397	0	67.84%	XP_019754731.1	*Dendroctonus ponderosae*
TyunCYP6DE1	i1_HQ_TYUN_c29325/f3p0/1840	509	0	76.23%	XP_019755300.1	*Dendroctonus ponderosae*
TyunCYP315A1	Cluster-27834.2194	292	8×10^{-139}	72.26%	XP_019755328.1	*Dendroctonus ponderosae*
TyunCYP315A2	Cluster-27834.2193	208	2×10^{-20}	70.15%	XP_019755336.1	*Dendroctonus ponderosae*
TyunCYP4G2	i1_HQ_TYUN_c24270/f27p4/1844	563	0	92.11%	XP_019755432.1	*Dendroctonus ponderosae*
TyunCYP4BG1	Cluster-27834.11078	501	0	78.09%	XP_019756327.1	*Dendroctonus ponderosae*
TyunCYP410A1	i1_LQ_TYUN_c6617/f1p1/1851	273	5×10^{-82}	53.52%	XP_019756499.1	*Dendroctonus ponderosae*
TyunCYP411A1	i2_LQ_TYUN_c22094/f1p0/2786	490	0	76.22%	XP_019758316.1	*Dendroctonus ponderosae*
TyunCYP411A2	i2_LQ_TYUN_c11742/f1p1/2726	185	6×10^{-77}	71.43%	XP_019758318.1	*Dendroctonus ponderosae*
TyunCYP345F1	Cluster-26110.0	500	0	82.20%	XP_019759785.1	*Dendroctonus ponderosae*
TyunCYP307B1	Cluster-27834.15214	399	1×10^{-153}	77.06%	XP_019760073.1	*Dendroctonus ponderosae*
TyunCYP307A1	i3_LQ_TYUN_c19449/f1p0/3461	492	0	96.54%	XP_019760634.1	*Dendroctonus ponderosae*
TyunCYP393A2	i0_LQ_TYUN_c4806/f1p0/966	112	5×10^{-17}	71.93%	XP_019760717.1	*Dendroctonus ponderosae*
TyunCYP9AP1	i1_LQ_TYUN_c81629/f1p2/1918	483	0	72.57%	XP_019760793.1	*Dendroctonus ponderosae*
TyunCYP9AN1	i2_LQ_TYUN_c15846/f1p0/2129	523	0	79.31%	XP_019761014.1	*Dendroctonus ponderosae*
TyunCYP433A1	i1_LQ_TYUN_c80202/f1p0/1820	274	2×10^{-128}	70.45%	XP_019761671.1	*Dendroctonus ponderosae*
TyunCYP9Z3	i2_LQ_TYUN_c27749/f1p0/2479	151	2×10^{-44}	73.33%	XP_019764934.1	*Dendroctonus ponderosae*
TyunCYP345E1	i1_HQ_TYUN_c62660/f2p0/1795	304	4×10^{-159}	72.33%	XP_019765603.1	*Dendroctonus ponderosae*
TyunCYP347D1	Cluster-20450.0	498	0	78.93%	XP_019767652.1	*Dendroctonus ponderosae*
TyunCYP303A1	Cluster-27834.21448	484	0	76.80%	XP_019768066.1	*Dendroctonus ponderosae*
TyunCYP18A1	i2_LQ_TYUN_c27030/f1p0/2265	159	8×10^{-82}	79.87%	XP_019768143.1	*Dendroctonus ponderosae*
TyunCYP18A2	i2_HQ_TYUN_c25448/f2p0/2291	525	0	90.50%	XP_019768147.1	*Dendroctonus ponderosae*
TyunCYP393A1	Cluster-27834.3112	461	0	69.78%	XP_019768810.1	*Dendroctonus ponderosae*
TyunCYP334E1	Cluster-27834.2023	583	0	85.74%	XP_019770320.1	*Dendroctonus ponderosae*
TyunCYP347E1	i2_LQ_TYUN_c3851/f1p0/2279	500	0	79.00%	XP_019771168.1	*Dendroctonus armandi*
TyunCYP6DG3	i3_LQ_TYUN_c17722/f1p0/3197	354	0	77.68%	XP_019771861.1	*Dendroctonus ponderosae*
TyunCYP9AZ1	i2_LQ_TYUN_c28708/f1p0/2957	134	2×10^{-70}	79.85%	XP_019773084.1	*Dendroctonus ponderosae*
TyunCYP334E2	i2_LQ_TYUN_c27105/f1p0/2563	498	0	83.77%	XP_019773300.1	*Dendroctonus ponderosae*
TyunCYP349B2	Cluster-40684.0	420	0	82.86%	XP_019773560.1	*Dendroctonus ponderosae*
TyunCYP434A2	i1_HQ_TYUN_c939/f2p0/1483	360	5×10^{-124}	50.00%	XP_019869691.1	*Aethina tumida*
TyunCYP6BW3	i2_LQ_TYUN_c30164/f1p0/2137	382	2×10^{-121}	48.28%	XP_023014703.1	*Leptinotarsa decemlineata*
TyunCYP410C1	i0_LQ_TYUN_c9810/f1p0/524	150	8×10^{-12}	32.69%	XP_030755100.1	*Sitophilus oryzae*

