Phylogenetic relationships of amphioxus in the West Pacific Ocean and a preliminary test on *Asymmetron lucayanum* regeneration

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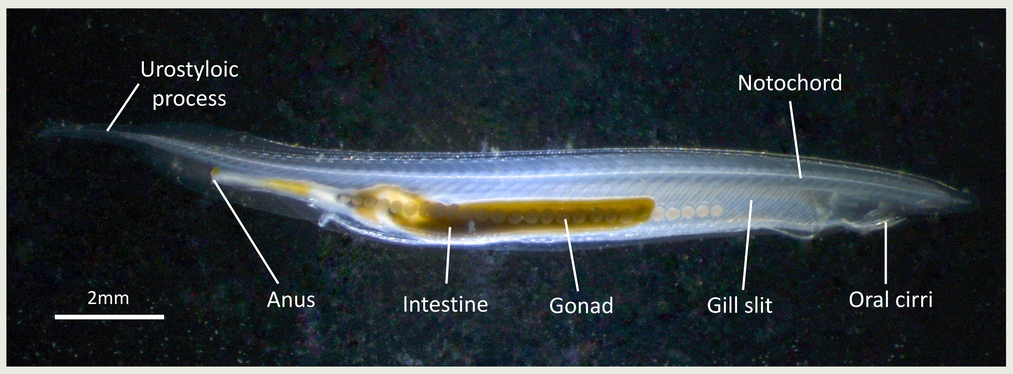
ABSTRACT

Amphioxus, also named as lancelet, is a chordate classified under subphylum cephalochordata. Amphioxus is very important in the study of vertebrate evolution, especially in the origin of vertebrates. There are 3 genera and 30 species of amphioxus which has been discovered. Previous studies have proposed the phylogenetic relationships of a few amphioxus species. In this study, we focused on the species in the West Pacific Ocean, including all the three genera, *Branchiostoma belcheri*, *B. japonicum*, *Asymmetron lucayanum* (2 cryptic species), *Epigonichthys australis*, *E. cultellus*, *E. maldivensis*. Among these species, phylogenetic relationship of *E. australis* has never been studied based on molecular markers. Moreover, some species were only analyzed based on a single molecular marker. Amphioxus has been known for its ability in regeneration. Even so, regeneration studies were mostly applied on genus *Branchiostoma*, but not in the basal genus *Asymmetron*. This study will be focusing on the regeneration of *Asymmetron lucayanum* at the anterior part, which still remains unknown.

SAMPLE COLLECTION

Amphioxi were captured at different sites by SCUBA diving and were brought back to land with sand. Mix the sand with seawater and ethanol in a pail thoroughly, the amphioxi would come out of the sand and stay in the seawater. Pour the seawater on a black cloth, use a brush to pick up the amphioxi which appeared translucent on the cloth. For the phylogenetic analyses, the samples were kept in 95% ethanol. For the regeneration test, each amphioxus was kept in a 50ml tube with some sand and seawater.



PHYLOGENETIC RELATIONSHIPS

DNA extraction

Total DNA was extracted from each individual using Tissue & Cell Genomic DNA Purification Kit (Biokit, Taiwan) following the manufacturer's instructions.

PCR amplification

PCR was performed to amplify the 12S rRNA mitochondrial gene with the primer set EMTT/Val (Lin, 2001). The PCR reaction was conducted under the following conditions: 3 min 30 sec at 94°C for initial denaturing, 35 cycles of 1 min at 94°C, 45 sec at 52°C, 1 min at 68°C with a final extension for 10 min at 72°C. The length of the PCR products was confirmed by running agarose gel. The PCR products were sent to the biotechnology company for DNA sequencing.

Sequence analyses

All sequences including the outgroup (two hagfish species) were aligned with CLUSTAL W and adjusted by eye in Geneious. The sequences were trimmed to 915bp. Maximum likelihood tree was constructed using Tamura-Nei model with 1000 replications in MEGA X.

