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A manual for age determination of Pacific bluefin tuna *Thunnus orientalis*

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Abstract: Age determination techniques for Pacific bluefin tuna *Thunnus orientalis* using sectioned otoliths have been developed in recent years and a protocol of age determination is described. Opaque zones close to the core in the sectioned otoliths are difficult to recognize and some criteria are provided. The distance from the core is useful to recognize the 1st and 2nd opaque zones. The 1st to ca. 9th opaque zones are formed with similar distances between opaque zones. After ca. 9th opaque zones are relatively easy to recognize. This information facilitates age and growth studies of Pacific bluefin tuna which is used for stock assessment of this valuable fish species.

Key words: age determination, Pacific bluefin tuna, sectioned otolith, *Thunnus orientalis*

Pacific bluefin tuna *Thunnus orientalis* is one of the most highly valued fishery resources and is caught over a wide area in the North Pacific Ocean. Stock assessment of the species is conducted every two years using age-related information such as growth parameters, age composition of the catch by fishing ground, and fishing mortality etc. Age and growth of the species have been estimated by vertebral ring counts (Aikawa and Kato, 1938), scale ring counts, modal progression analysis (Yukinawa and Yabuta, 1967), tag release-recapture analysis (Bayliff *et al*., 1991) and otolith micro-increment analysis (Foreman, 1996). These age and growth studies have obtained maximum ages up to 10 years old for fish up to 200 cm fork length (FL). After the development of age determination technique using sectioned otoliths for southern bluefin tuna *Thunnus maccoyii* (Kalish *et al*., 1996; Clear *et al*., 2000) and Atlantic bluefin tuna *Thunnus thynnus* (Neilson and Campana, 2008), the sectioned otolith method is recognized as the most reliable tool to age these tuna species (e.g. Gunn *et al*., 2008).

Initial age determination of Pacific bluefin tuna using sectioned otoliths referred to the manual for southern bluefin tuna (Anonymous, 2002). However, age-length plots of Pacific bluefin tuna were not smooth when criteria for identification of otolith opaque zones of southern bluefin tuna was used (Tanabe and Kai, 2007). Specific criteria for Pacific bluefin tuna otoliths was subsequently developed (Shimose *et al*., 2008, 2009), and age estimates from 1 to 26 years old were obtained. To share the age determination technique for Pacific bluefin tuna, the International Scientific Committee recommended the development of a technical manual, focusing on the interpretation of annual bands in the sectioned otolith. The international workshop “Pacific Bluefin and North Pacific Albacore Tuna Age Determination Workshop” was held during 13-16 November 2013, and age determination protocol for Pacific bluefin tuna was discussed. In this manual, current methods of sample collection, otolith preparation, criteria to identify annual opaque zones, and other technical notes are described as an output of the workshop.

Collecting individual data and otolith samples

In 1992, the National Research Institute of Far Seas Fisheries started collecting otolith samples from Pacific bluefin tuna. The collecting effort

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greatly increased from 2007 and approximately 200 to 1,000 (depending on the year) otolith samples are currently collected each year. The sampling protocol was divided into three methods (Fig. 1).

1) Whole fish is purchased at the fishing port, and taken to the laboratory where the length is measured and otoliths removed. The cost of purchasing the fish is relatively high, so this

