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AIP Conf. Proc. 2079, 020003 (2019)
https://doi.org/10.1063/1.5092381

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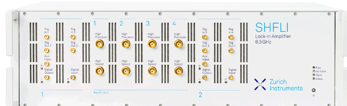

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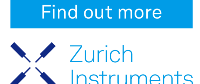

Gene Expression Analysis of the Tibetan Grassland Caterpillars (Lepidoptera: Lymantriinae: *Gynaephora*) in Response to High-Altitude Stress

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Abstract. Adaptation to high-altitude environments has been extensively studied in vertebrates, but studies on invertebrates, especially insects, are very limited. Insects show different characteristics in phenotype, physiology and behavior from vertebrates and thus may have evolved specific mechanisms of high-altitude adaptation. Here, we downloaded clean data/reads of *Gynaephora* species (*alpherakii* and *menyuanensis*) distributed in 3,000 m a.s.l (above sea level) and 4,800 m a.s.l on the Tibetan Plateau (TP), and performed a mixed assembly of these two transcriptomes to construct unigene set of *Gynaephora* to infer potential genetic mechanisms at gene expression level of high-altitude adaptation in insects. Subsequently, by comparing gene expression of these two *Gynaephora* species, we obtained 3,238 differentially expressed genes (DEGs), most of which were significantly enriched in mitochondrial function, ATP production and energy metabolism. This study is useful for understanding the adaptation, evolution and speciation of *Gynaephora* endemic to the TP and provides novel insight into high-altitude adaptation in TP animals.

INTRODUCTION

The adaptive evolution of animals to extreme environments is a key scientific question in evolutionary biology [1, 2]. The Tibetan Plateau (TP), with distinctive climate and geography characteristics, is the highest average altitude in the world. Hypoxia, cold climate and high levels of ultraviolet (UV) exposure are the most important environmental features of the TP. To adapt to high altitudes, animals on the TP have evolved adaptive characteristics in morphology, physiology and behavior [1, 3]. Therefore, TP organisms may be satisfactory materials for studying adaptive evolution to extremely ecological environments. In particular, closely related species provide natural model systems that could be used to explore adaptive mechanism of organisms to high altitudes. Currently, genetic basis of high-altitude adaptation in insects endemic to the TP remains largely unknown, particularly at gene expression plasticity.

Insects exhibit a series of adaptive behaviors under hypoxia [4, 5]. For example, insects exhibit drastically attenuated basal metabolic rates and increase tracheal system volumes under hypoxia. First, they increase the spiracle opening time, ventilation action and activity; stop these behaviors; and then switch from aerobic to anaerobic metabolic pathways [4]. Hypoxia is an important factor affecting insect morphology, including decreased body size, wing degeneration and increased tracheal system cross section [5]; the former two decrease the oxygen demand of insects, whereas the latter improves the efficiency of oxygen use. Although most previous studies have observed adaptive changes in the morphology, behavior, and physiology of insects under high-altitude and hypoxic

2018 International Conference on Biotechnology and Bioengineering (8th ICBB)
AIP Conf. Proc. 2079, 020003-1–020003-6; https://doi.org/10.1063/1.5092381
Published by AIP Publishing. 978-0-7354-1807-3/$30.00

environments [4. 5], the genetic basis underlying these adaptive phenotypes are largely unknown. To date, the exploration of high-altitude adaptation in insects has only been conducted for the migratory locust by analyzing gene expression and mitochondrial enzyme activity [6-8].

