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Reconsideration of skipjack otolith microstructural analysis for age and growth estimates in the WCPO

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Reconsideration of skipjack otolith microstructural analysis for age and growth estimates in the Western Central Pacific Ocean

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Abstract

Reliable estimate of growth curve of skipjack tuna in the western and central Pacific Ocean (WCPO) is an important not only to derive accurate spawning biomass estimates from stock assessment but also to evaluate underlying hypothesis of growth and spawning ecology of this species in the WCPO. Generally, otolith daily increment has been applied to estimate growth, however, large measurements errors were reported due to complexity of otolith microstructure of this species (Sardenne *et al*., 2015). In this document, we reconsidered a series of procedure for otolith analysis (e.g. extraction of otolith, preparation for interpretation of microstructure) targeting juvenile to adult in order to obtain reliable growth estimates of skipjack tuna in the WCPO.

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1 Introduction

Skipjack tuna *Katsuwonus pelamis* is a commercially important and highly migratory species distributing from tropical to temperate waters in the world (Matsumoto *et al*., 1984). In the Western Central Pacific Ocean (WCPO), tropical area is the main fishing ground of skipjack tuna for purse seine operated by various countries and Japanese distant water pole-and-line fisheries. Near Japanese waters are also important area for the Japanese small scale coastal troll, offshore and coastal pole-and-line, and offshore purse seines.

Estimating age and growth of this species is an important scientific role to assess this stock accurately because there still remains large uncertainties in the current stock assessment model (e.g. McKechnie *et al*., 2016; Ochi *et al*., 2016).

Generally, counting of daily and yearly rings formed in otolith has been considered for age determination in various fishes (Jones, 1986). The age determination in skipjack tuna has been also carried out based on the number of the otolith rings. In the early juvenile stage [ca. 10–40 mm in Standard Length (SL)], a method polishing the both sides of the embedded otolith were considered in several studies (Tanabe, 2002; Tanabe *et al*., 2003a). Tanabe *et al*. (2003a) suggested availability of the daily increments of otolith rings in juvenile skipjack tuna for age determination. From young to adult stages, following two methods were considered: 1) slicing method (read the micro-increments on the cross section cut along the transversal axis direction, Adam *et al*., 1996; Leroy, 2000; Sardenne *et al*., 2015); 2) etching method (dissolve the distal face of otolith with 10% HCl to expose the micro-increments, Wild and Foreman, 1980; Uchiyama and Struhsaker, 1981; Wild *et al*., 1995; Tanabe *et al*., 2003b; Kayama *et al*., 2007). Daily increment formation of otolith used by the slicing method is commonly recognized in some species of *Thunus* (Wild and Foreman, 1980; Wild *et al*., 1995; Stéquert and Conand, 2004; Sardenne *et al*., 2015). In young to adult skipjack tuna [250–570 mm in Fork Length (FL)], however, daily increments were not recognized on the marginal zone of otolith by the slicing method. Moreover, large measurement errors have been reported due to some complexity of otolith microstructure of this species, leading to the conclusion that the number of increments of otolith rings is not suitable for the age determination of skipjack tuna (Adam *et al*., 1996; Sardenne *et al*., 2015). On the other hand, etching method targeting young to adult skipjack tuna (180–710 mm FL) has been validated for use of daily formation of the microstructures on the marginal zone by observation of oxytetracycline (OTC) marked otoliths extracted from recaptured individuals (Tanabe *et al*., 2003b; Kayama *et al*., 2007). However, no documents about the procedure for otolith analysis targeting juvenile to adult skipjack have been reported previously. In addition, it

is very difficult to expose and identify daily ring of otolith uniformly by the etching method and it needs experienced technique based on long-term training.

In this document, we reconsidered a series of procedure for otolith analysis (e.g., extraction of otolith, exposure, identify and count daily ring) of skipjack tuna targeting juvenile to adult stages in order to obtain reliable growth estimates of this species in the WCPO.

2 Terminology for otolith

Terms used for each part of otolith in skipjack tuna follows Secor *et al*. (1991), and all otoliths used in this document indicates sagitta.

Rostrum (**Fig. 1A, 2A**): Anterior-most projection
Postrostrum (**Fig. 1B, 2B**): Posterior-most projection
Antirostrum (**Fig. 1C, 2C**): Anterior projection, located dorsal to the rostrum
Core (**Fig. 1D, 2D**): Central part (calcified area occurring within the earliest deposited increment)
Sulcus (**Fig. 2E**): Sculptured groove along the otolith medial face

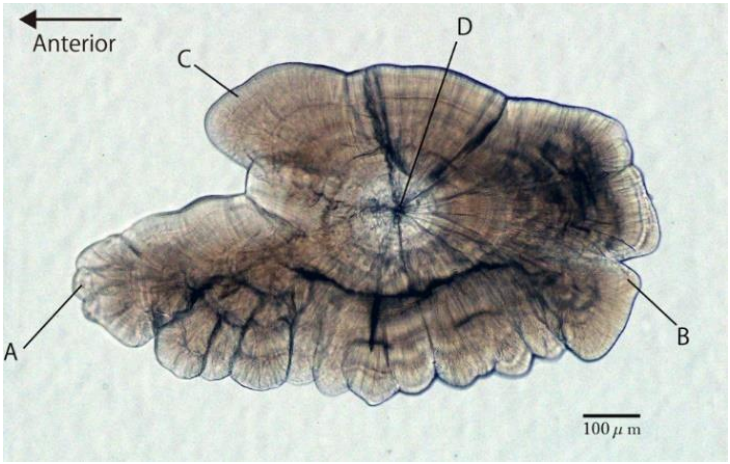


Fig. 1. Lateral view of otolith of juvenile skipjack tuna (A: Rostrum; B: Postrostrum; C: Antirostrum; D: Core).

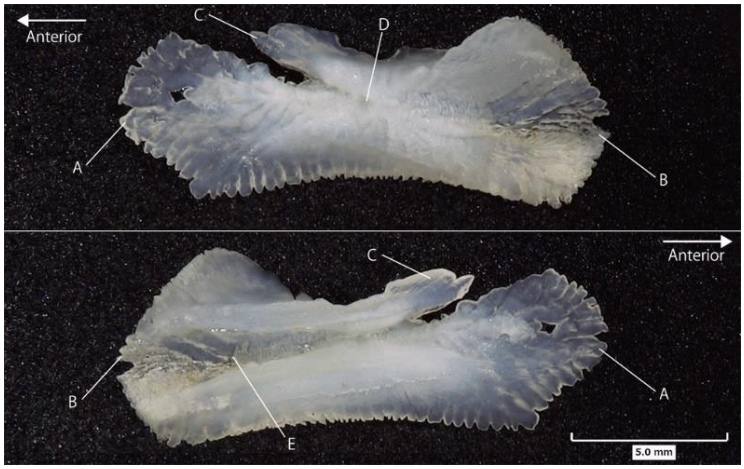


Fig. 2. Lateral (upper) and medial (lower) views of otolith of adult skipjack tuna (A: Rostrum; B: Postrostrum; C: Antirostrum; D: Core; E: Sulcus).

3 Definition of growth stages

Definition of each growth stage is as follows (Tanabe, 2002; Ashida *et al*., 2007).

Juvenile: 10–100 mm SL (Fig. 3A)
Melanophores seen in larval stage disappeared, body color is dark dorsally, but silvery white ventrally, and the number of each fin-ray reaches the fixed number.

Young: 100–200 mm FL (Fig. 3B)
Morphological characteristic (e.g. shape of each fin, bulge of caudal peduncle) of the skipjack tuna begins to appear, and the number of gill rakers reaches the invariable.

Sub-adult: 200–400 mm FL (Fig. 3C)
Abdominal four to six black longitudinal stripes begin to appear. Morphological features are similar to the adult, but not yet matured.

Adult stage: > 400 mm FL (Fig. 3D)
All morphological features are fully developed and some individuals attain sexual maturity.