A total of 33 candidate GSTs were identified from the total transcriptome of the different developmental stages of *T. yunnanensis*. The sequence identities of these candidate GSTs with other dipteran insects ranged from 51.96% to 97.14% in the NCBI database (Table 5). After removing short sequences (aa < 150) [32], 18 GSTs were chosen for the phylogenetic analysis. A neighbor-joining tree was subsequently constructed using our identified putative GST proteins and the sequences from four other Coleoptera species, *D. ponderosae*, *T. castaneum*, *D. valens*, and *A. planipennis* (Figure 4). Ten GSTs were identified in the transcriptome of *T. yunnanensis*, including six delta/epsilon class GSTs, four omega class GST, five sigma class GSTs, two theta class GSTs, and one microsomal class GST. The phylogenetic tree showed that TyunGST had a high homology with DarmGST.

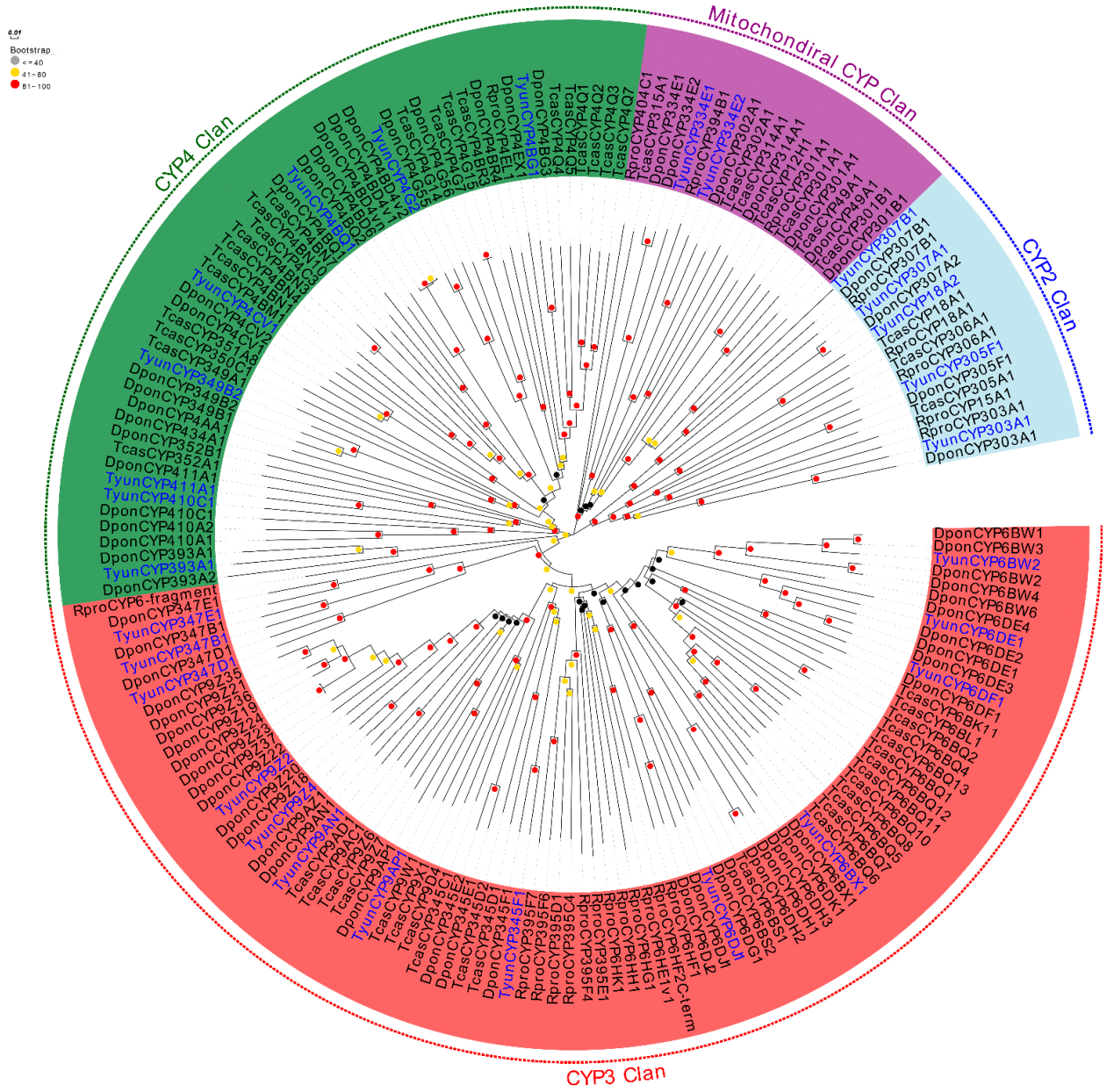

Figure 3. Neighbor-joining tree of candidate CYPs. Bootstrap values after 1000 replications. Dpon, *Dendroctonus ponderosae*; Rpro, *Rhodnius prolixus*; Tcas, *Tribolium castaneum*; Tyun, *Tomicus yunnanensis*.