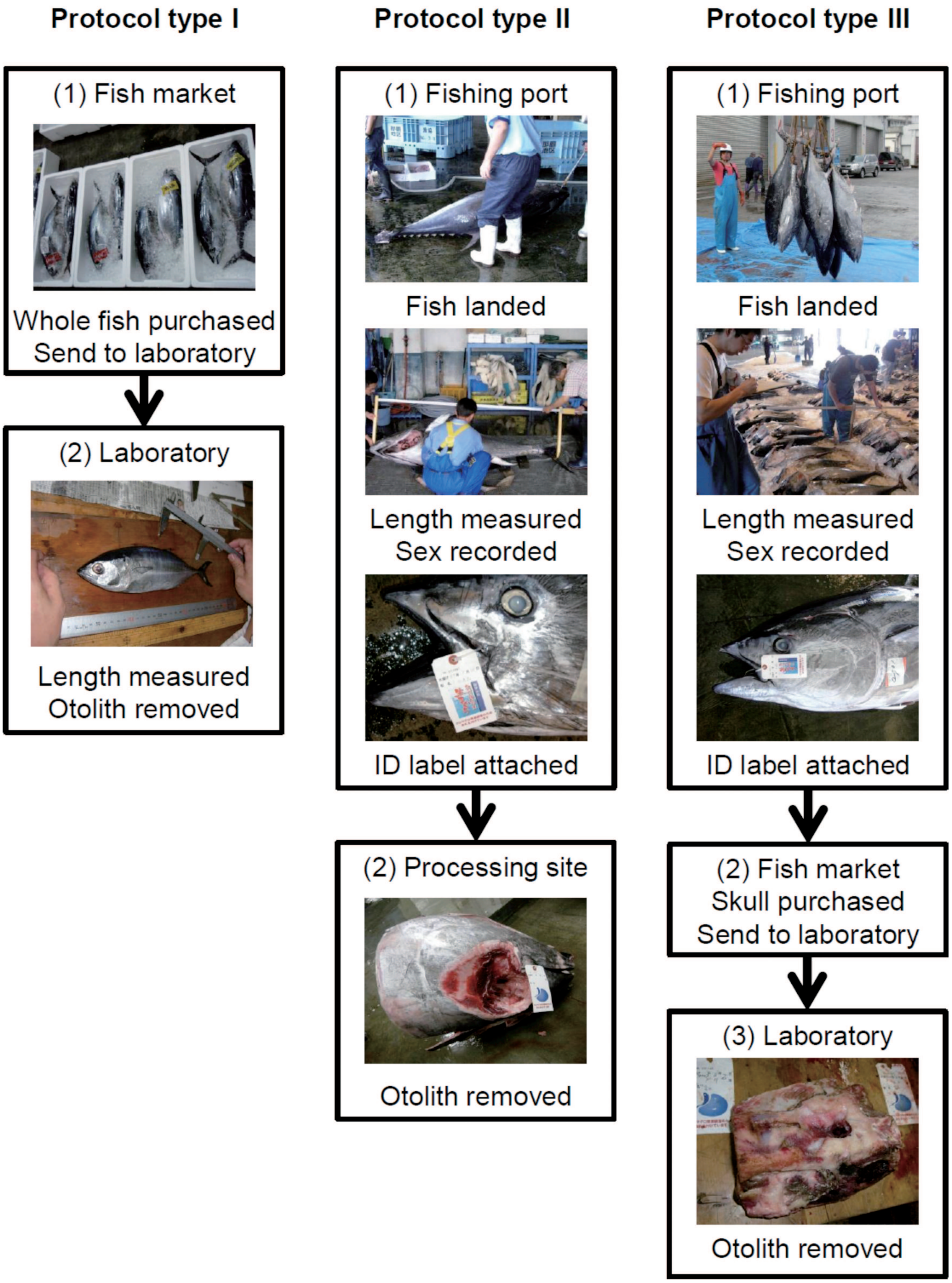

Fig. 1. Three types of sampling methods to collect length data and otoliths of Pacific bluefin tuna *Thunnus orientalis*.

method is generally only applicable to small individuals (ca. <100 cm FL).

2) Landed fish is measured, sexed and labeled at the fishing port, and then taken to the public fish processing site where the otolith is collected from the labeled skull a few days later.
3) Landed fish is measured, sexed and labeled at the fishing port, and then sent to Tsukiji fish market. The labeled skull is purchased from the fish buyer or processor at the market. This method is costly but less so than for the first method for large individuals.

To remove the otoliths, the skull of Pacific bluefin tuna can be cut using a large saw at the top of head using either a flat or V-shaped cut. The brain tissues are removed and the otoliths are extracted with the otolith membrane still attached. Otoliths should be cleaned using distilled water before storing dry in vials. Note that otoliths should not be cleaned/stored in tap water (which includes chlorine), seawater, or formalin because they may damage the otolith. There are three pairs of otoliths found in the skull but only the largest "sagittal pair" is used for age determination (Fig. 2).

Sampling strategies relating fish size

Two sampling strategies are suggested for southern bluefin tuna; i.e. length stratified sampling and random sampling (Anonymous, 2002). Length stratified sampling is preferable to derive growth curves or develop age-length keys for all length classes, while random sampling is useful to estimate the age composition of the catch of a specific fishery or area (Anonymous, 2002). Small Pacific bluefin tuna (<100 cm) can be selected by length and purchased as whole fish. Middle- (100–200 cm) or large- (>200 cm) sized fish can be sampled independently, because the landing ports of these two size classes are slightly different from each other. However, only random sampling is possible within each of these two size classes because specific buyers send the skulls and which skulls will be taken cannot be predicted. Selecting otoliths from those collected for age estimation can be undertaken using a stratified approach.