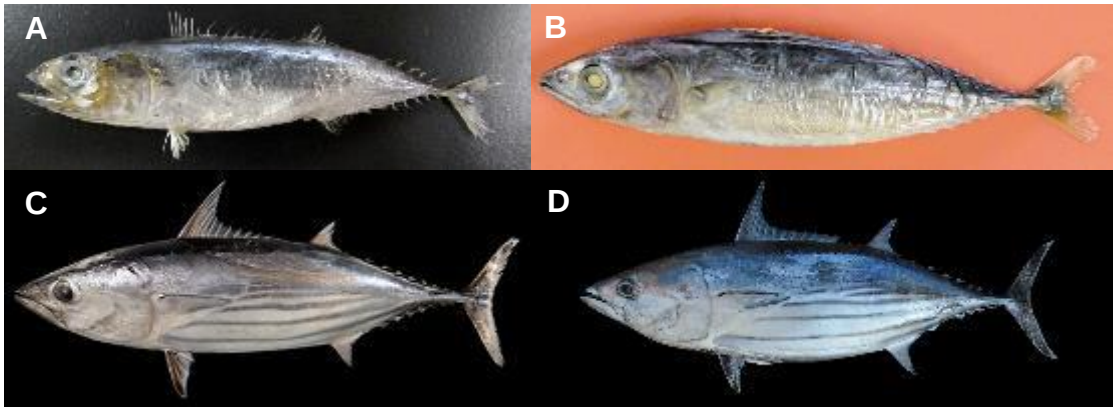
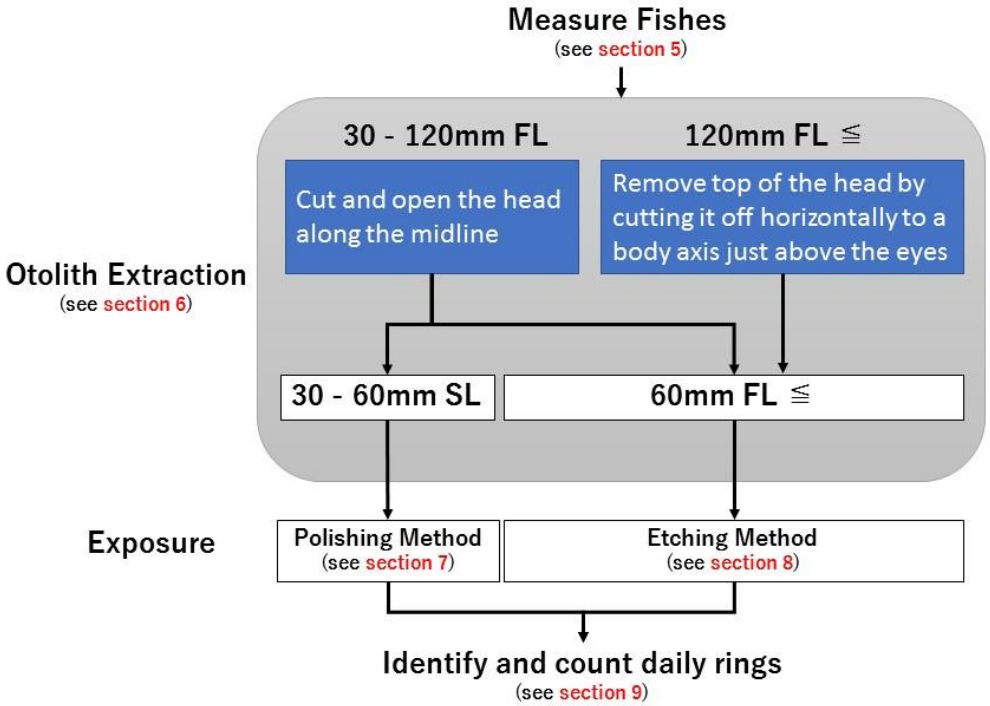


Fig. 3. Specimens of skipjack tuna. A: 70 mm FL, B: 120 mm FL, C: 380 mm FL, D: 550 mm FL.

4 A series of procedure for otolith treatment (Flow chart)



5 Measurements of specimens

For specimens of skipjack tuna, length and weight are measured before extraction of otolith. Standard length (SL) is measured for juvenile length and fork length (FL) is measured for young to adult length (**Fig. 4**).

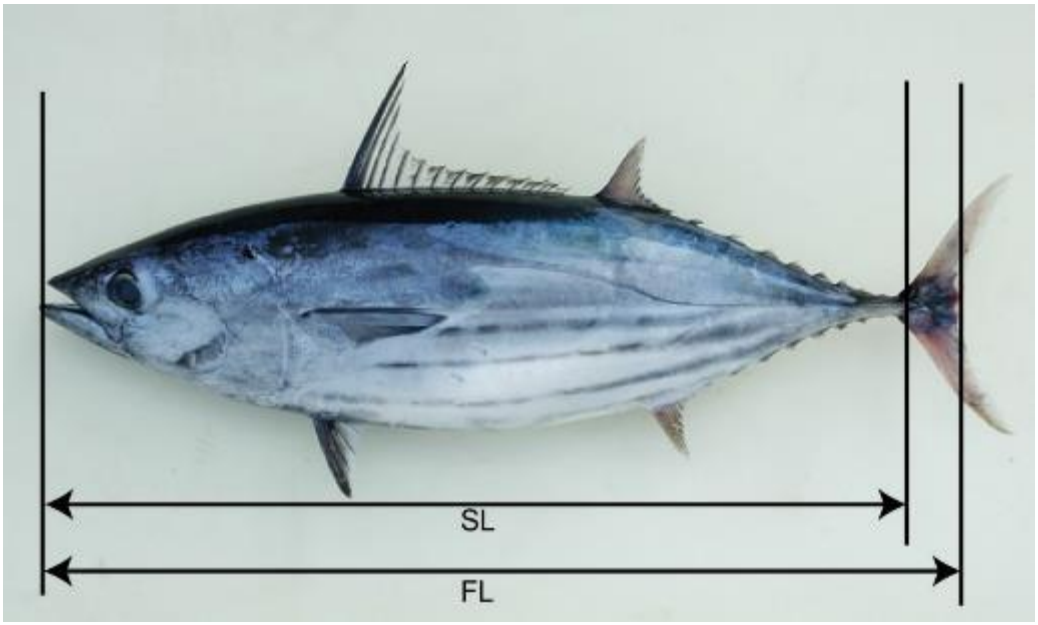


Fig. 4. Methods of measurement for standard length (SL) and fork length (FL) in skipjack tuna.

Employed items

Calipers [for small size (**Fig. 5A**); for large size (**Fig. 5B**)] and weight scale (**Fig. 5C**).

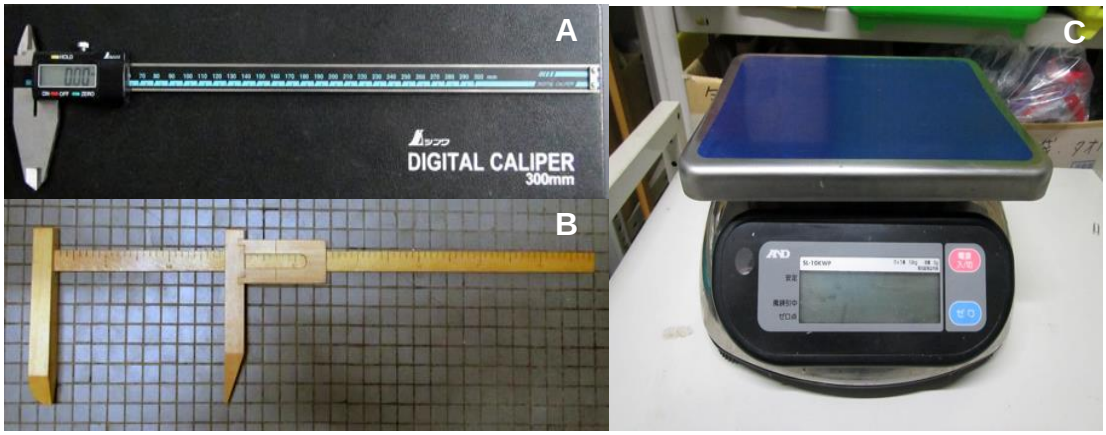


Fig. 5. Employed items for measurements of skipjack.

6 Extraction of otolith

Employed items

Dissecting needle (**Fig. 6A**), precise tweezers (**Fig. 6B**), petri dishes (**Fig. 6C**), clear microscope slide (**Fig. 6D**), razor's edge (**Fig. 6E**), stereomicroscope and microscope illuminator (**Fig. 6F**), quarterfold wiper (Kimtowel) (**Fig. 6G**), 96-well microplate (**Fig. 6H**), ethanol (**Fig. 6I**), and kitchen knife (**Fig. 6J**).

Fig. 6. Employed items used for otolith extraction.

Procedures (30–60 mm SL and 60–120 mm FL)

1. Cut off the head with a razor's edge, and cut open the head along the midline (**Figs. 7, 8**).
2. Remove the brains with a dissecting needle and precise tweezers under the stereomicroscope, and extract otolith from the cranial cavity with precise tweezers (**Fig. 9**)
3. Remove incrustation with dissecting needle and precise tweezers under stereomicroscope and clean the otoliths (**Figs. 10, 11**).
4. Pick up the cleaned otolith with precise tweezers and put it on 96-well microplate with a drop of 99% ETOH to make it easy to put and let it dry immediately (**Fig. 12**).

Procedure considerations

・Be careful not to pinch an otolith strongly with precise tweezers.
・Remove brains carefully while confirming the position of the otoliths.

Figures

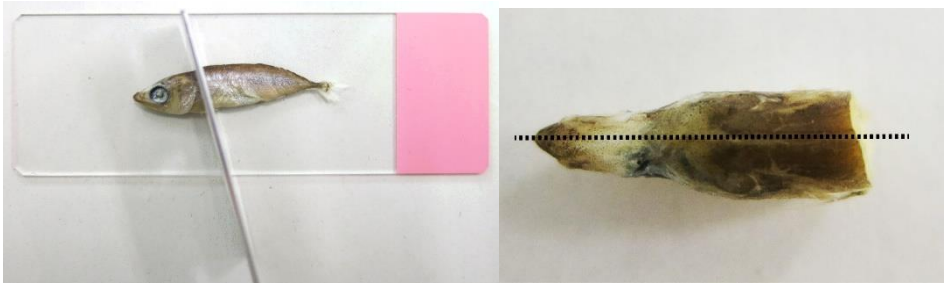


Fig. 7. Position to separate head and body (left), and showing cutting line of the head along the midline (right).