We identified 29 transcripts encoding CCEs in the *T. yunnanensis* transcriptome by a bioinformatics analysis and all of them were more conserved across other CCEs variants, with 42.86 to 93.03% amino acid identity (Table 6). To guarantee the reliability of the phylogenetic tree, 13 CCEs encoding short sequences wereremoved (aa < 480) [33], and 16 CCEs in our transcriptomes were aligned with CCEs from other coleoptera species (Figure 5). A total of 16 CCE genes were identified in the transcriptome of *T. yunnanensis*, including nine xenobiotic metabolizing enzymes class CCE, three microsomal and alpha-esterases class CCE, two beta- and pheromone esterases class CCE, one JHE class CCE, and one CO class CCE.

Table 5. Best BLASTX matches of *T. yunnanensis* GSTs.

Gene Name	Gene ID	aa	E-Value	Identity	Accession	Species Name
TyunGST1	i2_LQ_TYUN_c36781/f1p0/2077	217	2×10^{-145}	91.71%	AIC76455.1	*Dendroctonus armandi*
TyunGST2	Cluster-27834.9472	70	6×10^{-41}	97.14%	AJE61306.1	*Dendroctonus armandi*
TyunGST3	i0_LQ_TYUN_c12133/f1p0/748	141	3×10^{-48}	57.25%	AJE61307.1	*Dendroctonus armandi*
TyunGST4	i2_LQ_TYUN_c18657/f1p0/2385	112	3×10^{62}	76.74%	AJE61308.1	*Dendroctonus armandi*
TyunGST5	i3_LQ_TYUN_c11135/f1p0/3582	79	5×10^{-33}	82.35%	AJE61309.1	*Dendroctonus armandi*
TyunGST6	Cluster-27834.6624	230	2×10^{-146}	83.48%	AJE61311.1	*Dendroctonus armandi*
TyunGST7	Cluster-27834.8595	244	8×10^{-160}	88.52%	AJE61312.1	*Dendroctonus armandi*
TyunGST8	i1_LQ_TYUN_c149780/f1p1/1152	245	2×10^{-150}	82.04%	AVR54955.1	*Sitophilus oryzae*
TyunGST9	i3_LQ_TYUN_c12086/f1p0/3529	218	2×10^{-142}	88.58%	AVR54957.1	*Sitophilus oryzae*
TyunGST10	i2_LQ_TYUN_c14505/f1p9/2537	52	6×10^{-15}	71.43%	AVR54966.1	*Sitophilus oryzae*
TyunGST11	Cluster-27834.9551	105	9×10^{-41}	64.42%	AVT42177.1	*Lissorhoptrus oryzophilus*
TyunGST12	i0_LQ_TYUN_c7185/f1p0/508	148	6×10^{-48}	62.50%	AVT42178.1	*Lissorhoptrus oryzophilus*
TyunGST13	i2_LQ_TYUN_c7650/f1p0/2527	118	3×10^{-64}	87.85%	AVT42182.1	*Lissorhoptrus oryzophilus*
TyunGST14	Cluster-27834.21190	219	3×10^{-69}	51.96%	AVT42185.1	*Lissorhoptrus oryzophilus*
TyunGST15	i2_LQ_TYUN_c8057/f1p0/2856	294	7×10^{-150}	67.12%	AVT42197.1	*Lissorhoptrus oryzophilus*
TyunGST16	Cluster-27834.9541	219	1×10^{-144}	89.50%	QFU14637.1	*Dendroctonus armandi*
TyunGST17	i4_LQ_TYUN_c8880/f1p0/4497	137	3×10^{-69}	93.69%	QFU14640.1	*Dendroctonus armandi*
TyunGST18	Cluster-1630.0	205	1×10^{-76}	56.78%	QFU14643.1	*Dendroctonus armandi*
TyunGST19	i2_LQ_TYUN_c12200/f1p0/2343	231	3×10^{-137}	77.92%	QFU14646.1	*Dendroctonus armandi*
TyunGST20	i3_LQ_TYUN_c2494/f1p0/3326	218	4×10^{-146}	90.41%	XP_019753833.1	*Dendroctonus ponderosae*
TyunGST21	i2_LQ_TYUN_c34577/f1p0/2723	78	7×10^{-41}	83.54%	XP_019753835.1	*Dendroctonus ponderosae*
TyunGST22	i2_LQ_TYUN_c23261/f1p0/2987	124	2×10^{-73}	93.86%	XP_019755130.1	*Dendroctonus ponderosae*
TyunGST23	i2_LQ_TYUN_c24320/f1p0/2660	150	2×10^{-35}	84.72%	XP_019755704.1	*Dendroctonus ponderosae*
TyunGST24	i7_LQ_TYUN_c267/f1p0/7646	72	7×10^{-22}	67.21%	XP_019755792.1	*Dendroctonus ponderosae*
TyunGST25	i3_LQ_TYUN_c4401/f1p0/3564	111	4×10^{-53}	86.27%	XP_019755793.1	*Dendroctonus ponderosae*
TyunGST26	i3_LQ_TYUN_c26609/f1p0/3037	203	3×10^{107}	72.00%	XP_019755830.1	*Dendroctonus ponderosae*
TyunGST27	i0_LQ_TYUN_c24616/f1p0/906	205	1×10^{-129}	85.37%	XP_019760728.1	*Dendroctonus ponderosae*
TyunGST28	i2_LQ_TYUN_c11257/f1p0/2424	559	0	75.62%	XP_019764595.1	*Dendroctonus ponderosae*
TyunGST29	Cluster-27834.9273	224	4×10^{-124}	73.66%	XP_019766435.1	*Dendroctonus ponderosae*
TyunGST30	i1_LQ_TYUN_c146891/f1p0/1112	237	3×10^{-158}	89.83%	XP_019768505.1	*Dendroctonus ponderosae*
TyunGST31	i0_LQ_TYUN_c13481/f1p0/385	70	4×10^{-32}	82.86%	XP_019770972.1	*Dendroctonus ponderosae*
TyunGST32	i1_LQ_TYUN_c153362/f1p1/1072	206	3×10^{-77}	57.28%	XP_023313068.1	*Anoplophora glabripennis*
TyunGST33	i1_LQ_TYUN_c150961/f1p1/1003	206	2×10^{-78}	58.54%	XP_023313069.1	*Anoplophora glabripennis*

