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Reclassification of velvet antler portions following transcriptomic analysis

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Abstract

Context. Commercially, velvet antlers along the longitudinal axis are divided into four portions, namely, wax-like (WL), blood-colour (BC), honeycomb-like (HL) and bone (B) slices from the top to the base. However, there is no evidence at a molecular level showing the accuracy of this classification.

Aims. The aim of the present study was to take transcriptional approach to assess the accuracy of the traditional classification for these four portions of velvet antler, and to link the expressed mRNAs of each portion with possible functions by using bioinformatics analysis.

Methods. Three sticks of three-branched velvet antlers of sika deer were harvested from three anaesthetised 4-year-old sika deer. On the basis of the traditional methods used commercially, the velvet antler sticks were divided into the four portions of WL, BC, HL and B. Transcriptome sequencing was performed using Illumina HiSeq × Ten at BGI (Shenzheng, China).

Key results. In total, 5647 genes were obtained from the four portions. Spearman correlation analysis grouped these four portions into three clusters (WL, BC, HL+B). C-means analysis further confirmed a similar trend, indicating the accuracy of the new classification based on transcriptome analysis. Further functional analysis showed that highly expressed genes in WL, BC and HL+B were mainly related to cell cycle, cartilage development, and bone development respectively.

Conclusions. Four-portion classification based on traditional methods should be replaced by three-portion classification based on the mRNA expression levels.

Implications. We believe that this new classification can contribute to velvet antler industry, providing more accuracy in the use of velvet antlers as pharmaceuticals.

Additional keywords: blood-colour slice, bone slice, honeycomb-like slice, RNA-seq, wax-like slice.

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Introduction

Velvet antlers are the only mammalian bone accessory organs capable of full renewal in yearly cycles (Li *et al*. 2009). At the same time, velvet antlers are a precious traditional Chinese medicine that has been used for thousands of years in China, Korea, and Southeast Asian countries (Jeon *et al*. 2009; Zhou and Li 2009; Wu *et al*. 2013). Different tissue portions along the longitudinal axis of velvet antlers contain different amount of crude proteins, amino acids, water-soluble polysaccharides, monosaccharides, fatty acids and other substances; these substances show a gradually decreasing trend from the top to the base, while the distribution of minerals (mainly calcium) shows the opposite trend (Gong *et al*. 2018, 2019). Different levels of organic and inorganic substances in different portions of velvet antler lead to diversified pharmacological effects, which results in different commercial values. Generally, the price of different portions of velvet antlers decreases from the top to the base. The three-branched velvet antlers of a sika deer are used for description of four defined portions (Fig. 1). (1) Wax-like (WL) portion is characterised by its waxy appearance, with no visible blood vessels distributed. WL portion is located at the tip and ~2–3 cm long, roughly accounting for 1/25 of the total length of an antler. (2) Blood-colour (BC) portion is characterised by the blood colour appearance, which is achieved by rich blood content within evenly distributed blood vessels. BC portion is distal to

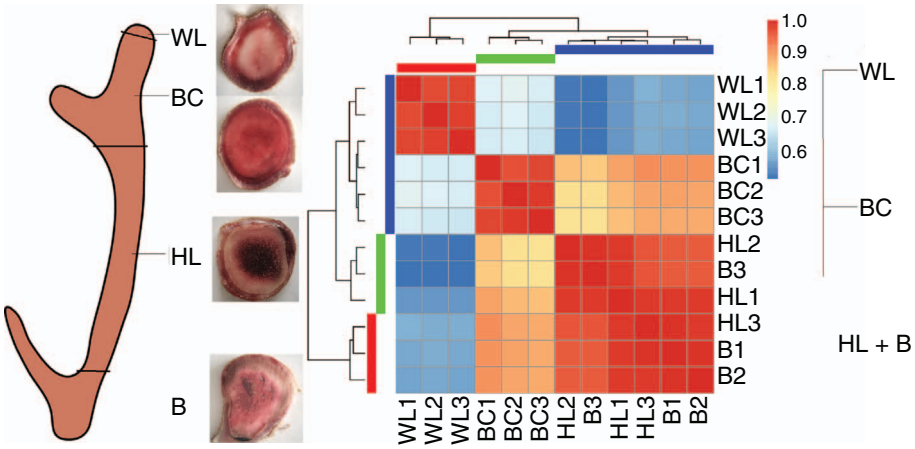

Fig. 1. Spearman correlation analysis. The results suggest that the four classic antler portions should be replaced with three portions. Colour in each block represents gene correlation level, ranging from negative (blue) to positive (red) values. WL, wax-like; BC, blood-colour; HL, honeycomb-like; B, bone.

the WL portion and ~8–10 cm long, roughly accounting for 1/6 of the total length of the antler. (3) Honeycomb-like (HL) portion is characterised by the typical honeycomb-like appearance, where the peripheral edge of this portion has begun to ossify. HL portion is distal to the BC portion and ~32–40 cm long, roughly accounting for 6/10 of the total length of an antler. (4) Bone (B) portion is located at the base and characterised by a fully calcified cortical bone ring and a central cavity. B portion is distal to the HL portion and ~8–10 cm long, accounting for 1/6 of the total length of the antler.