Fig. 8. Head cut open along the midline.

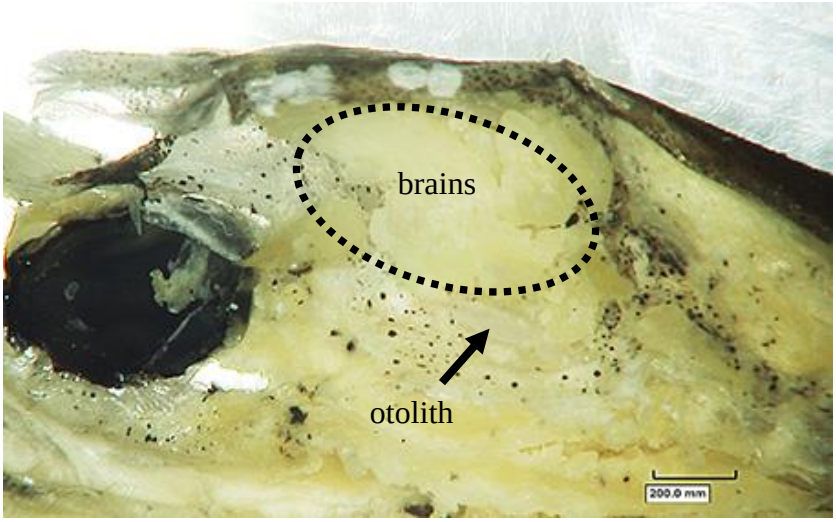


Fig. 9. Position of otolith and brain in skull.

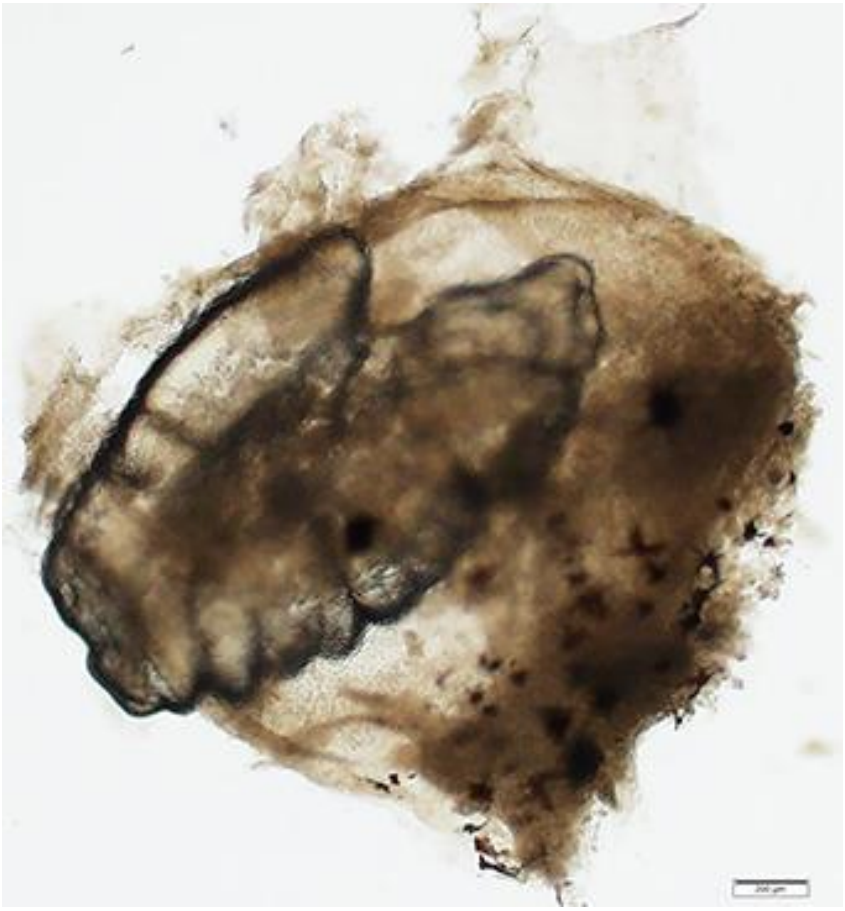


Fig. 10. Otolith with incrustation.

Fig. 11. Cleaned otolith.

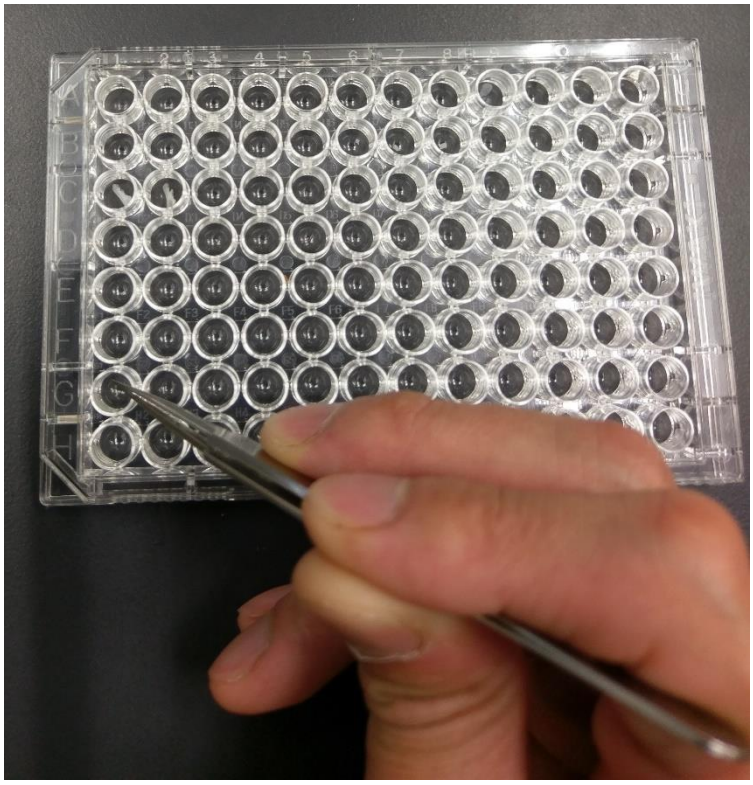


Fig. 12. The picture of putting otolith in a 96-well microplate with a drop of 99 % ethanol.

Procedures for young to adult stages (≧ 120 mm FL)

1. Cut off the head with a kitchen knife and remove the top of the head by cutting it off horizontally to a body axis just above the eyes (**Fig. 13**).
2. Hold the head as facing the snout and remove the brains carefully using precise tweezers (**Fig. 14**).
3. After removing the brains, a part of the semicircular canals is exposed. Pick up the semicircular canals with precise tweezers and draw them out carefully (**Figs. 15, 16**).
4. Keep a removed otolith in a meshed plate with water temporarily (**Fig. 17**).
5. Remove incrustation with a dissecting needle and precise tweezers under the stereomicroscope (**Fig. 18**) and clean the otoliths (**Fig. 19**).
6. Pick up the cleaned otolith with precise tweezers and put them in a 96-well microplate, and then let them dry at ambient temperature (**Fig. 20**).

Procedure considerations

・Remove the brains carefully while confirming the position of the semicircular canals.
・Be careful not to pinch rostrum side of otolith with precise tweezers because it breaks easily.

Figures

Fig. 13. The pictures of showing the cutting line just above the eyes horizontally to a body axis.

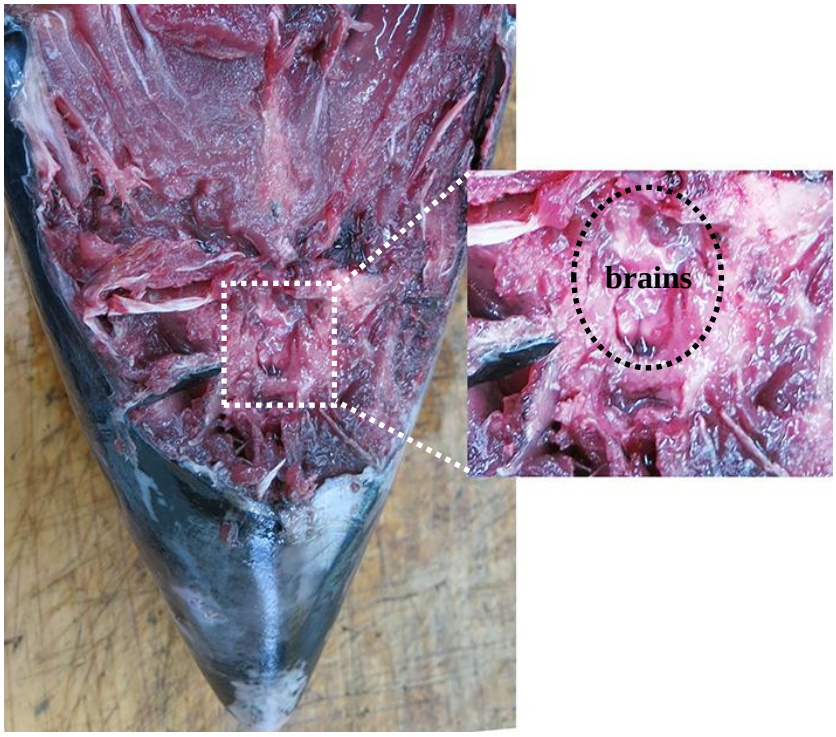


Fig. 14. Dorsal view of the head after cutting the top of the head off horizontally, showing position of the brains.

