Selection of Reliable Reference Genes for Real-time qRT-PCR Analysis of Zi Geese (Anser anser domestica) Gene Expression

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ABSTRACT: Zi geese (Anser anser domestica) belong to the white geese and are excellent layers with a superior feed-to-egg conversion ratio. Quantitative gene expression analysis, such as Real-time qRT-PCR, will provide a good understanding of ovarian function during egg-laying and consequently improve egg production. However, we still don’t know what reference genes in geese, which show stable expression, should be used for such quantitative analysis. In order to reveal such reference genes, the stability of seven genes were tested in five tissues of Zi geese. Methodology/Principal Findings: The relative transcription levels of genes encoding hypoxanthine guanine phosphoribosyl transferase 1 (HPRT1), β-actin (ACTB), β-tubulin (TUB), glyceraldehyde-3-phosphate-dehydrogenase (GADPH), succinate dehydrogenase flavoprotein (SDH), 28S rRNA (28S) and 18S rRNA (18S) have been quantified in heart, liver, kidney, muscle and ovary in Zi geese respectively at different developmental stages (1, 2, 4, 6 and 8 months). The expression stability of these genes was analyzed using geNorm, NormFinder and BestKeeper software. Conclusions: The expression of 28S in heart, GAPDH in liver and ovary, ACTB in kidney and HPRT1 in muscle are the most stable genes as identified by the three different analysis methods. Thus, these genes are recommended for use as candidate reference genes to compare mRNA transcription in various developmental stages of geese. (Key Words: Reference Genes, Real-time qRT-PCR, Zi Geese, Gene Expression)

INTRODUCTION

The research on improving egg production is always the focus on poultry breeding and management, especially for geese because of their poor laying performance and reproductive seasonality (Shi et al., 2008; Kang et al., 2009). Zi geese (Anser anser domestica) are excellent layers with a superior feed-to-egg conversion ratio. This species breeds only in northeast area of China including Heilongjiang and Jilin provinces. In domestic fowl breeding programs, the traditional method to improve egg production is to select those with the biggest egg-laying amount or the rate of lay (Kuhnlein et al., 1997). With the development of molecular biotechnologies such as genomics and proteomics analysis, alternative methods including selection of breeders with egg-laying or other important trait marker genes or proteins are incorporated. Recent progress in molecular breeding technologies has provided tools to study complex biological traits under different physiological conditions using quantitative genes expression analysis (Chen et al., 2007). Among them, selection of breeders by comparing mRNA transcription between different domestic fowl samples has become a very important molecular biological protocol for improving egg production (Kuhnlein et al., 1997; Yen et al., 2006; Chen et al., 2007; Kang et al., 2009). In order to improve production performance of geese, researches on breeding and genetics should be focused on molecular genetic markers mapping, genome analysis and identification of candidate genes for specific performance.