3.4. Tissue Expression Profile of the CYP, GST, and CCE Genes

In order to explore the expression profiles of detoxification genes in different tissues, we screened all 113 genes from the DEG library. In total, 64 genes were found, and 49 genes were missing from the DEG data. The expression profiles based on FPKM values revealed that several detoxification genes (TyunCYP410A1, TyunCYP9AN1, TyunCYP393A1, TyunGST22, TyunGST29, TyunGST31, TyunCCE8, and TyunCCE17) are highly expressed in the antenna (FPKM > 100) and the expression levels of these genes are all higher in male antennae than in female antennae (Figure 6). TyunCYP4G2 is highly expressed in the residue. Tyun315A1, TyunCYP6BK1, TyunCYP6DF1, and TyunGST3 are expressed in all tissues; TyunCYP6DF1 expression in antennae is higher than in other tissues. TyunCYP345E1 and TyunCYP345F1 are little expressed in all tissues (Figure 6a). TyunGST11 and TyunGST33 are expressed in all tissues, the expression of TyunGST11 in foot and antennae is higher than other tissues, and the expression level of male feet is higher than that of female legs. The antenna expression of TyunGST33 is significantly higher than that of other tissues and the male antenna expression is higher than that of the female (Figure 6b). TyunCCE21 is expressed in all tissues. TyunCCE16 is strongly expressed in the head and carcasses, and expression in these two parts of the females is higher than that of the males (Figure 6c).

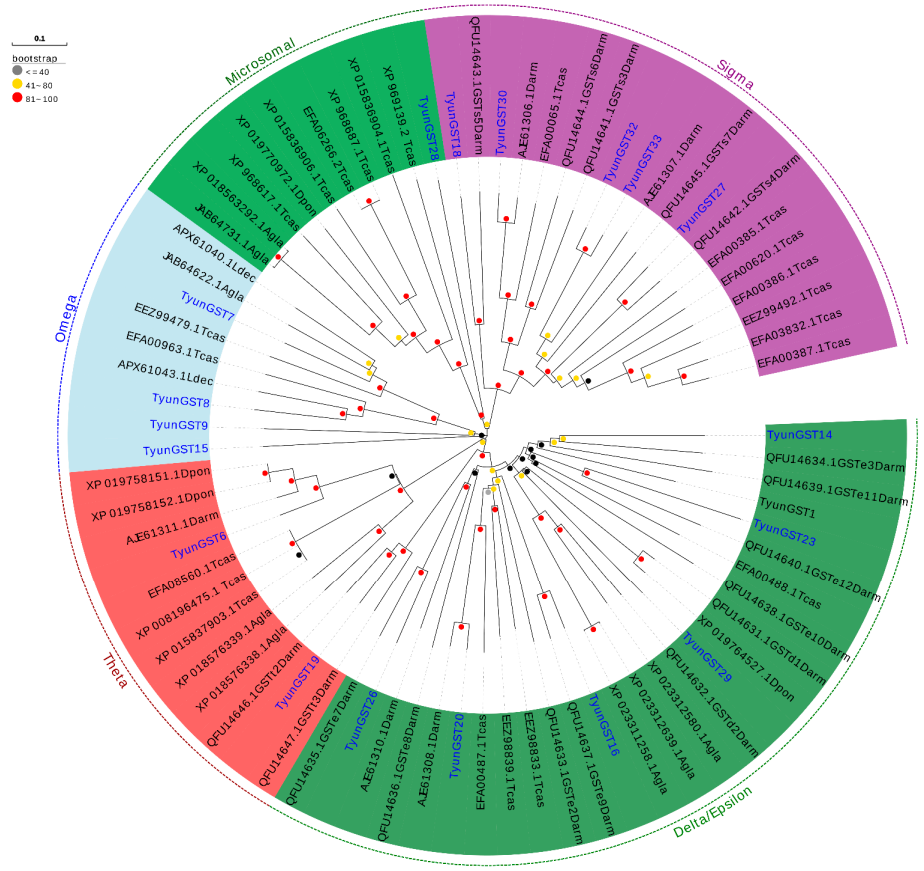

Figure 4. Neighbor-joining tree of candidate GSTs. Bootstrap values after 1000 replications. Dpon, *Dendroctonus ponderosae*; Darm, *Dendroctonus armandi*; Agla, *Anoplophora glabripennis*; Ldec *Leptinotarsa decemlineata*; Tcas, *Tribolium castaneum*; Tyun, *Tomicus yunnanensis*.