The genus *Gynaephora* (Insecta: Lepidoptera: Erebidae: Lymantriinae), also named as grassland caterpillars, consists of 15 nominated species in the worldwide. Seven species are mainly distributed in mountainous areas of the Northern Hemisphere and the Arctic tundra, and the other eight species endemic to the TP have been reported in China [9]. The TP *Gynaephora* species are major pests in alpine meadows, not only devouring high quality forages and leading to serious shortage of fodder vegetation, but also causing mouth mucous membrane canker and broken tongue disease in domestic animals and wildlife [9]. Over long-term evolution, TP *Gynaephora* species have gained adaptive characteristics in morphology and behavior [9]. These adaptive features could therefore make TP *Gynaephora* valuable model species for studying the mechanisms of insect adaptation to high-altitude environments. Currently, almost each TP *Gynaephora* species occupies a different high-altitude habitat, showing typically local distributions [9]. For example, *G. alpherakii* is mainly distributed in the Naqu region of Tibet with ~5000 m above sea level (masl), whereas *G. menyuanensis* is distributed in the northern Qinghai Province and the southwestern Gansu Province at ~3000 masl [9]. These two *Gynaephora* species are distributed in the highest and lowest altitudes among all eight endemic grassland caterpillars on the TP. Thus, the TP *Gynaephora* species also provide an ideal material for studying diverse adaptation to different high-altitude environments.

MATERIALS AND METHODS

G. alpherakii from Naqu County of Tibet (4,800 m asl, 31°48′N/92°04′E), and *G. menyuanensis* from Menyuan County, Qinghai Province (3,000 masl, 37°62′N/101°19′E), on the TP [10]. Clean reads of the TP *Gynaephora* species were downloaded from NCBI Sequence Read Archive (SRA) repository [Accession Number: SRR4242137 and SRR4242138]. Clean reads of each species contained data from respective nine different developmental stages, including egg, 1-6 larva instar, pupa and female adults. The de novo transcriptome assembly of these clean reads, including *G. alpherakii*, *G. menyuanensis*, *Gynaephora* (combining all reads of these two species), was performed using Trinity software [11]. To gain annotation information for the unigenes, the blastx tool with default parameters was used to hit unigenes to public protein databases, including non-redundant (NR) protein database, Swiss-Prot, and Kyoto Encyclopedia of Genes and Genomes (KEGG). According to the NR annotation information, the gene ontology (GO) information of the unigenes was extracted using in-house scripts.

To identify differentially expressed genes (DEGs), we firstly mapped all reads of each of *G. alpherakii* and *G. menyuanensis* to the referenced unigene set of *Gynaephora* using Bowtie software [12], and expression level of each unigene was then calculated using RSEM (RNA-Seq by Expectation Maximization) based on reads per kilobases per millionreads (RPKM) value [13]. DEGs between these two *Gynaephora* species were detected using EBSeq [14]. If fold change (FC) of unigene expression was ≥ 2 and false discovery rate (FDR) < 0.01, these unigenes were considered DEGs. Next, functional enrichment analysis, GO and KEGG, for a set of DEGs was conducted according to our previous methods [10]. Then, the significance levels of all GO and KEGG terms were corrected with the Benjamini and Hochberg (BH) methods by using the *p.adjust* module in R package [15] to control the FDR in multiple pairwise comparisons.

To confirm the RNA-seq results, quantitative real-time PCR (qRT-PCR) was performed for eleven randomly selected DEGs. Gene-specific primers for the qRT-PCR were designed using Beacon Designer 7 (Table 1). A portion of the pooled total RNA in RNA-seq was also used for the qRT-PCR analyses. Namely, total RNA was used to generate first-strand cDNA with the SYBR® Prime Script™ RT-PCR Kit II (Takara, Japan). The cDNA samples were diluted to 100 ng/μl with free-RNase water. qRT-PCR analyses were performed on the ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems, USA) using SYBR® Premix Ex Taq II Kit (Takara, Japan). Each qRT-PCR analysis was performed with triplicate. The elongation factor-1A (*EF1A*) gene was selected as the endogenous control, and the relative expression level of each gene was calculated according to the $2^{-\Delta\Delta CT}$ method [16].

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TABLE 1. Primers used in qRT-PCRs.