Real-time quantitative reverse transcription polymerase chain reaction (Real-time qRT-PCR) is a major development of PCR technology that enables reliable detection and measurement of DNA (cDNA) generated during each cycle of PCR process (Arya et al., 2005). Real-time qRT-PCR, with its capacity to detect and measure very small amount of nucleic acids in a wide range of samples from numerous sources, has been used extensively in molecular biology, e.g., molecular diagnostics, life sciences, agriculture, and medicine. Real-Time qRT-PCR represents a
rapid and reliable method for the detection and quantification of mRNA transcription levels of a selected gene in various biological specimens, or at different developmental stages or different physiological status (McCurley and Callard, 2008; Beekman et al., 2011). However, recently, a growing body of research have demonstrated that these genes expression can change in different tissues, during growth and differentiation, in response to biochemical stimuli, and in disease states (Janovick-Guretzky et al., 2007; Wen and Mao, 2007). The expression levels of the ideal endogenous reference genes should be constant in different experimental conditions. So the limitation for the application of qRT-PCR is the need for suitable internal reference genes which reduce the specimen differences and allow the quantification of this gene expression to be comparable (Huggett et al., 2005). It is well-known that normalization is critically important to reduce sample-to-sample variations, which includes the RT efficiency and RNA integrity, cDNA sample loading, instrumental errors, and the presence of PCR inhibitors etc. (Stahlberg et al., 2004; Bustin et al., 2005). Normalization of target gene expression levels must be performed before doing relative comparisons (Pfaffl et al., 2001). Several normalization strategies have been proposed, and the use of endogenous reference genes is currently the preferred one. An ideal endogenous reference gene used for normalization of data in quantitative real-time PCR should have the following features: constant expression levels among all individuals, organs and cells, during different developmental stages, and various experimental treatments (Jin et al., 2004). Usually, the well-known housekeeping genes are chosen such as the glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actins (ACTB), hypoxantine phosphoribosyltransferase (HRPT), β-tubulin (TUB), elongation factor 1 alpha (EF1A) and 18S, 28S rRNA (Shu et al., 2004; Fernandes et al., 2008; Øvergard et al., 2010). Several commonly used reference genes in studies of domestic fowl gene expression include the ACTB, GAPDH and 18S rRNA. Shu et al. (2004) used ACTB as a reference gene to study the expression level of three novel expressed sequence tags (ESTs) in hypothalamus, pituitary, muscle, liver and fat tissues of Shaoxing ducks (Shu et al., 2004). Scholz chose 18S rRNA as the endogenous reference to analyze the sex-dependent gene expression level in early brain development of chicken embryos by Real-time PCR (Scholz et al., 2006). Yen used ACTB as a reference gene for normalization of data in transcript analysis of pituitary gland genes in laying geese (Ding et al., 2007). Chen used ACTB as a reference gene to analyze relative mRNA expression levels in the hypothalamus/pituitary glands in the Red-feather Taiwan country chicken which show significantly different reproductive performance (Chen et al., 2007). Ding chose 18S rRNA as a reference gene for normalization of data in transcriptional analysis of Vitellogenin I, apoVLDL-II, ethanolamine kinase, G-protein gamma-5 subunit, and leucyl-tRNA synthase expression level in the livers of the laying and pre-laying geese (Ding et al., 2007).

Thus far, the genes encoding GAPDH, ACTB and 18S rRNA have been used as endogenous reference genes for qRT-PCR in geese (Chen et al., 2007; Ding et al., 2007; Kang et al., 2009), but the stability analysis of these candidate genes in geese has not yet been reported. Based on earlier gene expression studies in domestic fowl, we have tested the stability of expression of seven housekeeping genes, including GAPDH, ACTB, HPRT1, TUB, succinate dehydrogenase flavoprotein A (SDH), 18S rRNA and 28S rRNA in this study. The geNorm (Vandesompe et al., 2002), NormFinder (Andersen et al., 2004), BestKeeper (Pfaffl et al., 2004), and the comparative ΔCt method are popular algorithms to determine the most stable endogenous reference genes from a set of tested candidate reference genes in a given experimental condition. Hence three different software tools were used to validate the stability of the selected housekeeping genes in different developmental stages (1 d, 2, 4, 6 and 8 months old) of Zi geese tissues (heart, liver, kidney, muscle, ovary) using real-time qRT-PCR.

**MATERIALS AND METHODS**

**Geese and tissue collection**

The study protocol was approved by the Animal Care Committee of Jilin University. Thirty female Zi geese were randomly selected from one hundred geese in a local breeding farm and raised according to the standard program used at the farm (Daqing, China). Six geese were sacrificed at the age of 1 d, 2, 4, 6 and 8 months respectively. Geese were sacrificed by electrical stunning followed by exsanguination. Heart, liver, kidney, muscle and ovary samples were rapidly removed, wrapped in foil, frozen in liquid nitrogen, and then stored at -70°C until analysis.

**Total RNA isolation**

Total RNA was isolated from the Zi geese tissues (hearts, livers, kidneys, muscles and ovaries) at 1 d, 2, 4, 6 and 8 months respectively according to the Trizol® reagent (Invitrogen, USA) manufacturer’s instructions. Total RNAs were treated with DNase I (RNase Free, Takara, Japan) according to the manufacturer’s instructions to remove contaminations of genomic DNA. Total RNA concentration and purity was determined using a SmartSpec™ Plus spectrophotometer (Bio-Rad, USA). The optical density (OD) ratio A260/A280 nm was measured with the spectrophotometer was 1.95±0.12 (OD A260/A280 ratio± SD).
Reverse transcription

Total RNA (1.5 μg) from Zi geese, 500 ng/μl of random hexamers primer (Promega, USA), 10 mM deoxynucleoside triphosphate (dNTP) Mix (Takara) and sterile MilliQ water (to a total volume of 12 μl) were heated to 65°C for 5 min in order to disrupt possible secondary structures and then quickly chilled on ice. Thereafter, 5×First-Strand Buffer was combined with 0.1 M dithiothreitol (DTT) and 40 units/μl of RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen). The mixture was mixed gently and incubated at 37°C for 2 min. Then a total of 200 units of M-MLV reverse transcriptase was added and incubated at 25°C for 10 min. Reverse transcription was performed at 37°C for 50 min, and the reaction mixture was heated to 70°C for 15 min. The final cDNA products were diluted 10-fold prior to use in real-time PCR.