Table 6. Best BLASTX matches of *T. yunnanensis* CCEs.

Gene Name		aa	E-Value	Identity	Accession	Species Name
TyunCCE1	i2_LQ_TYUN_c19726/f1p0/2240	580	0	79.41%	AYN64423.1	*Dendroctonus armandi*
TyunCCE2	i1_LQ_TYUN_c11797/f1p0/1951	405	0	93.03%	AYN64424.1	*Dendroctonus armandi*
TyunCCE3	Cluster-27834.6014	133	3×10^{-43}	61.42%	AYN64428.1	*Dendroctonus armandi*
TyunCCE4	Cluster-27834.16668	243	5×10^{-156}	88.48%	AYN64429.1	*Dendroctonus armandi*
TyunCCE5	Cluster-27834.3878	550	5×10^{-164}	45.34%	XP_019754206.1	*Dendroctonus ponderosae*
TyunCCE6	Cluster-27834.11182	570	0	81.05%	XP_019754592.1	*Dendroctonus ponderosae*
TyunCCE7	Cluster-27834.18917	577	0	87.19%	XP_019755320.1	*Dendroctonus ponderosae*
TyunCCE8	i1_LQ_TYUN_c11402/f1p0/1940	563	0	58.70%	XP_019755320.1	*Dendroctonus ponderosae*
TyunCCE9	Cluster-27834.6015	557	0	64.16%	XP_019755963.1	*Dendroctonus ponderosae*
TyunCCE10	i1_HQ_TYUN_c4678/f4p0/1715	558	0	82.08%	XP_019756055.1	*Dendroctonus ponderosae*
TyunCCE11	i1_LQ_TYUN_c2026/f1p0/1764	559	0	85.15%	XP_019756056.1	*Dendroctonus ponderosae*
TyunCCE12	Cluster-27834.3444	122	5×10^{-75}	90.98%	XP_019756397.1	*Dendroctonus ponderosae*
TyunCCE13	Cluster-27834.5714	268	2×10^{-169}	88.01%	XP_019756739.1	*Dendroctonus ponderosae*
TyunCCE14	i1_LQ_TYUN_c29743/f1p0/1703	545	0	50.54%	XP_019757154.1	*Dendroctonus ponderosae*
TyunCCE15	i1_LQ_TYUN_c35851/f1p0/1947	228	8×10^{-114}	75.60%	XP_019758307.1	*Dendroctonus ponderosae*
TyunCCE16	i1_LQ_TYUN_c30292/f1p1/1995	566	0	88.18%	XP_019761960.1	*Dendroctonus ponderosae*
TyunCCE17	i1_LQ_TYUN_c16028/f1p0/1766	347	0	81.23%	XP_019764466.1	*Dendroctonus ponderosae*
TyunCCE18	i1_LQ_TYUN_c9615/f1p0/1928	532	0	76.15%	XP_019765893.1	*Dendroctonus ponderosae*
TyunCCE19	i1_LQ_TYUN_c37855/f1p0/1526	414	0	83.09%	XP_019766062.1	*Dendroctonus ponderosae*
TyunCCE20	Cluster-27834.3692	531	0	85.50%	XP_019766553.1	*Dendroctonus ponderosae*
TyunCCE21	Cluster-27834.10155	569	0	86.12%	XP_019769801.1	*Dendroctonus ponderosae*
TyunCCE22	i3_LQ_TYUN_c17625/f1p0/3579	266	2×10^{-136}	89.52%	XP_019769801.1	*Dendroctonus ponderosae*
TyunCCE23	i1_LQ_TYUN_c34869/f1p0/1995	577	0	69.46%	XP_019769830.1	*Dendroctonus ponderosae*
TyunCCE24	Cluster-27834.16736	569	0	75.66%	XP_019772474.1	*Dendroctonus ponderosae*
TyunCCE25	i2_LQ_TYUN_c30644/f1p0/2041	337	0	78.34%	XP_019773718.1	*Dendroctonus ponderosae*
TyunCCE26	Cluster-27834.18773	65	9×10^{-7}	42.86%	XP_023727217.2	*Cryptotermes secundus*
TyunCCE27	Cluster-27834.13066	308	7×10^{-127}	60.06%	XP_030749177.1	*Sitophilus oryzae*
TyunCCE28	Cluster-27834.7029	572	0	47.51%	XP_030754488.1	*Sitophilus oryzae*
TyunCCE29	i2_LQ_TYUN_c24874/f1p0/2585	308	3×10^{-107}	55.89%	XP_030768230.1	*Sitophilus oryzae*

