Manuscript Review

Manuscript Number: #28089

Full Title: **Evaluation of potential reference genes for 1 quantitative RT-PCR analysis in spotted sea bass (*Lateolabrax maculatus*) under normal and salinity stress conditions**

Authors: Wang et al.

The study reported to validate 9 candidate reference genes (HPRT, GAPDH, EF1A, TUBA, RPL7, RNAPol II, B2M, ACTB and 18S rRNA) under normal physiological condition and different chronic salinity-stress conditions in spotted sea bass (*Lateolabrax maculatus*). All the 9 genes have been validated using four different algorithms (geNorm, NormFinder, BestKeeper, and comparative ΔCt method) and overall ranking has been assigned through RefFinder tool. Although, it's a well-planned attempt to validate the reference genes in this species, the manuscript lacks clarity in different sections such as experimental design, discussions and need further improvement. There are several studies being reported in different teleosts on reference gene validation, hence it would have been more convincing if the authors used a gene expression model by selecting any stress-responsive gene to show a relative expression calculation how a best reference gene and least stable gene effect the expression pattern.

The major and minor concerns that arose while reviewing this manuscript are outlined below:

Major concerns:

- The authors have validated the reference genes under salinity stress after keeping the fish in a 30-day experimental condition. As, *Lateolabrax maculatus* is a euryhaline fish, it is understandable that the fish will try to cope up with the stress and they will get acclimatized to its new rearing condition. Hence, it would have been more useful if the authors tried to validate the reference genes under acute salinity stress conditions at initial hours or days after keeping the fish in different salinity conditions. Moreover, the authors have not mentioned in which salinity the fish were acclimatized for a week (Line no. 111-112 of Material and method section). For example, if the fish were acclimatized at seawater of 30 ppt, then the salinity stress condition of 30 ppt under the salinity stress experiment (Line no. 126-127) doesn't qualify as a stress condition.
- Under the Material method section 1, it is mentioned that the 10 tissues were taken from nine healthy fish, which constitute three separate pools. However, in contrast, only three fish were sacrificed from each salinity stress group and only gill tissue was taken for further RNA processing (Line No. 121-123). Further, under section 2 it has been mentioned that RNA was extracted from all the ten tissues of salinity stress groups from 3 pools. So, it is really hard to understand which exact experimental plan the authors have followed.
- The authors did not provide any details regarding removal of trace genomic DNA that might be present during RNA isolation, especially when it is a non-column based purification

method like Trizol. This information is very important for performing qRT-PCR and more precisely when validating the reference genes as genomic information can be detected and quantified using these methods, which will be misleading.

- The discussion section would have been more meaningful, had the result obtained in the present study been discussed or compared with the already reported studies of reference gene validation in related euryhaline species, more precisely, other seabasses such as European seabass (Mitter et al., 2009) and Asian seabass (Paria et al., 2016).
- Line No. 249: It has been mentioned RefFinder assign the overall ranking by accumulating four algorithms (geNorm, NormFinder, BestKeeper, and the comparative ΔCt method), while in Line No. 280 it says raw Ct values were used for computation through RefFinder. So, it is difficult to understand what is the input authors have used while predicting the overall ranking. Otherwise, authors could have compared the individual ranking obtained from individual algorithm and compiled the overall ranking.

Minor Comments:

- Line No. 55: It will "gene expression" instead of "genes expression"
- Line No. 121: "After breeding for 30 days". Is it rearing for 30 days?
- Line No. 149, 169: It should be either Applied Biosystems 7500 Real-Time PCR system or StepOne Plus Real-Time PCR system. In two places two different machines are mentioned
- In the abstract, results and material and method section (Line No. 45, 157, 190), it is mentioned that four algorithms and one online program (RefFinder) were used, whereas in several places of discussion and conclusion it is mentioned as five algorithms (Line no. 269, 312 ....). It should be uniform.
- Line No. 152: Concentration of the primer should be mentioned.
- Line No. 315: "may be is an appropriate" It will be "may be an appropriate"

Recommendation

- The manuscript cannot be recommended for publication in the present form.