Primer design

All primers designed for all reference genes were based on sequences published in Genbank (http://www.ncbi.nlm.nih.gov/Table 1). Primer pairs for qPCR amplification were designed using Primer premier 5.0 (http://www.premierbiosoft.com). BLAST searches were performed to confirm the total gene specificity of the primer sequences (http://www.ncbi.nlm.nih.gov/BLAST/). The specificity of all primers was checked by electrophoresis of RT-PCR products on the 1% agarose gel (Figure 1a).

Table 1. Information of the seven candidate genes selected

| Gene name | GenBank accession number | Primer sequences (forward/reverse) | Tm (°C) | Amplicon length (bp) | Amplification efficiency (%) | SD (E) | R² | Average Ct value |
|-----------|--------------------------|-----------------------------------|---------|----------------------|-----------------------------|--------|----|-----------------|
| GAPDH     | AY436595.1               | CTGTCAAGGGCTGAGAATG CAAGGGCATCTGCTGACAA | 55      | 280                  | 97.63                       | 0.007  | 0.998 | 19.83           |
| HPRT1     | NM_204848.1              | TGACTTACCGACCATTGCG CATAGAAGTCCGGTCGAGTTT | 55      | 102                  | 101.35                      | 0.014  | 0.988 | 28.08           |
| SDH       | NM_001080875.1           | ATCCATCGAGCCCTAACGTTACG CATAGAAGTCGAGGTCCAGTTT | 55      | 101                  | 91.28                       | 0.010  | 0.988 | 28.14           |
| ACTB      | M26111.1                 | CCATCTTGGAGGCTTACGTTAT TTAGCAGGACCTGTA TTGCT | 55      | 149                  | 93.43                       | 0.010  | 0.996 | 36.91           |
| TUB       | NM_001080860.2           | GAAGGAGCCAGGGAAACCAGG CCAATCCACACCACAAGA | 55      | 151                  | 96.84                       | 0.014  | 0.998 | 32.67           |
| 28S rRNA  | EF552792.1               | ATTTTCCTGTTCCCTACCTCT ATCTTCTATCTCTTACACCCACCTCTCT | 55      | 144                  | 98.03                       | 0.016  | 0.994 | 33.53           |
| 18S rRNA  | L21170.1                 | ACACCGACAGGATGCAACGCA GTCTCCGGTGCTGACAGG | 55      | 199                  | 103.54                      | 0.008  | 0.998 | 30.10           |

For each reference gene, gene name, GenBank accession number, primer sequences, Tm value, amplicon length, amplification efficiencies (E) and its standard deviation (SD (E)), Pearson’s coefficients of determination (R²) and average cycle threshold values are indicated.

Real-time qPCR

The qRT-PCR was performed on the first strand cDNA using the Line-Gene K Real-time PCR Detection System and software (Bioer, China) with SYBR® Premix Ex Taq™ (Takara). Briefly, each reaction (50 μl) consisted of 1 μl 10-fold diluted cDNA template, 25 μl of SYBR® Premix Ex Taq™ (2×Concentration), 0.5 μl of 20 μM of PCR Forward Primer and PCR Reverse Primer, and 23 μl of nuclease-free water. Thermal cycling was performed with an initial denaturation step of 10 s at 94°C, followed by 45 cycles of 5 s at 94°C, and 56°C for 30 s, and then a final extension at 72°C for 20 s. Finally, a dissociation curve was generated by increasing temperature starting from 65 to 95°C to determine the specificity of the reactions. The crossing cycle number (Cp) was automatically determined for each reaction by the Line-Gene K Real-Time PCR Detection System and software (Bioer) with default parameters using the second derivative method. As a control for genomic DNA contamination, an equivalent amount of total RNA without reverse transcription was tested for each reaction. A no-template control (NTC) was also included in each reaction. Relative quantitation of gene expression was performed using three replicates for each sample. The quality of standard curves was judged by the slope of the standard curve and the square of the Pearson correlation coefficient (R²). The PCR amplification efficiency of each primer pair is calculated from the slope of a standard curve using the following equation: Efficiency % = (10^(-1/slope)-1)×100% (Bustin et al., 2009).