Unigene name	Primer name	Sequence (5'-3')	Size (bp)
Unigene_BMK.31940	GynP1F	GTAGGAGCAATCATACGA	98
	GynP1R	CCAGAATACAACAACAAGAT	
Unigene_BMK.11162	GynP2F	CGGTGATTCAGTAGTTAG	96
	GynP2R	GGATGGATAATGATGGATT	
Unigene_BMK.15364	GynP3F	TGAAGAATTGTGAACCTA	175
	GynP3R	GCAGTTATTGTGTAATGAT	
Unigene_BMK.16355	GynP4F	AGTGGTCATACATCCTTA	152
	GynP4R	CCTTATCTGATTGGACAC	
Unigene_BMK.10404	GynP5F	ATCATTACGCACCATTCC	116
	GynP5R	ATGTAAGCACTACGAAGAAG	
Unigene_BMK.7047	GynP6F	GATTGGTATCATAGTATCTTG	90
	GynP6R	GTACTGGTACTACTGGTA	
Unigene_BMK.10613	GynP7F	ATGATGGTGATGATGATGAT	87
	GynP7R	GAAGGACACAAAGGACAT	
Unigene_BMK.31822	GynP8F	GTTAGATGCCACTATACAC	122
	GynP8R	ACACGGACTAAGAATACA	
Unigene_BMK.12785	GynP9F	CTTACTCCGCTTACACATAC	107
	GynP9R	CATTGCTTTAATAGATTTGTTCG	
Unigene_BMK.22885	GynP10F	CCGTATAGTGTTCCAAGT	194
	GynP10R	TCCGCTGTATTCGTAATC	
Unigene_BMK.17312	GynP11F	CGTTGTAAGAGCCTTCAC	82
	GynP11R	CCACCACCTATTCCTGAG	

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RESULTS

By assembling jointly these two libraries, 45,185 unigenes were obtained, and the mixed library showed an N50 value of 1,482 bp and a mean sequence length of 855 bp. A total of 23,328 unigenes (51.63%) of the combined *Gynaephora* species were successfully annotated. The expression profiles of eleven randomly selected unigenes were revealed by qRT-PCR data, which were consistent with the RNA-seq results. Log2 (fold-change) of the gene expression ratios between the RNA-seq and qRT-PCR data showed a significantly positive correlation (Pearson correlation coefficients = 0.96, $p < 0.01$) by a linear regression analysis, indicating that the DEG results from the RNA-seq experiment were reliable. A total of 3,238 genes were differentially expressed between *G. alpherakii* and *G. menyuanensis*, of which 1,374 genes were up-regulated and 1,864 were down-regulated. Among the nine annotated genes of the top 10 up-regulated genes, three were chorion proteins, three were predicted to be leucine-rich repeat extensin-like proteins, and the other three genes have only reported in insects so far encoding the insects-specific proteins VMP30, retinol dehydratase and putative ecdysone oxidase. For the top 10 down-regulated genes, five were annotated to known genes: two were related to cuticular proteins, the other three were annotated to be genes encoding cytochrome P450, lipase and immulectin. The 10 down-regulated genes were obviously associated with resistance and immunity in insects. All of the DEGs that were obtained from this comparison were enriched to the three GO subcategories (Table 2). The results showed that DEGs under biological process (BP) were significantly enriched to 14 GO terms, and the top five terms were “positive regulation of growth rate”, “body morphogenesis”, “iron-sulphur cluster assembly”, “mitochondrial translation” and “oxidation-reduction process”. Among cellular component (CC), DEGs were significantly enriched to the top 10 GO categories, and the top five terms were “mitochondrial large ribosomal subunit”, “mitochondrion”, “proteasome complex”, “mitochondrial respiratory chain complex I” and “small nuclear ribonucleoprotein complex”. Within molecular function (MF), DEGs were significantly clustered to 12 GO terms, and “structural constituent of cuticle”, “peptidase activity”, “structural constituent of cytoskeleton”, “ATP binding” and “structural constituent of chorion” were ranked as the top five. KEGG pathway enrichment analysis of DEGs showed that all of the DEGs were significantly enriched to 26 KEGG terms, and we present the top 20 (Fig. 1), of which “oxidative phosphorylation”, “pentose phosphate pathway” and “proteasome” were the top three.