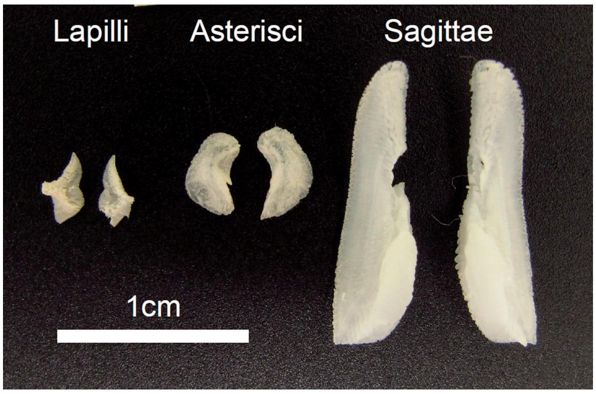

Fig. 2. Photograph of three pairs of otoliths of Pacific bluefin tuna *Thunnus orientalis* (141 cm FL, male). The largest “sagitta” is used for age determination.

There are many fishing grounds and fishing gear types for Pacific bluefin tuna in Japan. Some examples of major fishing areas and fisheries are follows (Fig. 3):

A) Tsugaru Strait: troll and longline vessels target

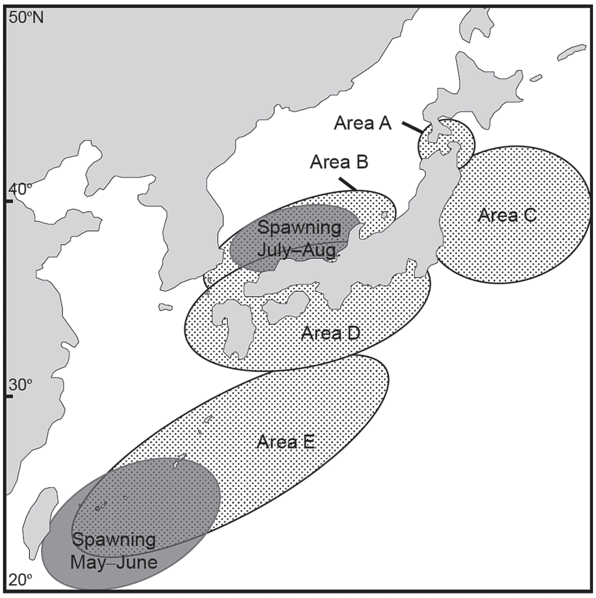

Fig. 3. Map of spawning grounds and fishing grounds of Pacific bluefin tuna *Thunnus orientalis*. Two spawning grounds are known and peak spawning seasons are shown. Major fishing grounds in Japan are divided into five areas; i.e. A) Tsugaru Strait: operating troll and longline fisheries during August to December, B) Sea of Japan: operating purse seine fishery during June to August, C) Off Sanriku: operating purse seine and set net fisheries year round, D) Southern Japan: operating troll fishery during July to December, E) Nansei Islands: operating longline fishery during April to July.

middle- to large-size fish during August to December, with a small number of landings in the other months.

B) Sea of Japan: purse seine vessels target small to middle-size fish during June to August, with minor landings in the other months. Most of middle-size fish are in spawning condition (Okochi *et al*., unpublished data).

C) Off Sanriku: purse seine and set net vessels occasionally catch small to middle-size fish.

D) Southern Japan: troll vessels target small-size fish in July to December, with minor landings in the other months.

E) Nansei Islands: longline vessels target large-size fish in April to July. These fish are in spawning condition (Okochi *et al*., unpublished data).

Although fork length frequency distributions differ among areas, the combined data and samples cover the full size range of fish to derive growth curves (Fig. 4). Although there are many other fisheries not only in Japan but also in other countries, details are not shown here.