PRELIMINARY RESULT

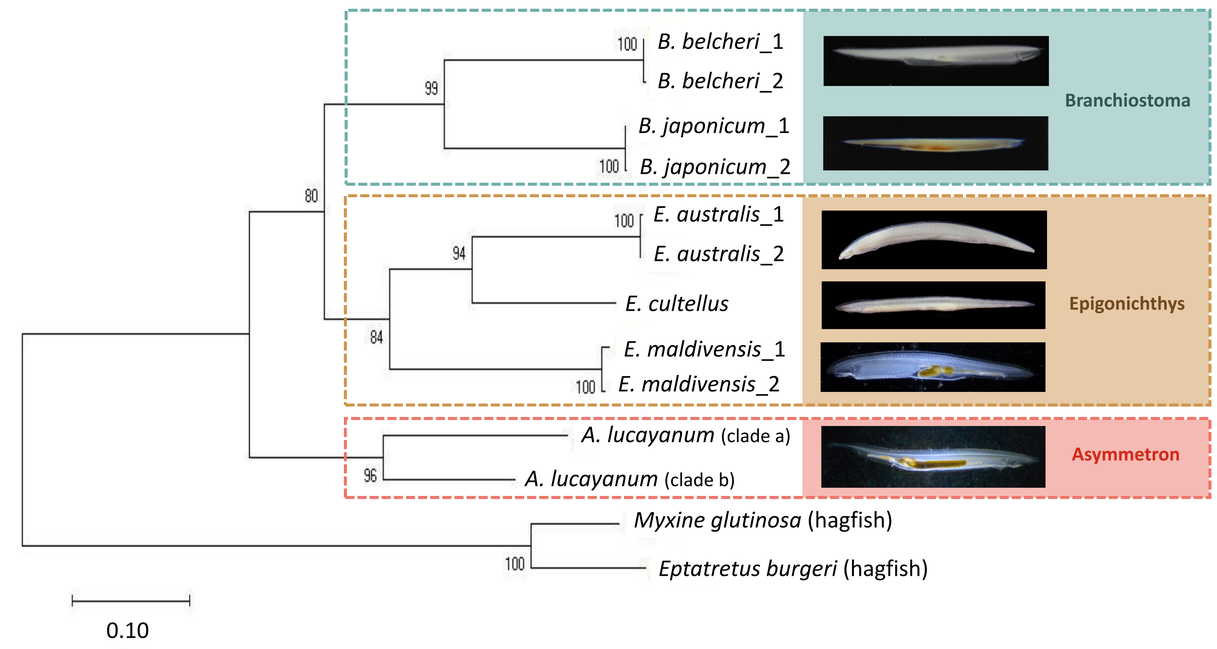


Fig. 1. 12S rRNA maximum likelihood tree using Tamura-Nei model with 1000 replications and two hagfish as outgroup. Value at the nodes are bootstrap values. Scale bar indicates number of expected substitutions per site.

REGENERATION OF *Asymmetron lucayanum*

LABORATORY CULTURE

Each amphioxus was placed in an 80ml beaker with 1cm depth of sand and sterilized seawater filled up to 80ml.

Diet - microalgae

Tetraselmis chui: *Nannochloropsis oculate*: *Skeletonema costatum*: *Isochrysis galbana*: *Chaetoceros gracilis* = 1:1:1:2.
6ml of 54,560 cells/ml algae mixture were fed daily.

Seawater

The seawater was changed daily. The feces was sucked away with 20ml of seawater. Sterilized seawater was then added to fill up to 80ml.

Air supply

Air bubbler was set up for each beaker with 24-hour air supply.

AMPUTATION OF THE ANTERIOR PART

An amphioxus was placed on a 9cm petri dish with seawater. The petri dish was then placed on ice for 120 to 150s. The water temperature started to drop and when the temperature reached 5-7°C, use a droplet to touch the amphioxus to check its consciousness. The amphioxus was ready to be cut when it has no response.

ANTERIOR PART REGENERATION

Fig. 2. The amphioxus was under healing process during the first week.

TAIL REGENERATION

Fig. 3. An amphioxus was injured at the posterior part during the capture process. It was observed for 110 days for the regeneration process.

FUTURE STUDY

We have successfully cultured amphioxus in the laboratory and observed the regeneration of the tail. The amphioxus has remained alive for a week and was in the wound healing process after the anterior amputation. We will do a longer-term observation of the anterior regeneration and increase the sample size to make a final conclusion. For the phylogenetic relationships, we will add sequence data from more nuclear markers in addition to mitochondrial markers, to make our result more robust.

REFERENCES

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Ildikó M. L. Somorjai, Rajmund L. Somorjai, Jordi Garcia-Fernàndez, and Hector Escrivà (2011) Vertebrate-like regeneration in the invertebrate chordate amphioxus. PNAS: Vol. 109, 517-522.