Comprehensive evaluation the stability of internal control genes for qRT-PCR normalization in cuttlefish *Sepiella japonica* using geNorm, NormFinder, and BestKeeper

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Abstract: Cuttlefish, *Sepiella japonica*, is an economically important cephalopoda species in East China Sea. Recently, a growing number of publications on biological research of this species have been made, and many of them were dealing with gene expression analysis. Quantitative real-time PCR (qRT-PCR) is a widely used method for analyzing gene expression, with its accuracy heavily reliant on selecting appropriate reference genes for normalization. So far, no studies on internal control gene screening have been reported in cuttlefish. Therefore, to obtain reliable results from qPCR analysis, six commonly used candidate reference genes were assessed in various tissues in both male and female cuttlefish, namely *EF-1γ*, *EF-1α*, *GAPDH*, *β-actin*, *18S rRNA*, and *Tubulin*. According to the results, although the Ct values of each gene varied across tissues, the Ct ranges of *EF-1α* and *EF-1γ* were comparatively narrow, indicating that these two genes had more constant expression levels. Furthermore, in a small number of identical tissues of males and females, a significantly different fluctuation range of Ct values for six genes except *Tubulin* was observed, implying that the corresponding gene may not be suitable as the internal controls when gene expression is evacuated between two genders. The online-tool RefFinder, which contains four algorithms—geNorm, Normfinder, BestKeeper, and Delta-Ct, was used to assess the stability of the candidate reference genes. The findings showed that both male and female cuttlefish displayed tissue-specific rankings in every algorithm. In each gender, when considering all tissues as one single group, almost four algorithms predicted that the two most recommended genes in both males and females were *EF-1γ* and *EF-1a*, followed by *GAPDH*. This conclusion was further supported by comprehensive ranking results. Besides, in most tissues, the optimal number of control genes was two; in a few, it was three. These findings suggested that, overall, *EF-1γ* and *EF-1a* were more suitable as the reference genes than others, with *GAPDH* coming in next.

①Expression profiling of candidate reference genes

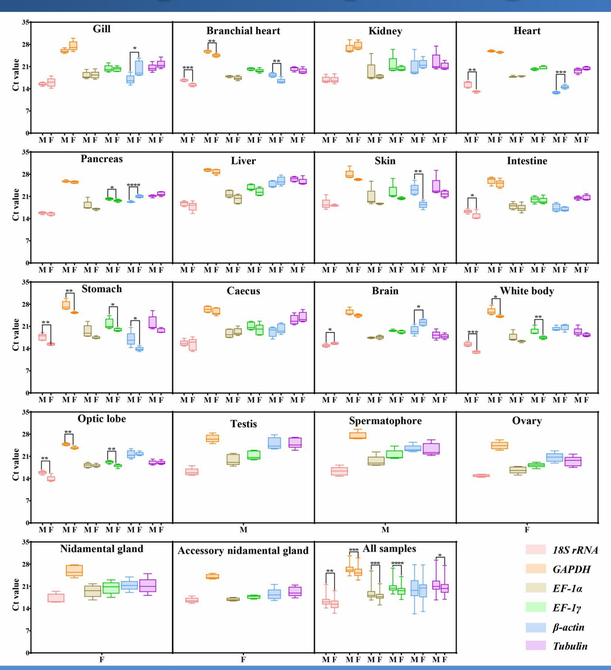

Figure 1. Range of Cycle threshold (Ct) values of the candidate reference genes in different tissues of cuttlefish. Medians are indicated by lines across the box. whisker caps denote the maximum and minimum values. Different colors indicate different candidate genes. M, male. F, female. n = 5. Statistical significance between male and female groups was performed using unpaired two-tailed *t*-test *, $p < 0.05$. **, $p < 0.01$. ***, $p < 0.001$. ****, $p < 0.0001$.