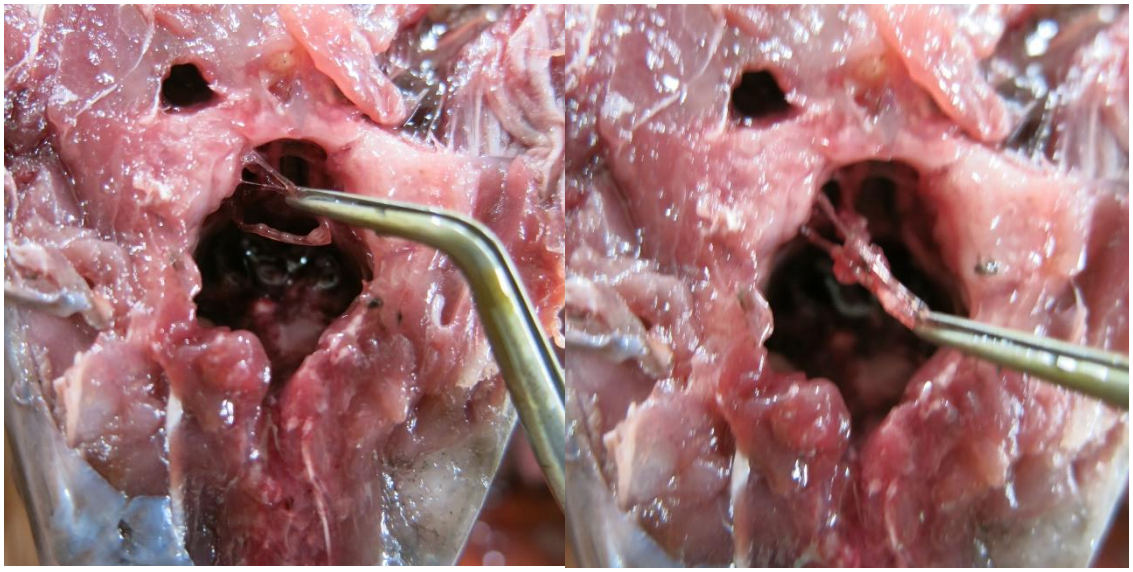


Fig. 15. The condition of picking up the semicircular canals with tweezers and draw them out.

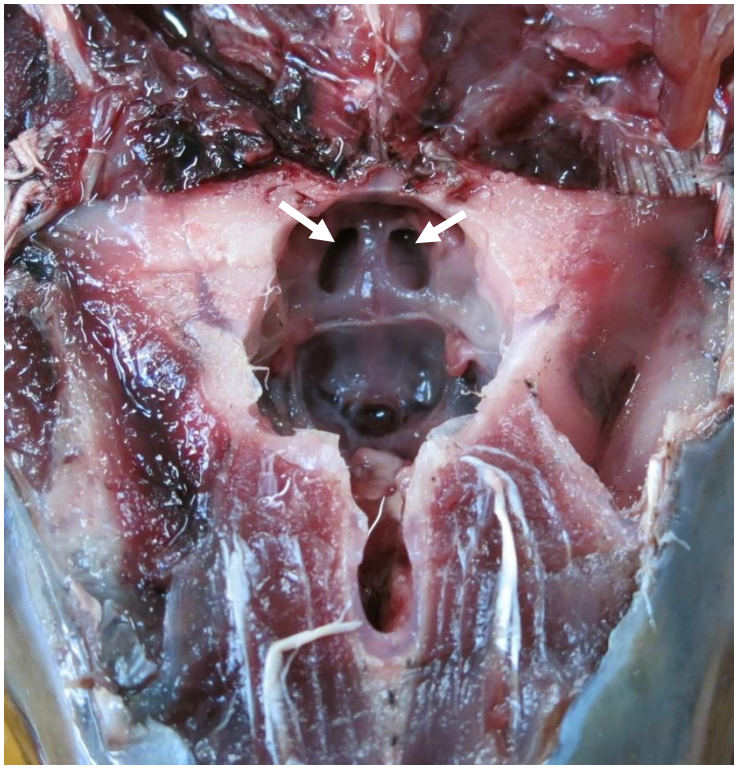


Fig. 16. The position of otolith cavity.

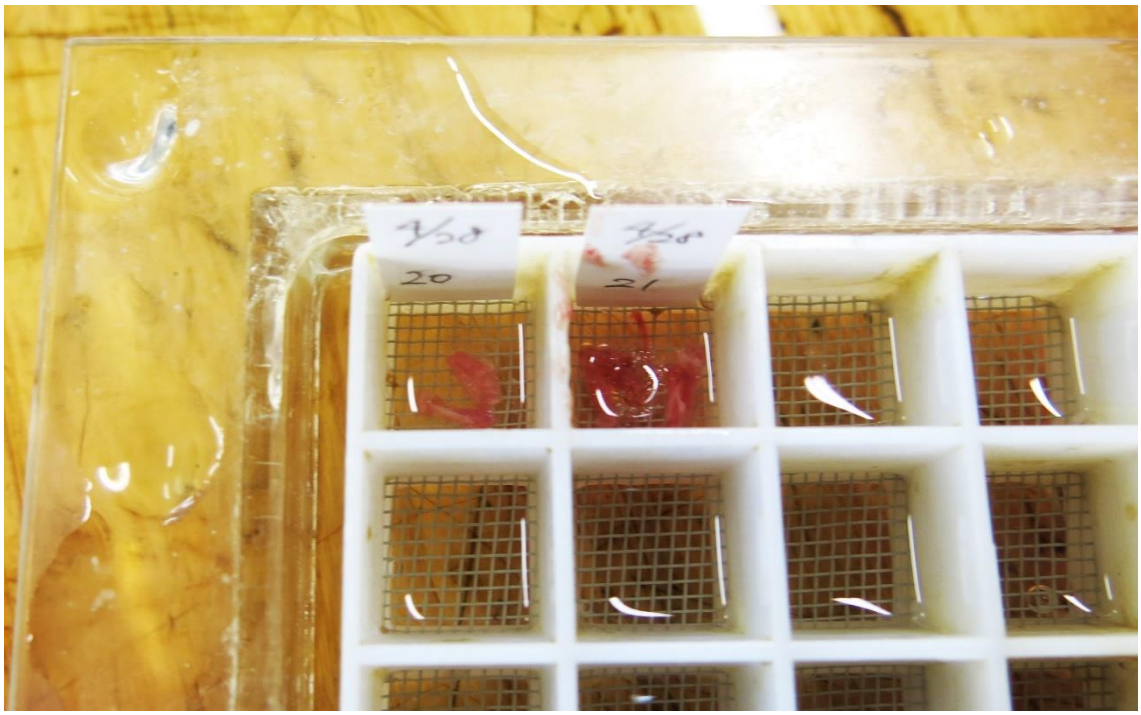


Fig. 17. Removed otolith stored in a meshed plate temporarily.

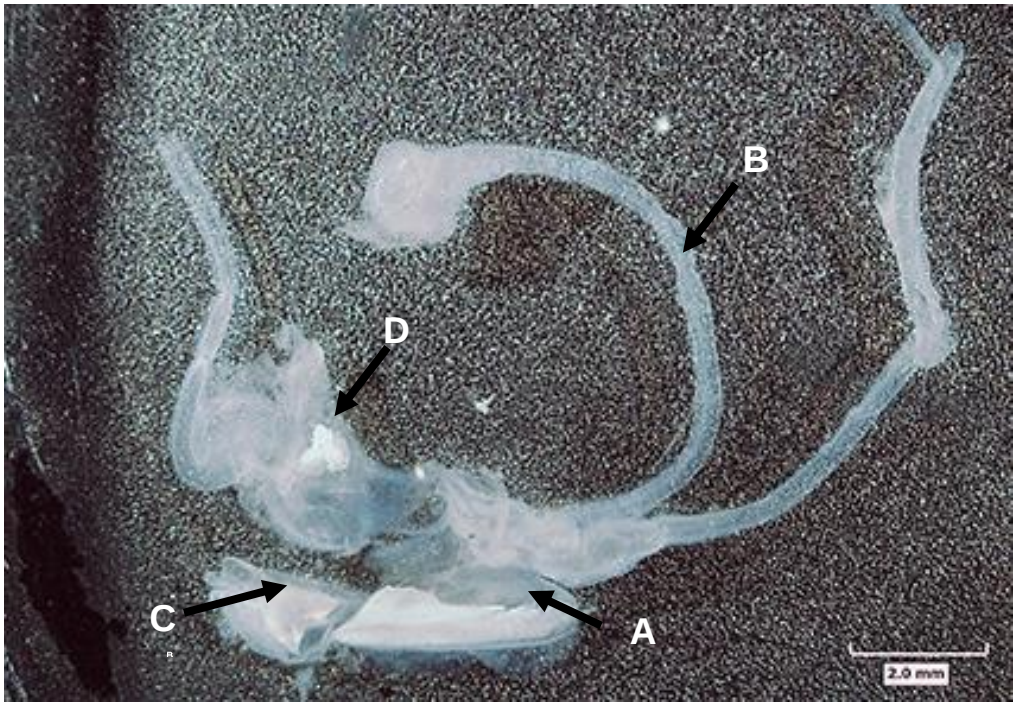


Fig. 18. Removed otolith (A), semicircular canal (B), asteriscus (C), and lapillus (D).