TABLE 2. List of enriched GO terms from differentially expresed genes (DEGs) between two *Gynaephora* species. BP: Biological process; CC: Cellular component; MF: Molecular function

Subcategories	Go terms (Id)	FDR
BP	positive regulation of growth rate (GO:0040010)	2.97E-07
BP	body morphogenesis (GO:0010171)	4.75E-05
BP	iron-sulfur cluster assembly (GO:0016226)	7.78E-05
BP	mitochondrial translation (GO:0032543)	9.28E-05
BP	oxidation-reduction process (GO:0055114)	1.50E-04
BP	embryo development ending in birth or egg hatching (GO:0009792)	2.06E-04
BP	mitochondrial electron transport, NADH to ubiquinone (GO:0006120)	3.07E-04
BP	mitochondrial electron transport, cytochrome c to oxygen (GO:0006123)	4.32E-04
BP	mitochondrion organization (GO:0007005)	1.68E-03
BP	multicellular organismal development (GO:0007275)	2.57E-03
BP	translation (GO:0006412)	3.01E-03
BP	cellular component organization (GO:0016043)	6.36E-03
BP	developmental process (GO:0032502)	8.70E-03
BP	single-organism cellular process (GO:0044763)	1.33E-02
CC	mitochondrial large ribosomal subunit (GO:0005762)	5.71E-08
CC	mitochondrion (GO:0005739)	1.24E-04
CC	proteasome complex (GO:0000502)	1.78E-04
CC	mitochondrial respiratory chain complex I (GO:0005747)	2.85E-04
CC	small nuclear ribonucleoprotein complex (GO:0030532)	7.64E-04
CC	endoplasmic reticulum (GO:0005783)	1.38E-03
CC	mitochondrial inner membrane (GO:0005743)	3.72E-03
CC	mitochondrial respiratory chain complex IV (GO:0005751)	4.42E-03
CC	membrane part (GO:0044425)	3.41E-02
CC	membrane (GO:0016020)	4.87E-02
MF	structural constituent of cuticle (GO:0042302)	3.65E-10
MF	peptidase activity (GO:0008233)	2.80E-04
MF	structural constituent of cytoskeleton (GO:0005200)	5.99E-04
MF	ATP binding (GO:0005524)	6.28E-04
MF	structural constituent of chorion (GO:0005213)	8.76E-04
MF	peptidyl-prolyl cis-trans isomerase activity (GO:0003755)	1.41E-03
MF	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen (GO:0016705)	1.61E-03
MF	chitin binding (GO:0008061)	2.03E-03
MF	NADH dehydrogenase (ubiquinone) activity (GO:0008137)	2.12E-03
MF	oxidoreductase activity (GO:0016491)	2.83E-03
MF	NADH dehydrogenase activity (GO:0003954)	1.71E-02
MF	oxidoreductase activity, acting on NAD(P)H (GO:0016651)	4.66E-02

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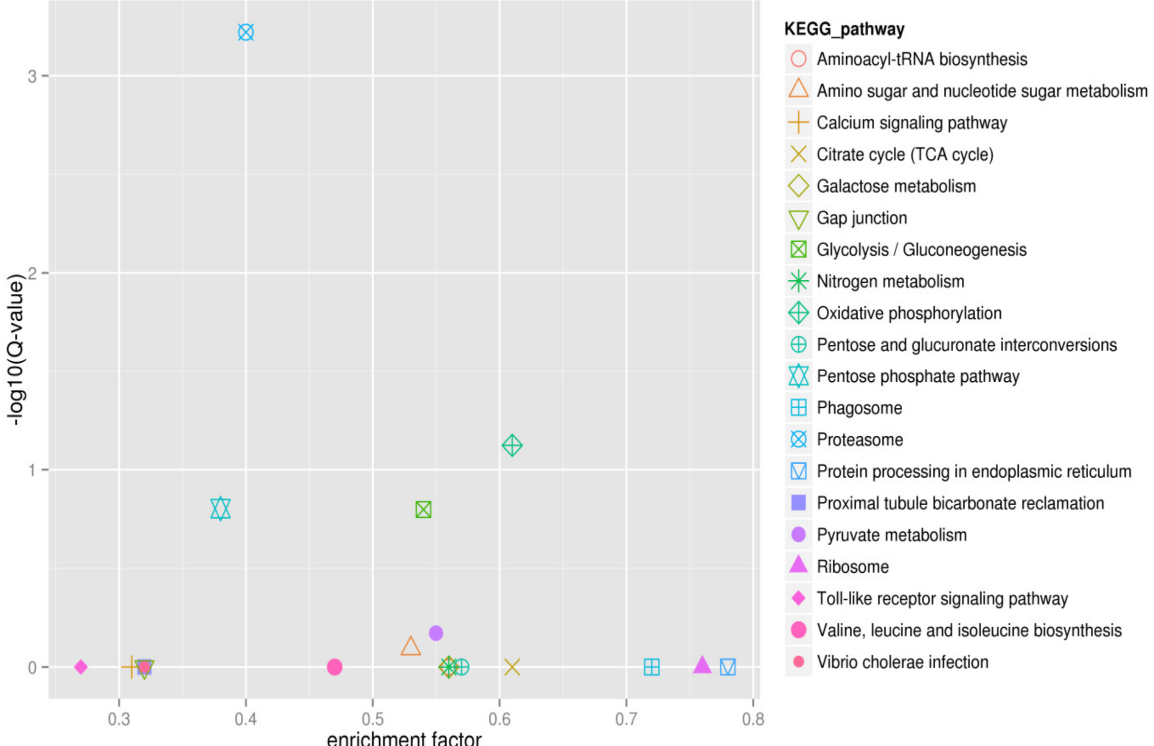