Statistical analysis

Differences in expression levels of GAPDH, ACTB, HPRT1, TUB, SDHA, 18S rRNA and 28S rRNA with developmental stage were examined by one-way ANOVA. The IBM SPSS statistics 17.0 package (IBM, USA) was used for all analyses. Significance levels were set at p<0.05. Determination of reference gene expression stability were calculated using geNorm, NormFinder and BestKeeper.
For evaluation of expression stability of the candidate reference genes, Ct values for all samples were calculated and the stability of the genes was determined utilizing three different software tools: geNorm, Normfinder and BestKeeper. The gene expression stability (M) and the optimal number of endogenous reference genes for normalization were determined by using the geNorm algorithm as previously described (Vandesompe et al.,
The second algorithm utilized was NormFinder as previously described by Andersen (Andersen et al., 2004). It is an algorithm that attempts to find the optimum reference genes out of a group of candidate genes. It can also, in contrast to geNorm, take information of groupings of samples into account, such as treatment/control,

![Graphs of ACTB, 18S rRNA, HPRT1, and TUB](image-url)
sick/healthy, or different developmental stages. The BestKeeper algorithm creates a pairwise correlation coefficient between each gene and the BestKeeper index (BI). This index was then compared to each individual candidate housekeeping gene by pair-wise correlation analyses, with each combination assigned a value for the Pearson correlation coefficient (r) and the probability (p) (Pfaffl et al., 2004). The gene with the highest coefficient of correlation with the BI indicates the highest stability.

RESULTS

Selection of candidate reference genes and primer design
We have investigated seven housekeeping genes commonly used as internal controls in expression studies, including the GAPDH, ACTB, HPRT1, TUB, SDHA, 18S rRNA and 28S rRNA. The primers were designed according to the Zi geese mRNA sequences which are available in GenBank (Table 1).

Quality control of the nucleic acids and qPCR
The optical density (OD) ratio A260/A280 of the RNA was 1.8 to 2.0. Agarose gel electrophoresis (Figure 1b) that 28S:18S ratio was approximately 2:1 indicated that the RNA was intact. Agarose gel electrophoresis (Figure 1a) and melting curve analysis (Figure 1c and Table 1) revealed that all primer pairs amplified a single PCR product with the expected size. Furthermore, sequence analysis of cloned amplicons revealed that all sequenced amplified fragments were identical or nearly identical to the sequences generated by the designed primers. In order to quantitatively determine the transcriptional level of each candidate gene, the average cycle threshold (Ct) value of each gene was calculated (Table 1). As expected, the average Ct value of different gene varied. A standard curve using a dilution series of the cloned amplicons was made to calculate the gene-specific PCR efficiency. The correlation coefficient (R²) of the slope of the standard curve, the PCR amplification efficiency (E) and the PCR efficiency standard deviation (SD) of each gene were listed in Table 1. All primer pairs utilized in this study presented amplification efficiency between 91 to 104% (Table 1).

Expression stability of candidate reference genes
Three different software tools were used to calculate the expression stability of the candidate reference genes: geNorm, NormFinder and BestKeeper.

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the least stable genes. In kidney tissues the best correlations were obtained for ACTB (r = 0.975), SDH (r = 0.943) and GAPDH (r = 0.936) with p value of 0.001. TUB are ranked as the least stable genes. In muscle tissues the best correlations were obtained for HPRT1 (r = 0.980) and 18S (r = 0.979) with p value of 0.001. TUB are ranked as the least stable genes. In ovaries tissues the best correlations were obtained for GAPDH (r = 0.914) and SDH (r = 0.876) with p value of 0.001. 28S are ranked as the least stable genes.