Fig. 19. Cleaned otolith.

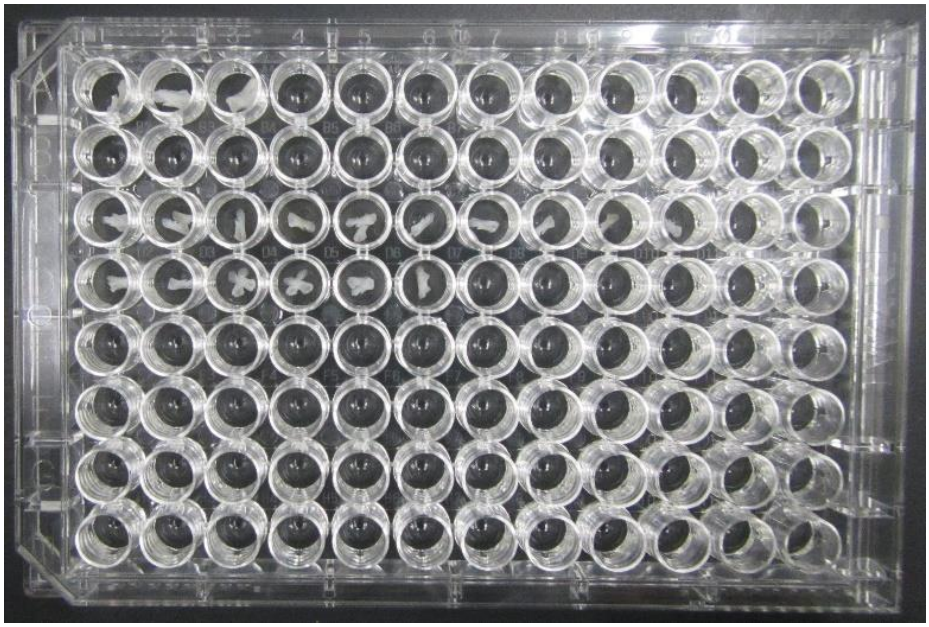


Fig. 20. Cleaned otolith stored in a 96-well microplate.

7 Polishing method

Employed items

Dissecting needle (**Fig. 21A**), accurate tweezers (**Fig. 21B**), clear microscope slide (**Fig. 21C**), stereomicroscope (**Fig. 21D**), enamel resin, enamel diluent (**Fig. 21E**), polishing paper (grit size: 9µm, 3µm, 1µm) (**Fig. 21F**), and optical microscope (**Fig. 21G**).

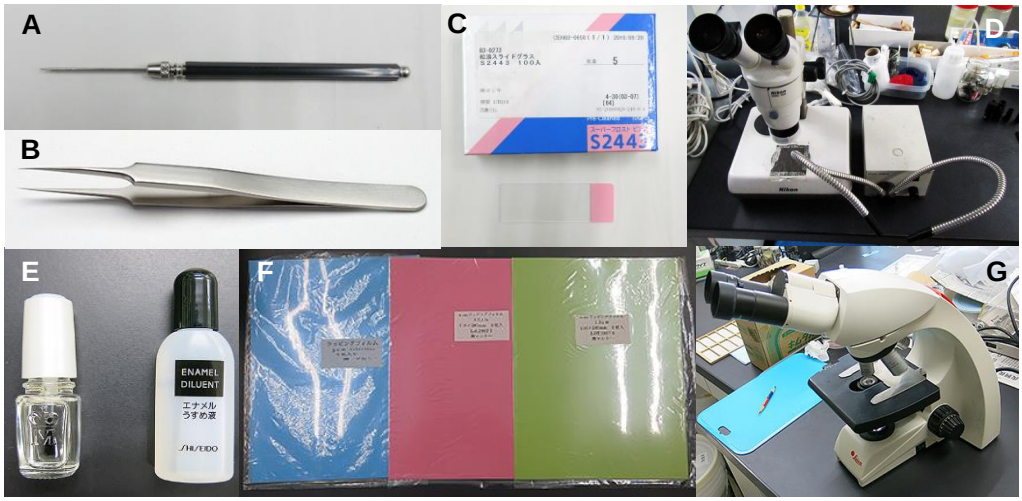


Fig. 21. Employed items used for polishing.

Procedures

1. Mix enamel resin and enamel diluent in the ratio of 1 to 2. Henceforth, it is called mixed enamel resin (MixER).
2. Use a dissecting needle covered with MixER and pick up an otolith with MixER's viscosity (**Fig. 22**). Put the otolith on a clear microscope slide as dropping MixER from the dissecting needle.
3. Put the otolith with the medial (sulcus) side up by a dissecting needle and precise tweezers under a stereomicroscope, and embed it completely in MixER on the clear microscope slide (**Fig. 23**). The mounted otolith needs to be set for one day.
4. After MixER completely dried, polish the mesial aspect of otolith with polishing paper at grit size of 9μm (**Fig. 24**).
5. Finish polishing when the sulcus disappears and the surface becomes smooth. Subsequently, polish the otolith with polishing papers at grit sizes of 1-3μm for finish (**Fig. 25**). The time recommended for polishing every step is approximately 30 minutes.
6. Dissolve the MixER with a few drops of enamel diluent and turn the otolith over under a stereomicroscope using a dissecting needle and precise tweezers. After that, mount the otolith and let it dry again.
7. Polish the other side of otolith with the same procedure as 5.
8. Finish the polishing procedure when the daily rings between the core and the edge can be observed. Cover the polished surface with MixER (**Fig. 26**).

Procedure considerations

・The degree of polishing should be frequently confirmed under the optical microscope.
・When turning the otolith over after polishing one surface, it is important to wait until the MixER completely dissolves and confirm if the otolith moves easily in order to prevent it from breaking.

Figures

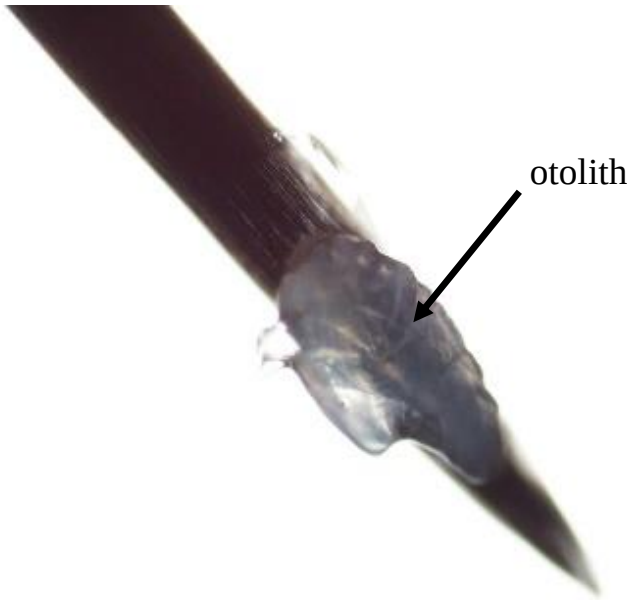


Fig. 22. Otolith attaching the tip of a dissecting needle with MixER.

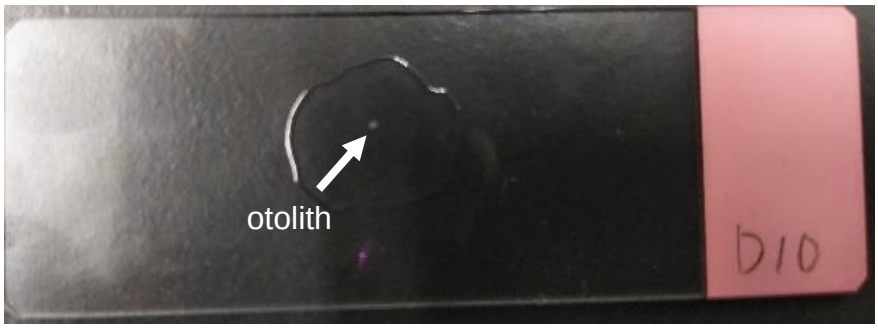


Fig. 23. Otolith embedded in MixER on a clear microscope slide.