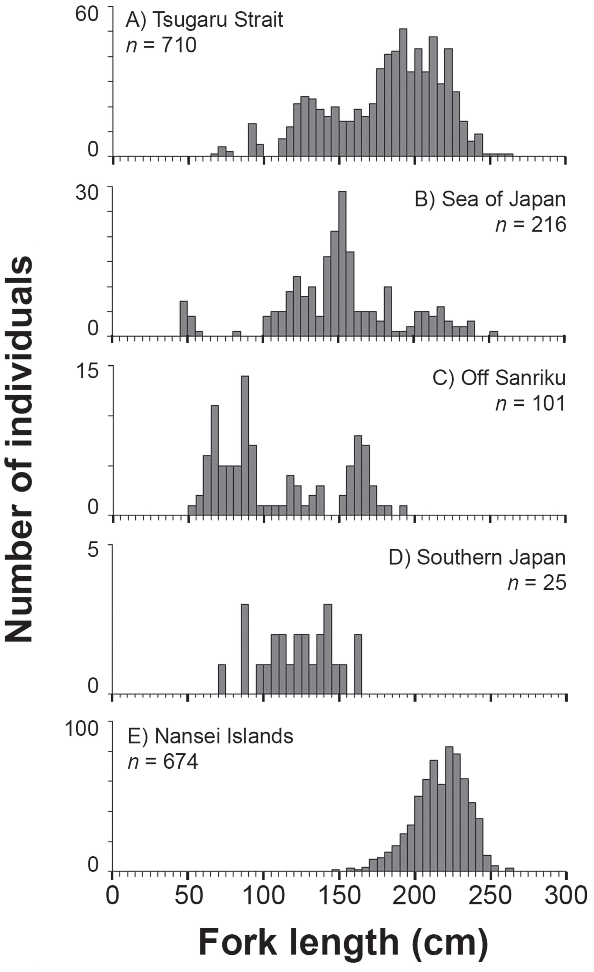

Fig. 4. Fork length frequency distribution of Pacific bluefin tuna *Thunnus orientalis* at five different fishing grounds. Data are from otolith samples collected in Japan between 1992 and 2011.

Preparation of otolith section

Structures and terminology for tuna otoliths are well described in the ageing manual for southern bluefin tuna (Anonymous, 2002), and also shown here using Pacific bluefin tuna otoliths (Fig. 5). The rostrum is situated on the ventral side of the otolith and extends anteriorly. The core is difficult to recognize on the otolith surface, but is located just anterior to distal lobe. The sulcus is situated on the inner side of the fish body between the dorsal arm and ventral arm on the otolith.

Each otolith is embedded in epoxy resin and allowed to harden for 24 hours before sectioning. Then it is sectioned ca. 0.3–0.5 mm thick in an area including otolith core region (Fig. 5). Sectioning should be conducted perpendicular to the longest

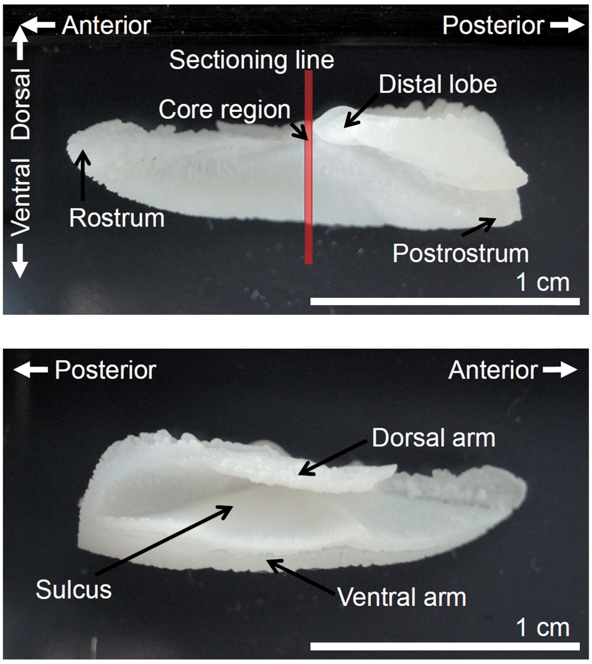

Fig. 5. Photographs of distal side (upper) and sulcus side (lower) for whole left otolith of Pacific bluefin tuna *Thunnus orientalis* (243 cm FL male).

axis of whole otolith as precise as possible. Preparation of serial sections around core region is recommended to refer to if there is low readability in a primary section. The thinner sections show the clearest view but they can be easily broken. Some sectioning methods are described in the manual for southern bluefin tuna (Anonymous, 2002). The section is attached on a glass slide using mounting medium, such as Crystal Bond (melts at low temperature heat) or Marinol (dissolves in solvent). The surface of the attached otolith should be polished flat using aluminum powder or polishing paper, or coated with a liquid mounting medium. The remaining otolith pieces encased in resin block should be kept after sectioning for possible use later.