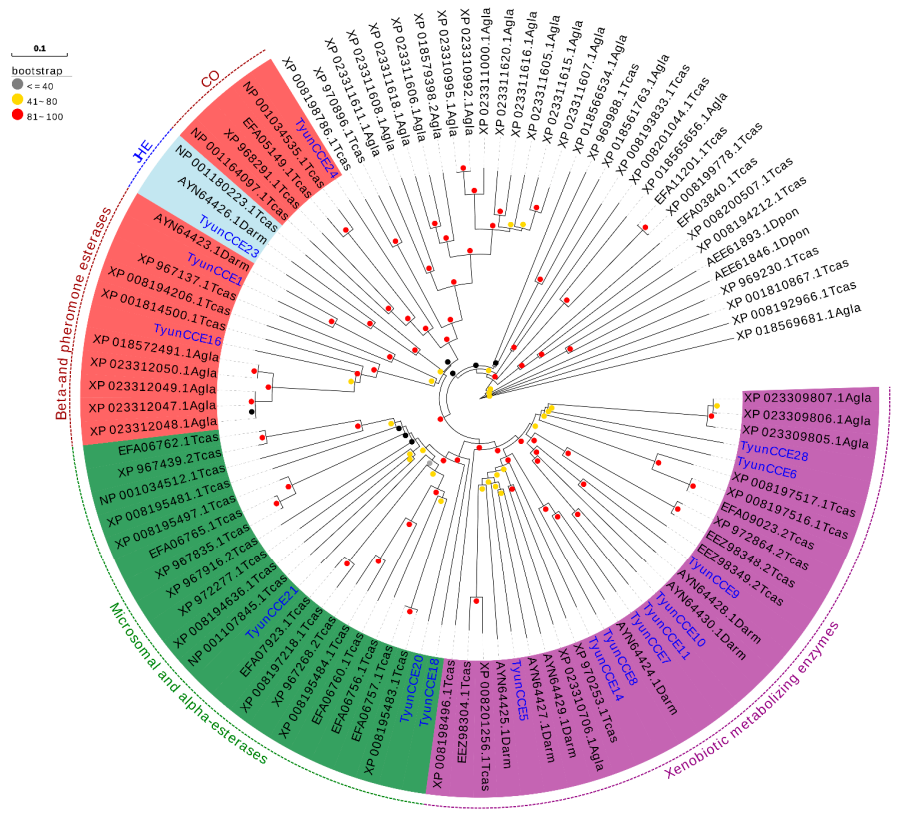

Figure 5. Neighbor-joining tree of candidate CCEs. Bootstrap values after 1000 replications. Dpon, *Dendroctonus ponderosae*; Darm, *Dendroctonus armandi*; Agla, *Anoplophora glabripennis*; Tcas, *Tribolium castaneum*; Tyun, *Tomicus yunnanensis*.

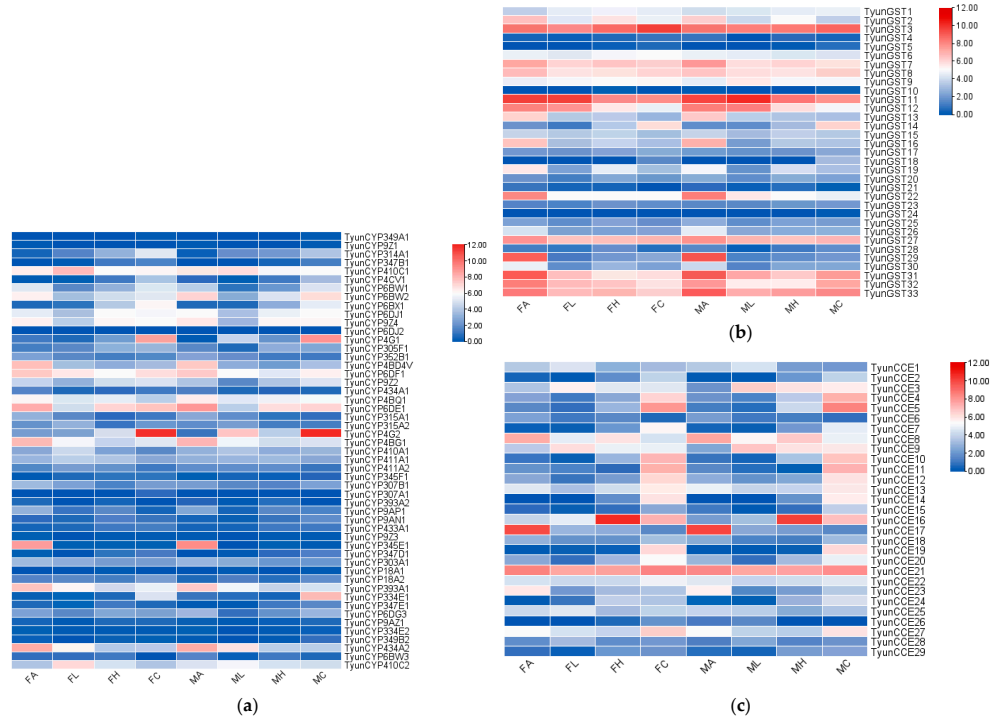

Figure 6. Expression profiles of detoxification genes in *T. yunnanensis*. (**a**) CYP; (**b**) GST; (**c**) CCE. MA: male antennae, MH: male head, ML: male legs, MC: male carcasses, FA: female antennae, FH: female head, FL: female legs, FC: female carcasses.