②geNorm analysis

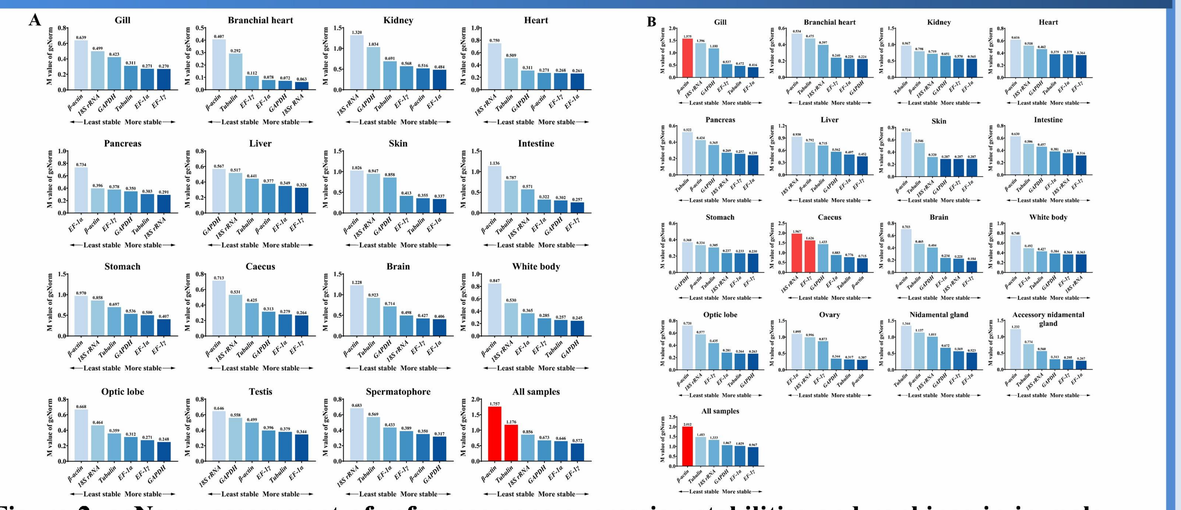

Figure 2. geNorm assessment of reference gene expression stabilities and rankings in in male (A) and female (B) cuttlefish.. Six candidate reference genes are ranked according to stability values (M values) assessed by geNorm algorithm. The darker the blue column, the more stable the reference gene. Gene with a M value greater than the cut-off value of 1.5 is not suitable as an internal reference gene, which is highlighted in red column.

③NormFinder analysis

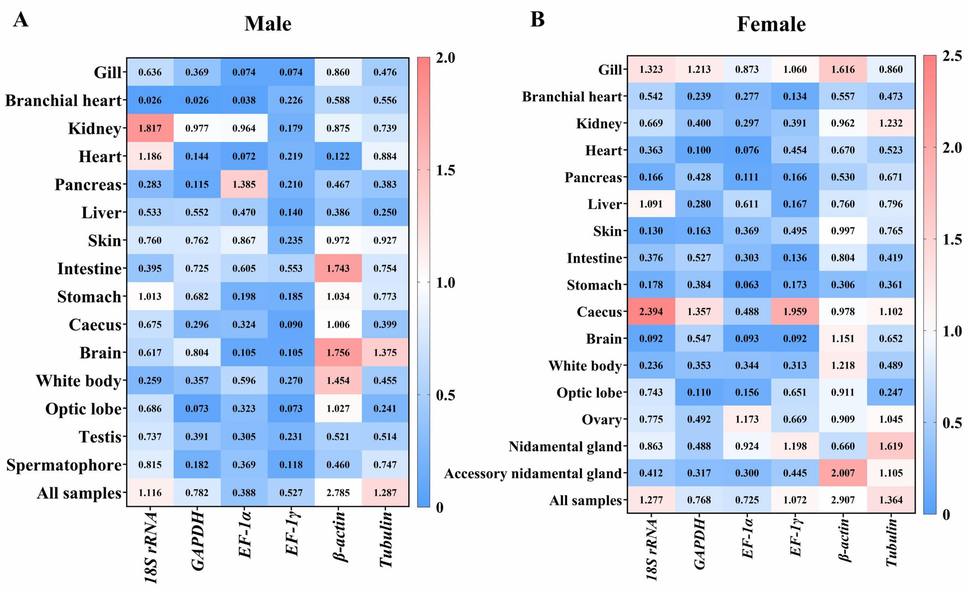

Figure 3. Expression stability and ranking of reference genes as determined by NormFinder in male (A) and female (B) cuttlefish. The stability values of each gene in different tissues are exhibited. Lower values (blue) indicate higher stability, higher values (red) indicate lower stability.

④BestKeeper analysis

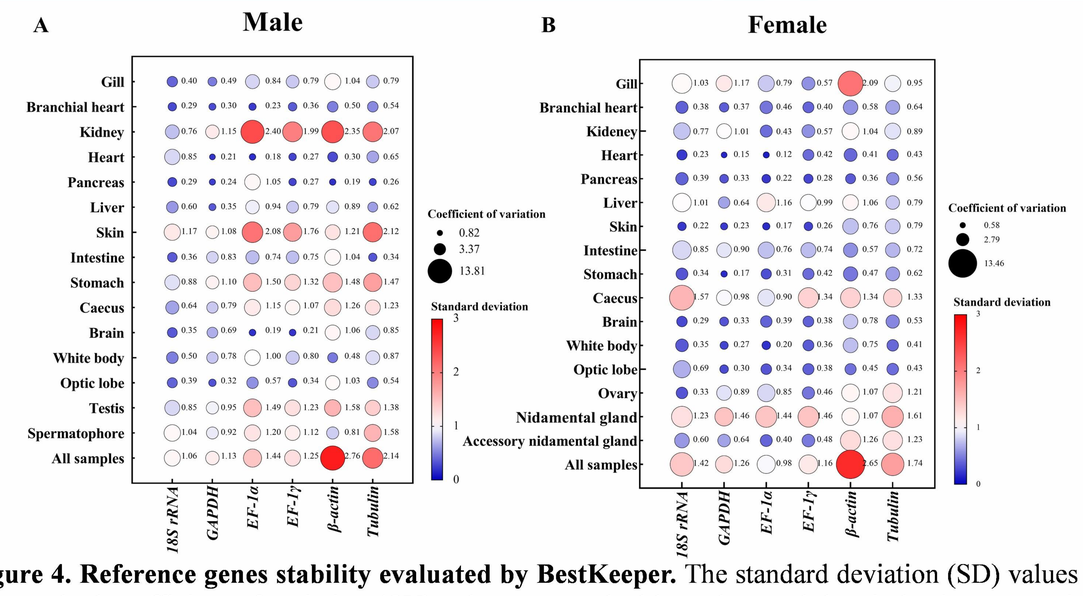

Figure 4. Reference genes stability evaluated by BestKeeper. The standard deviation (SD) values and the standard coefficient of variation (CV) values are marked by colors and the circle size, respectively. The values displayed are SD values. Lower values (blue) and smaller circle indicate higher stability, higher values (red) and larger circle indicate lower stability.