Observation of sectioned otolith

Prepared slides of otoliths are viewed under a stereo microscope using reflected light and a black background or using a compound microscope equipped with transmitted light. Using reflected light is beneficial for checking whether the otolith edge condition is opaque or translucent. Digital photographs of all sections at the same magnification are recommended to be taken and saved for counts of annual opaque zones on the PC monitor and for re-ageing if necessary.

Two or three arms/lobes are visible from the core area of the otolith. The distal lobe is not found in sectioned otoliths which including the core in most Pacific bluefin tuna otoliths. Opaque zones are found in both the dorsal arm and ventral arm of the sectioned otolith. The ventral arm grows longer and straighter with one or two flection points. Opaque zones are more distinct in this ventral arm and are recommended for opaque zone counts (Fig. 6).

Identification of otolith opaque zones

Opaque zones are counted following methods outlined in other tuna ageing studies (Anonymous, 2002; Farley *et al*., 2006). Opaque zones close to the core (ca. up to 10 years) are difficult to identify and counts should be based on zonation color and the distance between the core and each opaque zones. Early growth (ca. up to 2 years) of Pacific bluefin tuna has been accurately estimated by modal analysis of length frequency data and otolith micro-increment analysis, and fish are known to grow to 40–60 cm FL at age 1 year and 80–90 cm at two years (Yukinawa and Yabuta, 1967; Foreman, 1996). Using this information, individuals of 35–70 cm caught during April to June are thought to be one year old, and those of 70–95 cm are considered to be two years old (Fig. 7). As opaque zones form in April to July (see below) or even earlier for the first annual opaque zone (Shiao, unpublished data), the otoliths of individuals caught during these months are expected to possess one and two opaque zones, respectively.

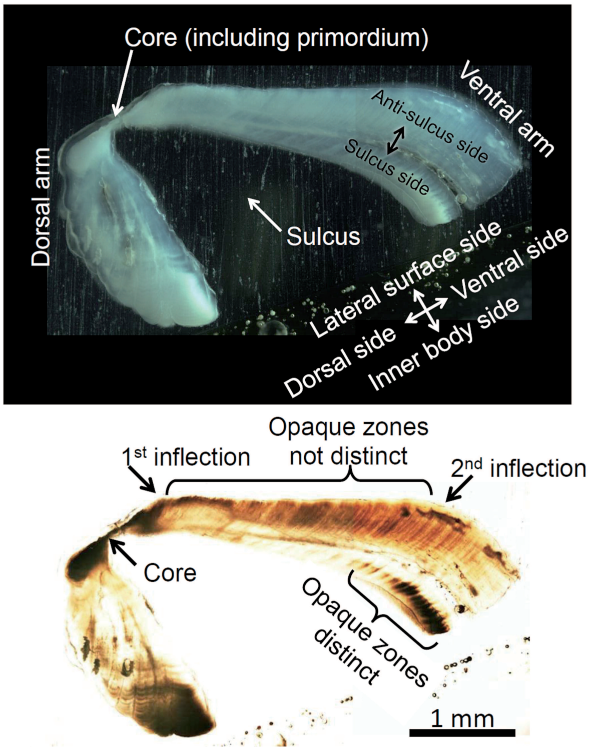

Fig. 6. Photographs of sectioned otolith of Pacific bluefin tuna *Thunnus orientalis* (243 cm FL, male). Reflected light (upper) and transmitted light (lower) show different images.

Using these 1 and 2 year-old individuals, frequency distributions of the distances from the core to the middle of the 1st opaque zone and to the 2nd opaque zone were found to be unimodal (Fig. 8). Measurements from the core to the first opaque zone was 0.61 to 1.02 mm (n = 90, mean = 0.79), but it will vary depending on the hatch month of the individual fish because the peak spawning season