According to the reports, mRNAs play important regulatory roles in the growth and their translated proteins are the bioactive substances for the pharmacological effects (Clark *et al*. 2006; Lai *et al*. 2007; Pita-Thomas *et al*. 2010; Yao *et al*. 2012*a*, 2012*b*; Yang *et al*. 2017). Therefore, mRNA can be utilised as a factor for assessment the accuracy of traditional classification of velvet antler portions. The purpose of the present study was to take transcriptional approach to assess the accuracy of the traditional classification of velvet antler portions, and, in conjunction with bioinformatics analysis, to link the expressed mRNAs in each portion with possible functions, and, ultimately, the pharmacological effects of each velvet antler portion.

Materials and methods

Collection of the four portions of velvet antlers in sika deer

Three sticks of three-branched velvet antlers of sika deer were harvested from three anaesthetised 4-year-old sika deer on the experimental deer farm of Institute of Special Wild Economic Sciences of CAAS, Changchun, China, on 17 July 2018. On the basis of the traditional method that is used commercially, we divided the antler into the following four portions (2–3-cm-thick slice/portion from the middle part of each portion): WL, 1 cm down from the tip; BC, 6 cm down from the tip; HL, 20 cm down from the tip; B, at the base of the antler. Each tissue slice was put into a beaker after the skin was peeled off. After being washed several times in PBS phosphate buffer at pH 7.2–7.4, the tissue slices were immediately placed in liquid nitrogen until further processing.

Table 1. Summary of RNA-seq data
B, bone portion; BC, bone-colour portion; HL, honeycomb-like portion; WL, wax-like portion

Sample	Raw data (million)	Clean data (million)	Clean data (%)	Mapped data (%)
WL	227.36	204.90	90.11	84.28
BC	224.88	201.59	89.63	84.92
HL	227.42	203.78	89.60	85.91
B	222.24	198.17	89.17	85.62

Transcriptome sequencing and analysis

The unskinned tissue slices from each of the four antler portions were rapidly ground into fine powder by using Freezer/Mill 6770 (SPEX CertiPrep Ltd, Edison, NJ, USA). Total RNA was extracted from each slice by using Trizol reagent (Invitrogen Inc., Camarillo, CA, USA), according to the manufacturer's instructions. The RNA concentration and quality (260 : 280 ratio) were measured using Evolution-300 (thermo, Waltham, MA, USA) with a RIN number of >7.0, according to the manufacturer's instructions.

To construct 12 RNA-seq libraries, ~5 μg of the total RNA per sample was used for the RNA sample preparations. The library for sequencing was generated using a TruSeq RNA Library Preparation Kit v3 (Illumina, San Diego, CA, USA). Transcriptome was paired-end sequenced with the Illumina HiSeq × Ten at BGI (Shenzheng, China), which generated ~150-bp paired-end raw reads. After filtering out adaptor sequences and low-quality reads, the selected clean reads from each sample were aligned and mapped to the sika deer (*Cervus nippon*) reference genome (Ba *et al*. 2017) using Bowtie (Langmead *et al*. 2009). The gene expression level was measured using RSEM (Li and Dewey 2011). The differentially expressed genes (DEGs) among the groups were filtered through run_DE_analysis.pl from Trinity package (https://github.com/

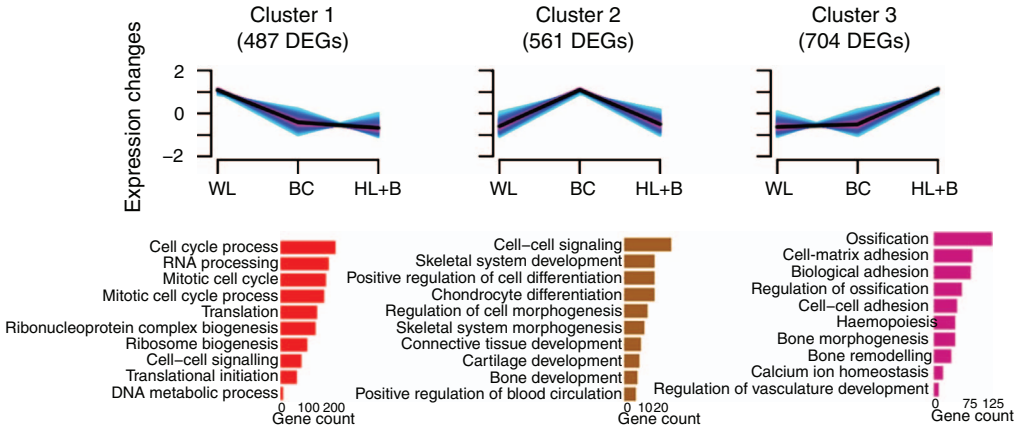

Fig. 2. Summary of highly differentially expressed genes (DEGs) and the corresponding functions in the three distinguishable portions of velvet antlers. The top three panels show that c-means analysis identified three patterns of gene expression across the three portions of velvet antler, and the three bottom panels show the GO functional analysis results of each cluster. WL, wax-like; BC, blood-colour; HL, honeycomb-like; B, bone.

trinity-rnaseq/trinityrnaseq/wiki, accessed 31 March 2020) with the min cpm (count per million) values of replicates of >1, and analysed by using the DEseq R package (Anders and Huber 2010). Spearman correlation was used to evaluate the grouping of antler samples on the basis of the expression levels of DEGs. Next, the DEGs were grouped using Fuzzy c-means clustering from Mfuzz R package (Kumar and Futschik 2007).