**DISCUSSION**

Real-time qRT-PCR, with its capacity to detect and
measure very small amount of nucleic acids in a wide range of samples from numerous sources, has been used extensively in molecular biology, e.g., molecular diagnostics, life sciences, agriculture, and medicine. Normalization is required to reduce the tube-to-tube variations caused by variable RNA quality or reverse transcription efficiency, inaccurate quantification, and pipetting etc (O’Connell, 2002). Endogenous reference genes are thus commonly used to normalize the expression levels of analyzed genes. ACTB, together with GAPDH, TUB, EF1A and 18S rRNA, are expressed constitutively and are involved in basic housekeeping functions required for cell maintenance. Because of this, they are commonly used as reference genes to normalize gene expression studies (Sturzenbaum and Kille, 2001; Jin et al., 2004; Wen and Mao, 2007; Øvergård et al., 2010). Recently, a growing body of research has demonstrated that these genes expression can change in different tissues, during growth and differentiation, in response to biochemical stimuli, and in disease states (Wen and Mao, 2007; Janovick-Guretzky et al., 2007). The expression levels of the ideal endogenous reference genes should be constant in different experimental conditions. The geNorm, Normfinder, BestKeeper, and the comparative ΔCt method are popular algorithms to determine the most stable endogenous reference genes from a set of tested candidate reference genes in a given experimental condition.

In this study, we have selected seven candidate reference genes from Genbank to analyze their candidacy to be used as reference genes. Also, we have developed a qRT-PCR method for GAPDH, HPRT1, ACTB, 18S, 28S, SDHA and TUB as the target gene. The specificity of the qRT-PCR primer pairs was confirmed by agarose gel electrophoresis, Tm analysis and sequencing of the amplicons. The PCR amplification efficiency was estimated, and the reference genes were ranked according to their expression level stability across various developmental stages in Zi geese tissues using geNorm, Normfinder and BestKeeper algorithms. When gene expression stability in Zi geese was analyzed by geNorm, which had been recently noted as one of the best methods to determine the most stably expressed genes for qRT-PCR analysis (10, 8), the most stable genes in the seven series were different as shown in Table 2 and Figure 2. In different developmental stages of Zi geese heart tissues, the stability rank was GAPDH, 28S>SDH>ACTB>18S>HPRT1>TUB, and the optimal number of reference genes was four. In order to avoid co-regulation, we have also determined the stability of the selected genes using Normfinder and BestKeeper. The 28S, SDH, GAPDH and HPRT1, as identified by both the NormFinder and the BestKeeper tools, were the four most stable genes, which supported the geNorm analysis in this experiment. The stability of the four genes, from the highest to the lowest, was 28S, SDH, GAPDH and HPRT1. In liver tissues, the stability ranking was GAPDH, HPRT1>SDH>TUB>28S>18S>ACTB, and optimal number of reference genes was three. In kidney tissues, the stability ranking was ACTB, SDH>GAPDH>HPRT1>28S>18S>TUB, and the optimal number of reference genes was three. In conclusion, the three algorithms did not rank the candidate reference genes in the same order, but all indicated that ACTB, SDH and GAPDH should be the most stably expressed genes in the experimental conditions applied in this study. In muscle, the stability ranking was HPRT1, 18S>GAPDH>28S>SDH>ACTB>TUB, and the optimal number of reference genes was two. In ovary, the stability ranking was GAPDH, HPRT1>SDH>TUB>18S>ACTB>28S, and the optimal number of reference genes was two. The NormFinder identified that GAPDH and HPRT1 to be the two most stable genes whereas, in contrast, the BestKeeper identified GAPDH and SDH to be the two most stable genes.

**IMPLICATIONS**

This research is the first attempt to validate a set of commonly used candidate reference genes in various tissues by applying different algorithms. The results show that the stability of reference genes varies among different tissues and developmental stages. This study highlights the importance of selecting appropriate reference genes for accurate gene expression analysis, especially in non-model organisms like Zi geese, where the use of endogenous reference genes is not well-established. The use of multiple algorithms ensures a more robust and reliable approach to reference gene selection. Further research is needed to validate the selected reference genes in different contexts and conditions to ensure their suitability for a wide range of applications.
developmental stages in Zi geese tissues for the normalization of gene expression using qRT-PCR. Analysis of stability using geNorm, NormFinder and BestKeeper reveals that the geometric mean of GAPDH, 28S, SDH and HPRT1 (in heart), GAPDH, HPRT1 and SDH (in liver), ACTB, SDH and GAPDH (in kidney), HPRT1 and 18S (in muscle), and GAPDH and HPRT1 (in ovary) are recommended to be used as reference genes in Zi geese. These methods may further be employed to identify the most stable reference genes in other tissues or under other experimental conditions in the future studies on geese. Also, this study may serve as a good foundation for further studies on how to improve the economic traits of geese, both in egg, fatty livers and meat production.

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