FIGURE 1. Scatter diagram of the enriched KEGG pathways (top 20) of DEGs between *G. alpherakii* and *G. menyuanensis*.

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DISCUSSION

Here, we compared the transcriptomic data for two grassland caterpillar species inhabiting the highest and lowest altitudes of the TP, in order to reveal the genetic basis of high-altitude adaptation by analyzing DEGs. The DEGs were mainly enriched to mitochondrion and mitochondrial electron transport GO terms. Our recent study showed that the response of mitochondrial gene expression to different high-altitude environments was sensitive by comparing the expression levels of nine protein-coding genes between the mitochondrial genomes of *G. alpherakii* and *G. menyuanensis* [17]. Here, the results showed that the expression of more mitochondrion-related genes (not only the 13 genes encoded by mitochondrial genomes) was sensitive to different high altitudes. In this study, the genes encoding ecdysone oxidase and cytochrome P450 showed the most expression changes and are common resistance-related genes in insects, suggesting their important function in the resistance of *Gynaephora* species to extreme environments. Moreover, the alteration of gene expression in metabolic pathways (KEGG) is a common strategy to maintain ATP production under hypoxia. For example, the ATP content in Tibetan locusts was significantly higher than that of lowland locusts after hypoxia treatment.[7] Key pathway involved in ATP production and metabolism, such as “oxidative phosphorylation”, “pentose phosphate pathway” and “proteasome”, showed the most significance in the enriched KEGG of DEGs. Specially, two typical metabolic pathway in ATP production, tricarboxylic acid cycle (TCA) and oxidative phosphorylation, were enriched in this study. This evidence indicates that change in gene expression of *Gynaephora* species can alter ATP production to conquer various hypoxia environments. In particular, the TCA pathway also was significantly enriched when locusts experienced hypoxia, and expression of the gene encoding pyruvate dehydrogenase subunit is not affected [6]. However, the key gene related to pyruvate dehydrogenase was dow-regulated in DEGs in the current study, indicating that the divergent expression of members in the same pathway was favorable for the high-altitude adaptation of different insect groups.

CONCLUSION

By comparing the gene expression profiling of two *Gynaephora* species from different high altitudes of the TP, we found that DEGs between the two *Gynaephora* species mainly involved in mitochondrial function, indicating functional importance of mitochondrion. This study provides genetic information for the two *Gynaephora* species endemic to the TP, which will open a window for further understands the high-altitude adaptation of insects at gene expression levels. In addition, the candidate genes identified in this study are needed to be further confirmed by several investigations in other TP insects.

ACKNOWLEDGMENTS

This study was supported by the National Natural Science Foundation of China (31201520, 31760042 and 31760713), and the Natural Science Foundation of Guangxi Province (2016GXNSFBA380156, 2016GXNSFCA380007).

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