To determine the biological processes of DEGs in different clusters, GO functional enrichment analysis was performed using the DAVID online software (https://david.ncifcrf.gov/summary.jsp, accessed 31 March 2020).

Results and discussion

In total, 227.36, 224.88, 227.42 and 222.24 million raw reads were obtained from the RNA-seq libraries, derived from WL, BC, HL and B antler portions respectively. After quality control and adaptor removal, the raw sequences were filtered to obtain the clean reads, which were then mapped to the reference genome of sika deer (Ba *et al*. 2017). In total, 191.61 (84.28%), 190.97 (84.92%), 195.38 (85.91%) and 190.28 (85.62%) million reads from the four portions respectively, were successfully mapped to the reference genome (Table 1). Finally, a total of 5647 genes was obtained from the four portions and used for further analysis. These results indicated that mRNA sequences were highly enriched in the libraries; therefore, the data would be well suited for expression profiling analysis of the mRNAs.

After filtering through the cpm value of >1 by using run_DE_analysis.pl from Trinity software, 1937 DEGs among these groups were identified from the filtered genes, by using the criteria $|\log_2\text{fold change}| \geq 2.0$ and an adjusted *P*-value of ≤ 0.001 (Table S1, available as Supplementary material to this paper). Spearman correlation analysis was performed on these identified 1937 DEGs. The results showed that the HL portion and the B portion were essentially not different from each other, thus clustering together as a composite group (Fig. 1). This finding may reflect the possibility of using the uniform regulatory genes in these two portions. In the subsequent analyses, these two portions were, therefore, put together, and named HL+B.

The highly expressed 1937 DEGs in our newly classified portions were analysed through GO functional analysis (Fig. 2). Fuzzy c-means clustering model was used to further group 90.45% of 1937 DEGs into three clusters (MS $\geq$ 0.5). The highly expressed genes in Clusters 1, 2 and 3 fell into WL, BC and HL+B portions respectively (Fig. 2). These results, collectively, demonstrated the accuracy of our new classification approach at the transcriptional level.

Next, GO functional analysis was used to identify key biological-process categories of three new groups (portions) of velvet antlers. The 487 DEGs in Cluster 1 were mainly related to the functions on RNA processing (e.g. NOLC1, RPL18), mitotic cell cycle (e.g. CDC45, CDK1) and translation (e.g. MAP2K2, EIF5B). WL portion is the growth centre of velvet antler, with vigorous metabolism and the rapid-growth status being achieved mainly through fast cell proliferation and a proper control of the cell cycle in the WL portion (Li *et al*. 2002, 2009). Previous studies have also found more crude proteins, amino acids, water-soluble polysaccharides, monosaccharides, fatty acids (Gong *et al*. 2018, 2019) and bioactive compounds in the WL portion than in the other three portions. Therefore, we speculate that WL portion may have a higher nutritional or pharmaceutical value. The 561 DEGs in Cluster 2 were mainly involved in positive regulation of cell differentiation (e.g. TGFBR2, PRKCH), chondrocyte differentiation (e.g. WNT5B, WNT2B) and skeletal system development (e.g. MMP13, COL2A1). The findings showed that BC portion was the region of endochondral ossification, undergoing the process of cartilage proliferation and differentiation (Li 2013). The 704 DEGs in Cluster 3 showed an opposite tendency, at the expression level, to Cluster 1. These genes are mainly related to ossification (e.g. IHH, SOX8), cell-matrix adhesion (e.g. COL13A1, COL5A3) and bone or cartilage morphogenesis (e.g. SOX9, RUNX2). The

fact that the HL and B portions were indistinguishable from each other in the present study may be due to the fact that these two portions are undergoing gradual ossification transition (Li *et al*. 2009). Consistent with the previous study (Tseng *et al*. 2014; Hu *et al*. 2019), we speculate that the HL+B portion may have a good efficacy in treating osteoporosis. Overall, gene-expression profiling across our newly defined three antler portions would play a unique role in matching each antler portion to its biological function, and lay the foundation for the development of neutraceutical or pharmaceutical/traditional Chinese medicine drugs on the basis of the antler portion-specific knowledge.

Conclusions

Transcriptome analysis found that the traditional classification with four antler portions should be replaced with a new classification into three portions at the mRNA expression level, and this new classification would have downstream implications for developing velvet antlers as a type of traditional Chinese medicine.

Conflicts of interest

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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