Further, qPCR was employed to validate the expression of some detoxification genes and to investigate their expression profiles. The qPCR results of TyunCYP4G2, TyunCYP6DF1, TyunGST11, TyunGST33, TyunCCE16, and TyunCCE17 are consistent with the FPKM value analysis results, but the qPCR results of TyunCCE21 differ from the FPKM value analysis, which may be caused by the inconsistency between the qPCR analysis samples and the sequencing samples (Figure 7).

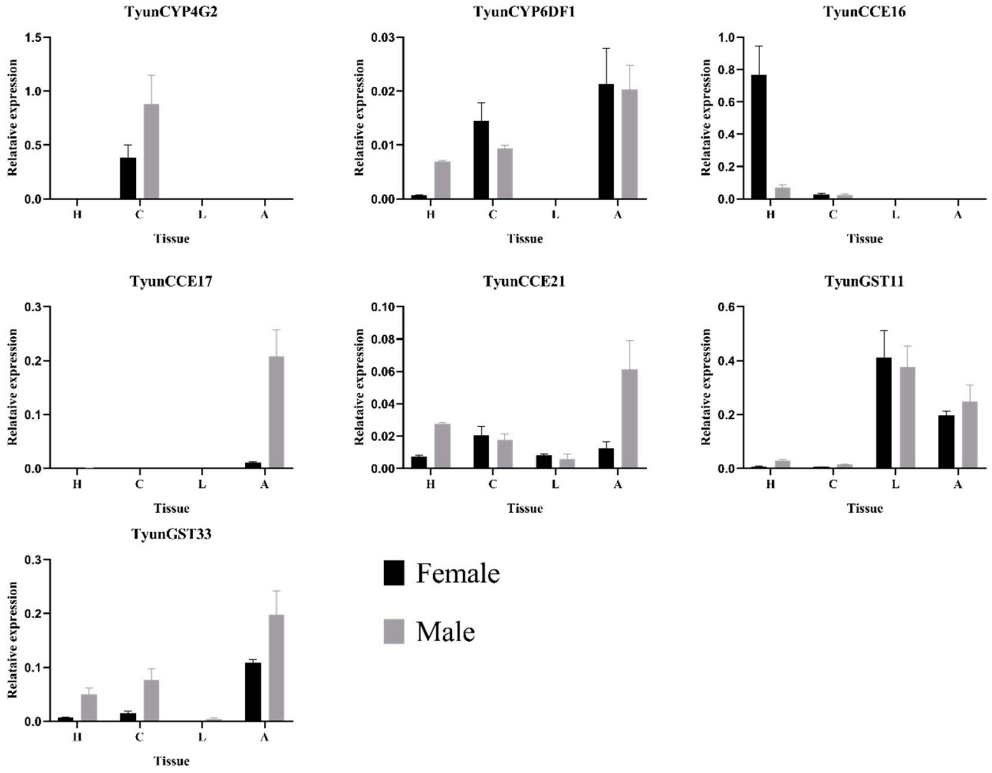

Figure 7. qPCR analysis of *T. yunnanensis* detoxification genes transcript levels in different tissues. H: head; C: carcasses; L: leg; A: antenna.

4. Discussion

Tomicus yunnanensis is one of the most important pests of *Pinus yunnanensis*. In most insect species, detoxification proteins play a key role in the degradation of plant secondary metabolites. In order to better understand the clues of how insects degrade plant secondary metabolites, we first identified candidate detoxification proteins in the transcripts of the antennae, heads, legs, and carcasses of *T. yunnanensis*, and studied some of the detoxification proteins and the expression profiles of four different organizations. Our research results provide new evidence for the molecular basis of the detoxification proteins of *T. yunnanensis* in the metabolic function of detoxification, which may help to develop better methods to control this pest. The identification of at least 51 CYP genes placed *T. yunnanensis* within the middle of the range of the P450 gene family size in insects for which the genome has been sequenced, ranging from a low of 46 in the honeybee (*Apis mellifera* Linnaeus, 1758 (Hymenoptera: Apidae)) to 143 in *T. castaneum* [34], and it was similar to the mountain pine beetle (*D. armandi*), which has 64 CYPs. Previous studies on *T. yunnanensis* were mainly in the direction of olfactory-related proteins, but there are few reports on the detoxification genes of *T. yunnanensis* [35]. In this study, 51 TyunCYPs were analyzed in different adult tissue transcripts, and we found that seven TyunCYPs are predominantly expressed in the antennae of both sexes, belonging to three CYP families, CYP2: TyunCYP305F1; mitochondrial CYP clan: TyunCYP315A1; CYP4: TyunCYP410A1, TyunCYP393A1, and TyunCYP305F1. CYP345E2, a member of CYP3, is an antenna-specific CYP from *D. pondersae* that has been proved to catalyze the oxidation of