⑤Comprehensive ranking of reference genes

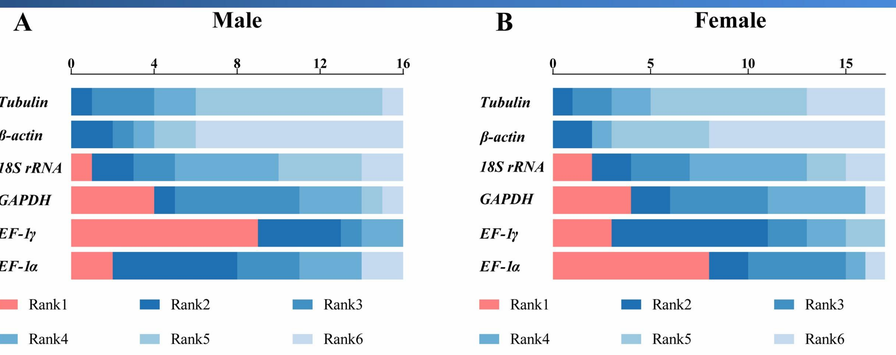

Figure 5. The proportion of each gene across the six rankings in all samples

⑥The optimal number of internal control genes

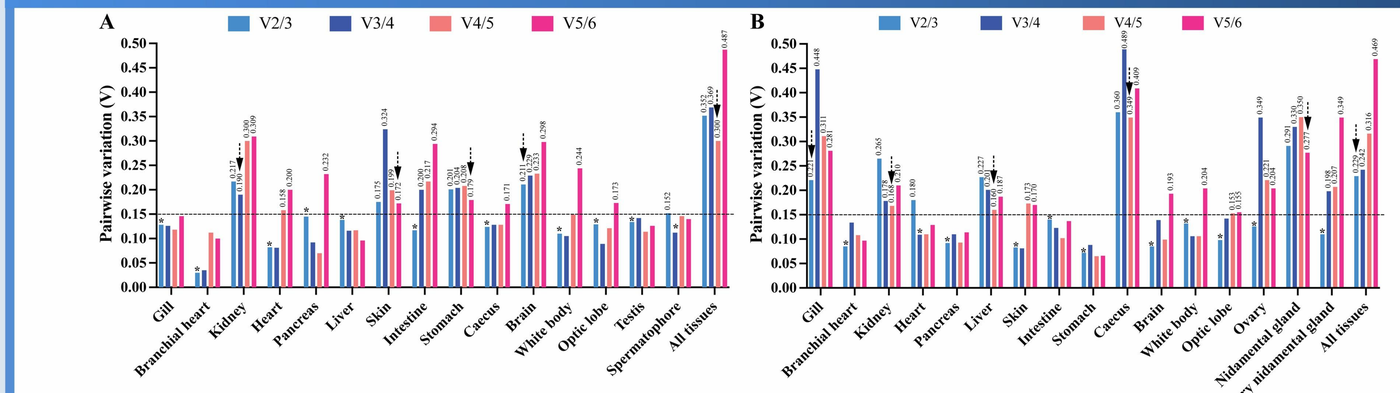

Figure 6. Determination of the optimal number of reference genes for normalization for male (A) and female (B). The dotted line represents the cut-off value of 0.15 for pairwise variation (V) analysis. When Vn/n+1 < 0.15, n indicates the ideal number of reference genes. The optimal number of reference genes for each group is marked with an asterisk. The dashed arrows indicate the Vn/v+1 values are closest to 0.15.

Conclusions

Among the six candidate reference genes, *EF-1α* and *EF-1γ* exhibited the most consistent Ct values across different tissues, indicating greater stability compared to *β-actin* and *Tubulin,* which showed significant variation. Additionally, except for *Tubulin*, the Ct values of at least one reference gene differed significantly between male and female cuttlefish within the same tissue. Stability rankings from four analysis methods showed both similarities and differences, but overall, *EF-1α* and *EF-1γ* were the most stable genes in both males and females. In male cuttlefish, the stability ranking was *EF-1γ* > *EF-1α* > *GAPDH* > *18S rRNA* > *Tubulin* > *β-actin*, while in females, it was *EF-1α* > *EF-1γ* > *GAPDH* > *18S rRNA* > *Tubulin* > *β-actin*. Two reference genes were optimal for most tissues, while three genes were required for the spermatophore and female heart. For tissues with Vn/n+1 values greater than 1.5, the optimal number of reference genes was based on the lowest Vn/n+1 value.