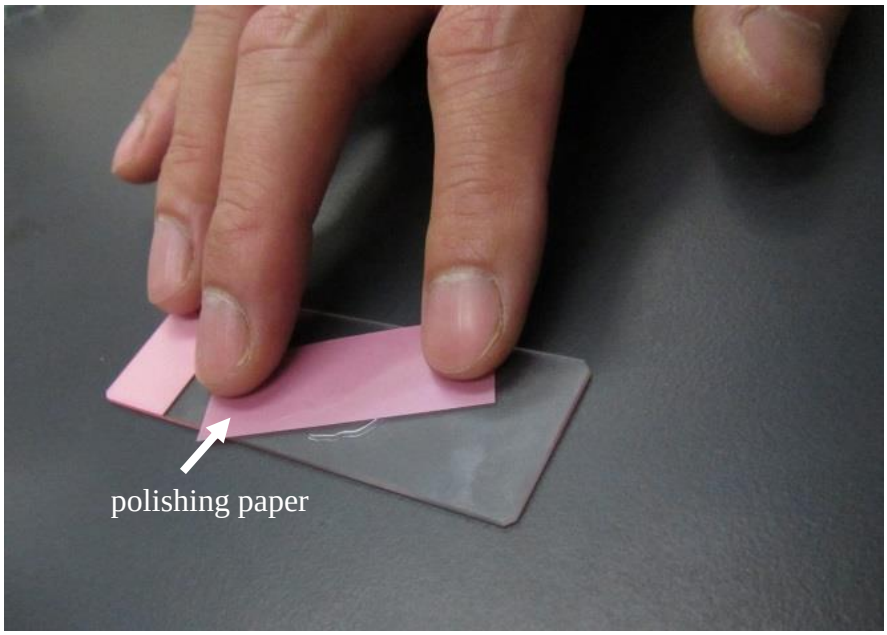


Fig. 24. Polishing an embedded otolith with polishing paper.

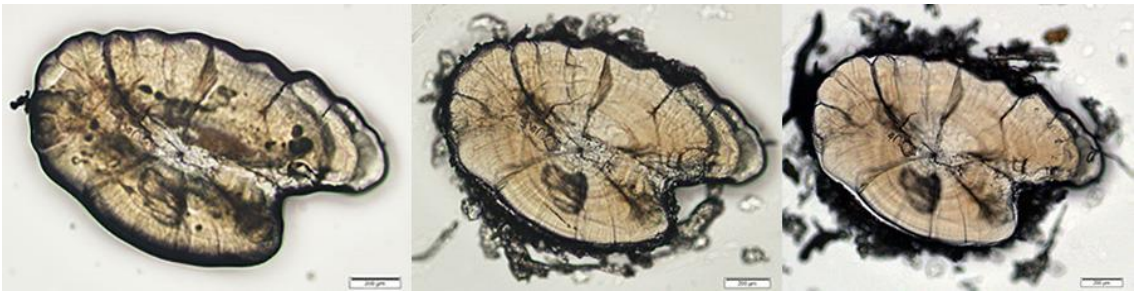


Fig. 25. The otolith polished with polishing paper (left: before polishing, center: in the middle of polishing, right: after polished).

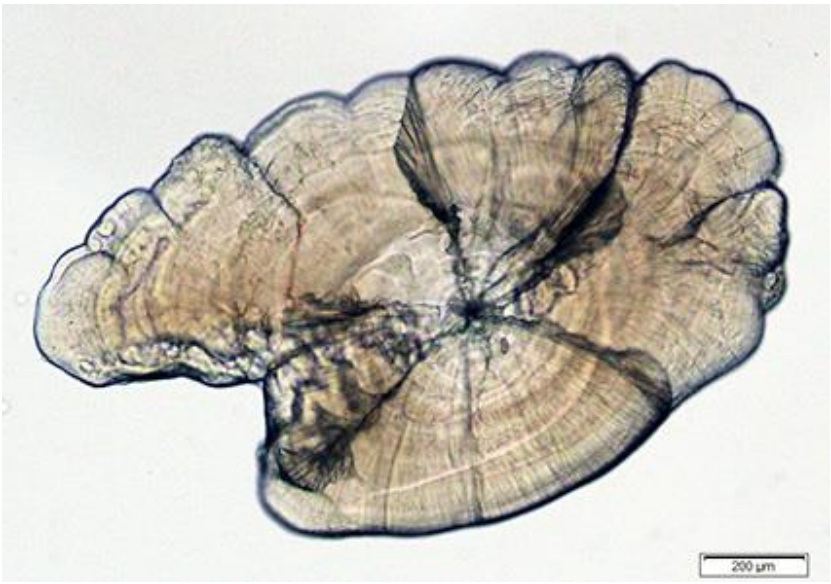


Fig. 26. Otolith completely polished on the both sides.

8 Etching method

Employed items

Dissecting needle (**Fig. 27A**), precise tweezers (**Fig. 27B**), clear microscope slide (**Fig. 27C**), enamel resin, enamel diluent (**Fig. 27D**), distilled water (**Fig. 27E**), optical microscope (**Fig. 27F**), 1 mol/l HCl (**Fig. 27G**), Kimwipes (**Fig. 27H**), resin mold (**Fig. 27I**), bamboo skewer (**Fig. 27J**), and epoxy resin (**Fig. 27K**).

Fig. 27. Employed items used for etching.

Procedures

1. Drop enamel resin onto clear microscope slide and put otolith lateral side (no sulcus face) up on there (**Fig. 28**). A half day is needed for enamel resin to be fixed.
2. After enamel resin completely dried, touch only around the core with a bamboo skewer soaked in HCL (1 mol/l) under an optical microscope and dissolve it (**Fig. 29**). After that, touch around the core with Kimwipes soaked in distilled water with precise tweezers in order to stop dissolution. Repeat these procedures until the daily rings between core and the fifth ring can be observed (**Fig. 30**).
3. Put the tip of bamboo skewer soaked in HCL (1 mol/l) on medial and marginal zones until rings can be observed (**Fig. 31**).
4. Finish the etching procedure when the rings between the core and the edge along the growth axis can be observed (**Fig. 32**). Dissolve the enamel resin on the clear microscope slide with several drops of enamel diluent, subsequently remove otolith from the clear microscope slide.
5. Put the otolith etched side down on the resin mold in the center and embed in epoxy resin (**Fig. 33**).
6. After the epoxy resin completely dried, remove the epoxy-resin block embedding an otolith from a resin mold.

Procedure considerations

・Be careful to avoid adhering enamel resin on the side for etching.
・The degree of the dissolution should be carefully confirmed under the optical microscope every time.
・The otolith after etching should be removed when the enamel resin is completely resolved to prevent it from breaking.

Figures

Fig. 28. The otolith on a clear microscope slide dropped in enamel resin.

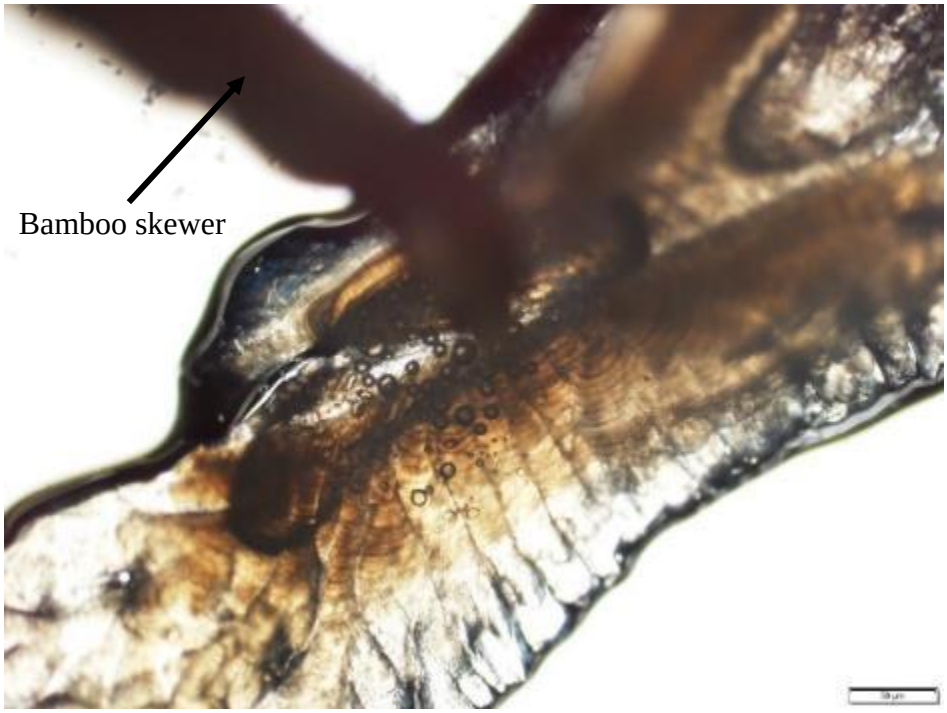


Fig. 29. Bamboo skewer soaked in HCL (1 mol/l) touching only around the core.