monoterpene volatile compounds in pine hosts [36]. In our study, we found two CYP345E2 homologues, TyunCYP345E1 and TyunCYP345F1 in *T. yunnanensis*, but the expression levels of TyunCYP345E1 and TyunCYP345F1 in the antennae were both low, which may be caused by differences between species. Some members of the CYP4 family are also involved in odor degradation, and most of them are involved in the synergistic reaction of detoxification and pheromone synthesis [37]. The expression level of TyunCYP393A1 in the antennae of females was higher than that of males, which might be related to the host location of females, while the expression level of TyunCYP410A1 in the antennae of males was higher than that of females, which may be because the male needs to degrade the secondary metabolites produced by the plant defense mechanism. TyunCYP4G2 is specifically and highly expressed in the residue, which may be related to the metabolism of toxic substances in *T. yunnanensis*. In the CYP6 family, few members have been reported to have odorant clearance functions [38]. In many (but not all) studies, genes from the CYP6 subfamily are shown to metabolize xenobiotics and plant natural compounds [27]. Several genes have been reported to have specific expression in the olfactory organs, such as CYP6B48, CYP6B42, and CYP6AE49 expressed in the male and female antennae of *Spodoptera litura* [39]. PxCYP6BG3 and PxCYP6BG6 were found in *Plutella xylostell*, which may be related to larval odor clearance [40]. In this study, we did not find any genes that were specifically and highly expressed in the olfactory organs of the CYP6 family.

The functions of GSTs in many insects' physiological functions, such as insecticide resistance and detoxification (plant secondary metabolites) have been fully demonstrated [40–42]. In this study, we identified 33 TyunGSTs from the *T. yunnanensis* transcriptome. According to some reports, A *Manduca sexta* olfactory specific GST called gstmsolf1 is reported as a degradable plant volatile trans-2-hexanal, belonged to the delta subfamily [40,43], in our study, we identified two new GSTs, TyunGST16 and TyunGST29, that were highly expressed in the antennae, and we found that they belonged to the delta/epsilon GST subfamily, Further family classification is needed for the functional speculation of TyunGST16 and TyunGST29. At present, we speculate that they may be related to odor degradation.. TyunGST30 and DaGSTs1 formed a linage in the sigma subfamily. DaGSTs1 may play a role in reducing the negative effects of terpenoids on beetles [44]. We speculate that TyunGST30 also has this function.

Insect CCE is a superfamily, which participates in many physiological processes and contains a variety of substrates [45]. Some of these are secreted enzymes, which refresh ORs by removing redundant esterase odorants from the surrounding area. Besides Z11-16: Ald, Z11-16: Ac is another major sex pheromone component reported in [46]. We found 29 CCE genes in the transcriptome of *T. yunnanensis*. The total number is higher than that of *D. armandi* (8) [47] and less than that of *T. castaneum* (63) [48]. The phylogenetic tree shows that the CCE genes of *T. yunnanensis* can be divided into five categories. Among them, TyunCCE5 and DarmCCE3 (AYN64425.1) cluster into the same branch, and DarmCCE3 is inferred to play a role in host detoxification [47], which we speculate that TyunCCE5 may also have. Using the FPKM value and a qPCR analysis, we found that three CCEs have a higher expression in the antennae: TyunCCE8, TyunCCE17, and TyunCCE21. TyunCCE8 and DarmCCE2 (AYN64424.1) are clustered into the same clade, whose most enzymes have a dietary detoxification function or ester odor degradation function, such as SlCXE10 [49] and SexCXE10 [50] with antennae dominant expression that can degrade ester plant secondary metabolites. Unlike these three CCEs, TyunCCE8, TyunCCE17, and TyunCCE21 have no obvious gender-biased expression. TyunCCE23, DarmCCE (AYN64426.1), and TcasCCE (NP_001180223.1) are divided into the JHE branch. TcasCCE (NP_001180223.1) has been confirmed to have the function of degrading juvenile hormones. We speculate that TyunCCE23 also has this function; of course, this inference needs to be further verified.

5. Conclusions

Our present work has characterized the detoxification gene families of *T. yunanensis* by transcriptome, together with bioinformatics-based analyses and molecular strategies of qPCR. Our study first lead to the identification of 113 genes associated with detoxification, with as many as 51 CYPs. Further, expression profile studies provided reference data for these genes to explore their potential roles in the detoxification of plant secondary metabolites. Together, this study has complemented the information of detoxification gene families in *T. yunanensis* and will allow for target experiments to screen potential attractants or repellents and to develop novel pest control strategies for controlling this beetle and protect the balance of the forest ecosystem.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d14010023/s1, Table S1: The primers designed for qPCR analysis; Table S2: Protein sequence of Tomicus yunnanensis.

Author Contributions: Conceptualization, W.L. and N.Z.; methodology, B.Y. and N.L.; software, J.Z. and Z.L.; validation, W.L., S.Z. and N.Z.; formal analysis, N.L.; investigation, J.Z. and Z.L.; resources, W.L. and B.Y.; data curation, J.Y.; writing—original draft preparation, W.L.; writing—review and editing, W.L. and N.Z.; visualization, N.Z.; supervision, N.Z.; project administration, N.Z.; funding acquisition, N.Z. All authors have read and agreed to the published version of the manuscript.

Funding: The research was supported by the National Natural Science Foundation of China (31760210), the Science Research Foundation of Yunnan Provincial Department of Education Project (2021Y269), and Key Project of Yunnan Applied Basic Research Program (grand No.202101AS070009; 2018FG001-010).

Institutional Review Board Statement: Not applicable.

Data Availability Statement: All Data are provided within the paper and supplementary material.

Conflicts of Interest: All the authors declare that they have no conflict of interest.

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