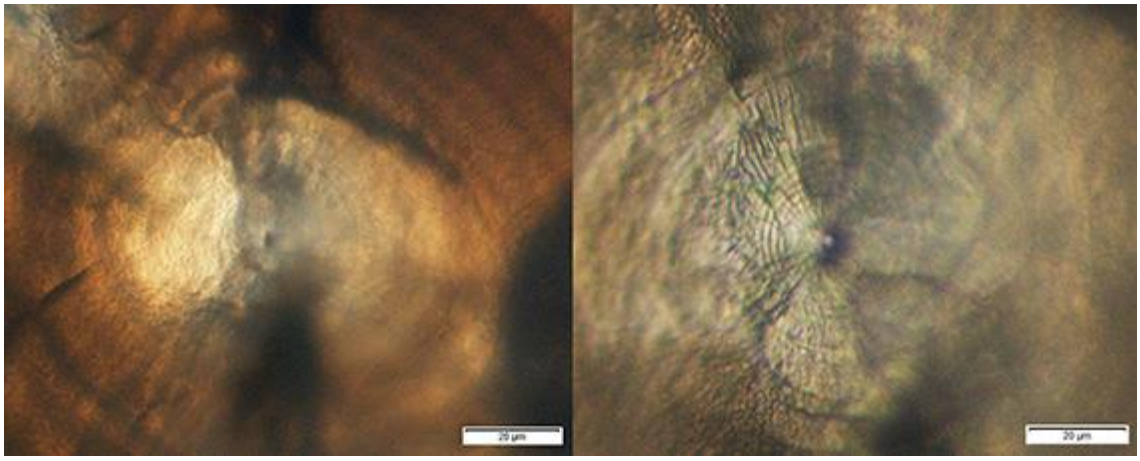


Fig. 30. The conditions of around the core before (left) and after etching (right).

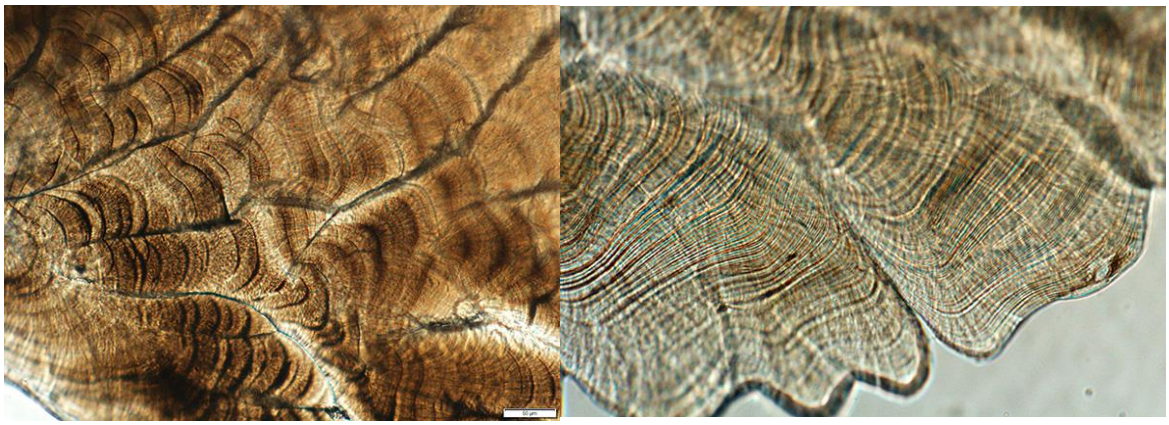


Fig. 31. The conditions of medial (left) and marginal (right) zones after etching.

Fig. 32. Anterior half of otolith after etching.

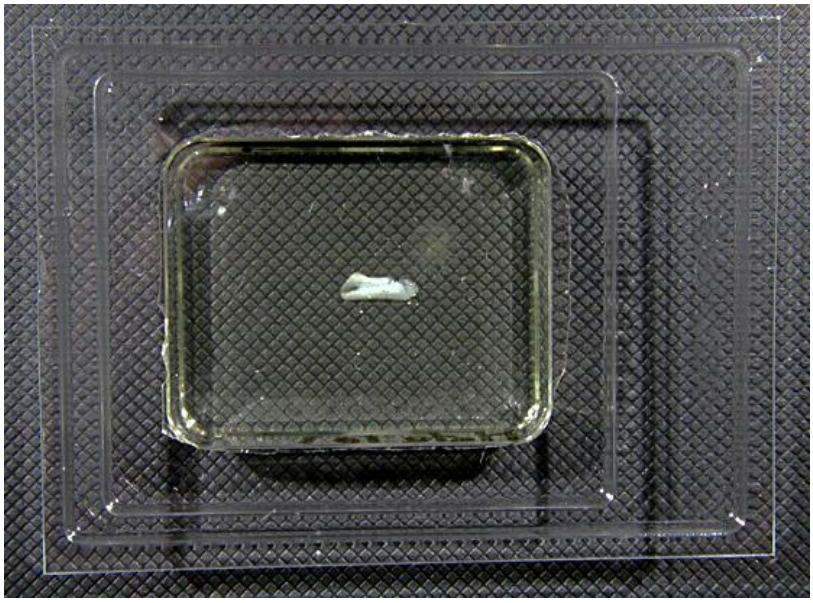


Fig. 33. Otolith embedded in epoxy resin on an epoxy mold.

9 Identify and count daily rings

Employed items

Light microscope (BX60-33; Olympus Optical), Charge coupled device (CCD)-camera (CS-580; Olympus Optical), image editing software (Adobe Photoshop), otolith-measurement system (ARP/W).

Procedures

1. With a CCD camera, take photographs of the otoliths that were treated in polishing and etching methods (**Fig. 34**). Set the magnification of the light microscope to be x25-50.
2. Bind pictures to make one full picture using image editing software (e.g., Adobe Photoshop) (**Fig. 35**).
3. Count the number of daily increments between the nucleus and the rostrum edge by using an otolith measurement system (ARP/W).

Fig. 34. Photographs of daily rings from a core to edge.

Fig. 35. Combined image of 196 pictures.

Procedure considerations

Skipjack otoliths are known to have following three different increment patterns between core and rostrum edge (Tanabe *et al*., 2003b; Kayama *et al*., 2007, **Fig. 36**): central area (C) for larval stage, medial area (MD) for juvenile to young stages, marginal area (MG) for the young to adult stages. In central area, daily periodicity in increment deposition during the first five days after hatching is validated in larval skipjack tuna (Radtke, 1983). The first fifth to seventh increments are usually formed as clear narrow daily ring, but the first one is sometimes obscure (**Fig. 37**). Distance from a core to the first increments is approximately 6–12 μm (Tanabe *et al*., 2003a). Hence, when the distance is longer than 15 μm, the first increment must be dismissed. In the medial area (ca. 10th–80th increments), interval of each increment is wider than the previous ones (**Fig. 38**). Sub-daily increments are usually observed in this area, including transitional period from the medial to marginal areas (**Fig. 39**). In the marginal area, increments are usually formed as clear but extremely narrow bands (**Fig. 40**).

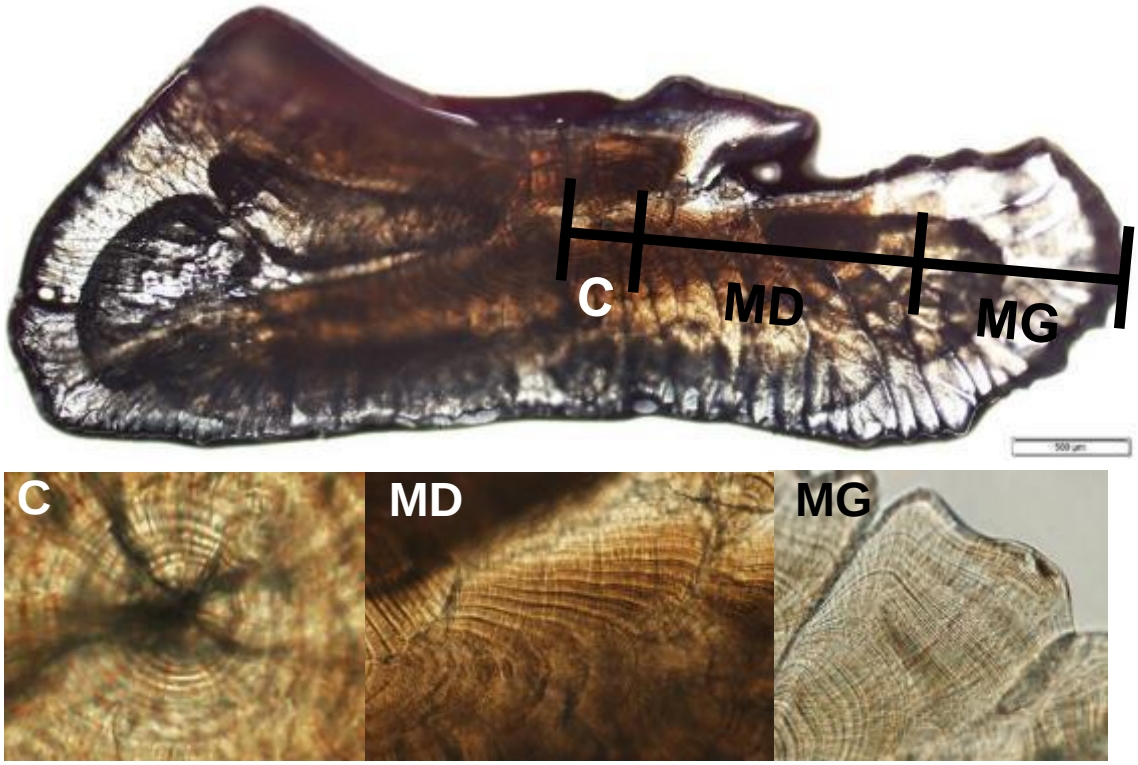


Fig. 36. Microstructures of three different areas in a sagittal otolith of adult skipjack tuna (416 mm FL). C: central area; MD: medial area; MG: marginal area.

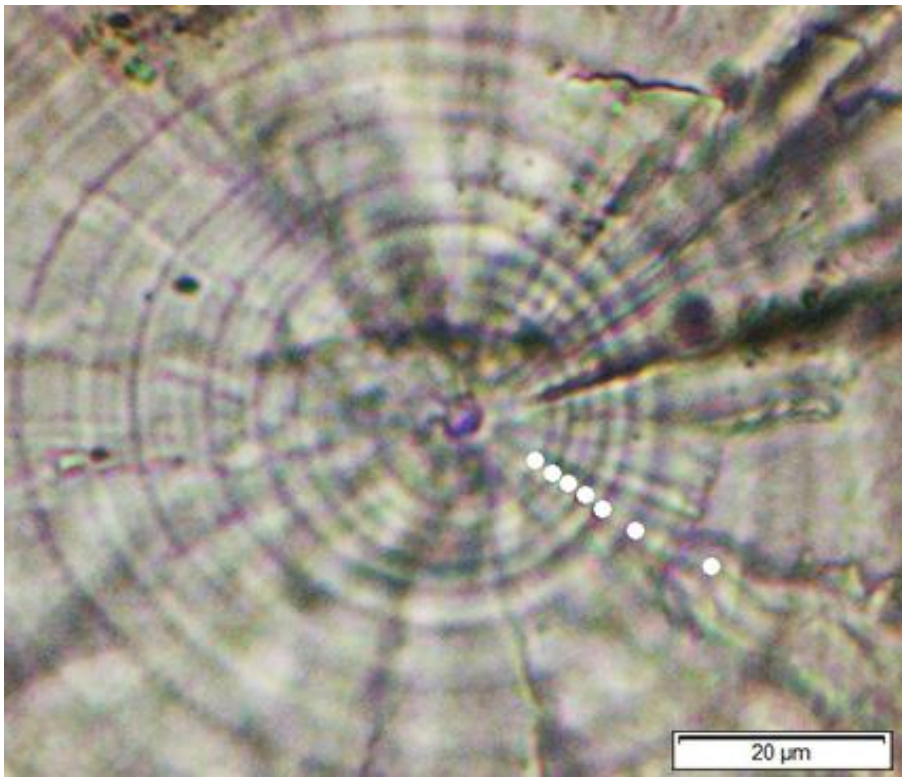


Fig. 37. First to seventh increments (white circle).

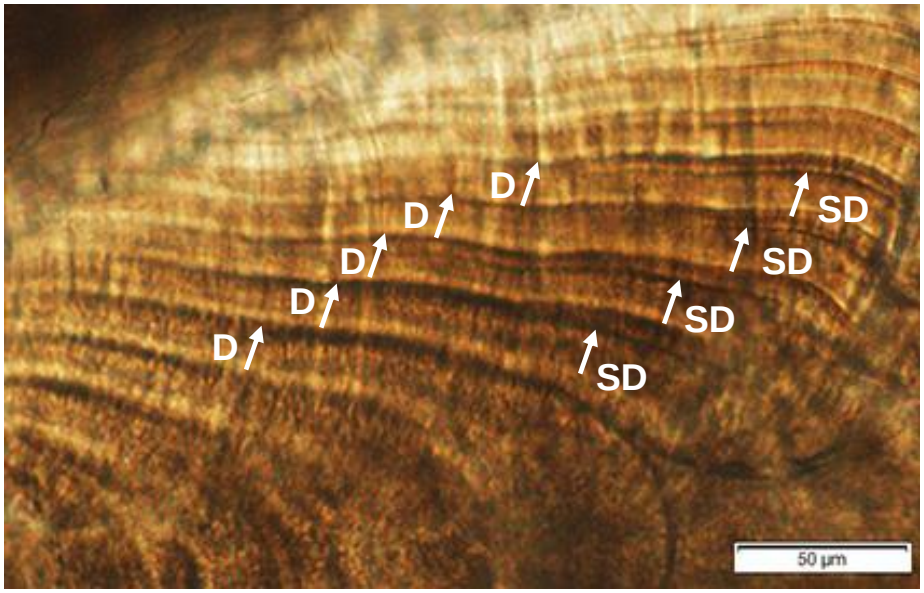


Fig. 38. Daily (D) and sub-daily (SD) rings in MD.

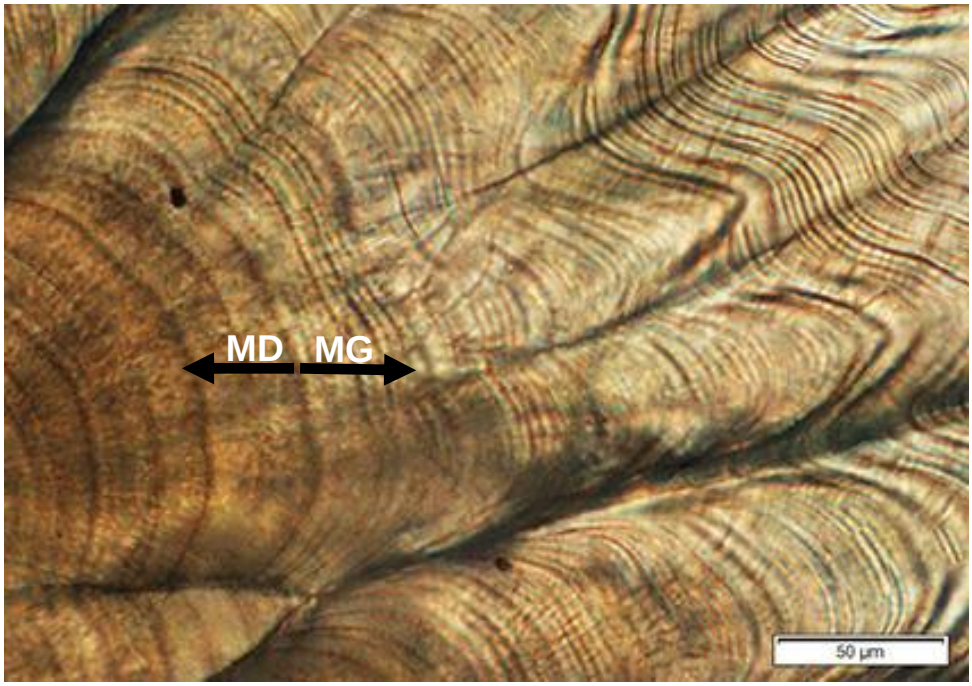


Fig. 39. Increments of transitional period from MD to MG.

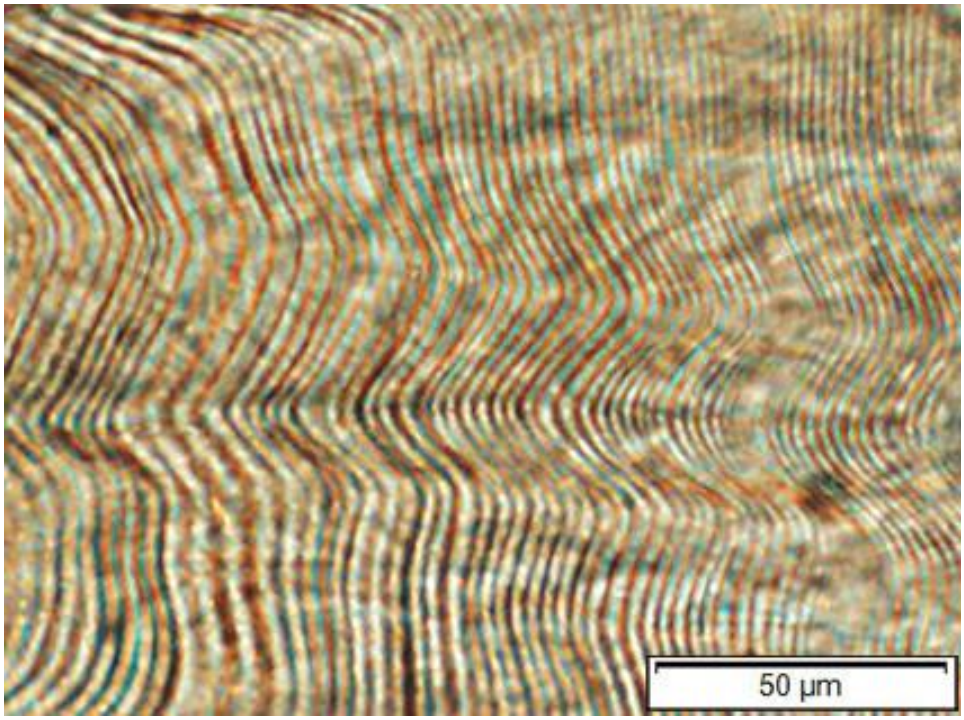


Fig. 40. Daily increments in MG.

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