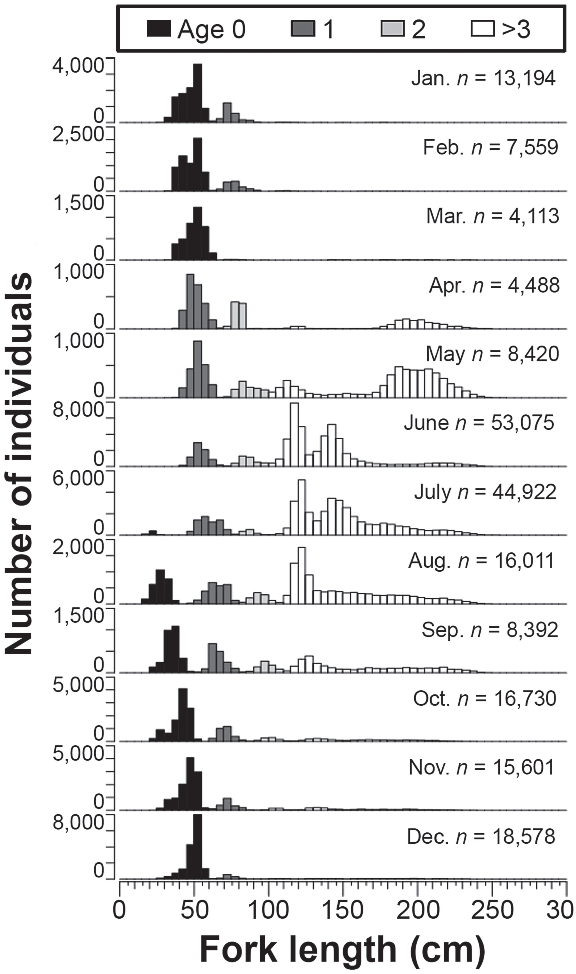

Fig. 7. Monthly changes in length frequency distribution of Pacific bluefin tuna *Thunnus orientalis*. Different age classes 0 to 2 years are recognized by their modes and plotted by different colors. Yearly age is assumed to increase on 1st April. Data are from RJB 2005 to 2010.

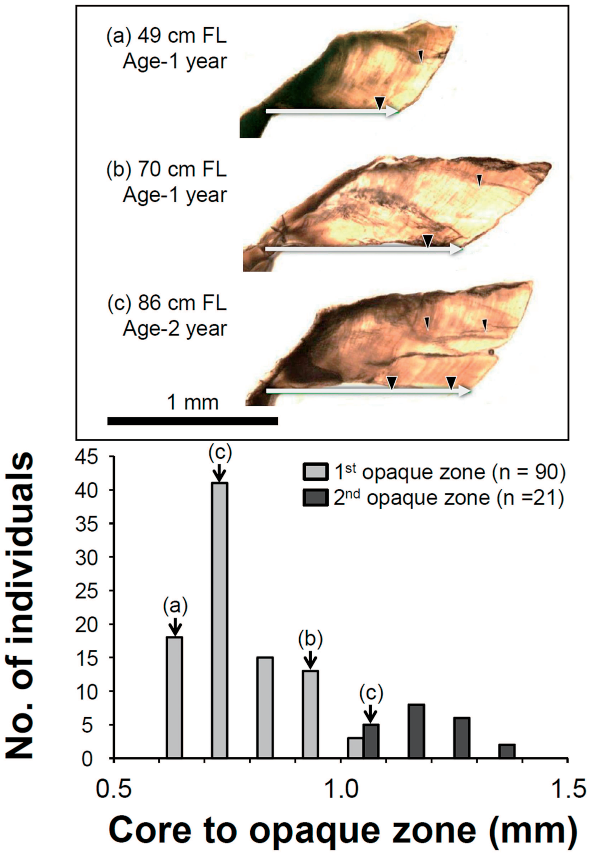

Fig. 8. Frequency distribution of distance from core to the 1st and the 2nd opaque zones of age 1 and 2 years Pacific bluefin tuna *Thunnus orientalis*. Measured parts of sectioned otolith from three different size fish are shown. (a) age-1 year small size (No. 2058, 49 cm FL, July), (b) age-1 year large size (No. 4898, 70 cm FL, July), (c) age-2 year (No. 4884, 86 cm FL, July). Arrows indicate recognized opaque zones.

extends from May to August (Itoh, 2009); i.e. early hatched fish (May-June) will have a longer distance from the core to the first opaque zone because they have more months to grow before the zone formed, while the late hatched fish (July-August) will have a shorter distance. The distance from the core to the middle of the second opaque zone was 1.07 to 1.39 mm (n = 21, mean = 1.18). These results provide useful criteria to identify the first and the second annual opaque zones.

The opaque zones between the first and second inflexion points are difficult to identify (i.e., the 1st to the ca. 9th opaque zones) (Fig. 6). The opaque zones form at similar distances apart, possibly because Pacific bluefin tuna grow almost linear until they reach ca. 200 cm FL (ca. 9 years old) (Shimose *et al.*, 2009), although measurements become slightly narrower as age increases. Opaque zones in this area should be counted referring to both the sulcus and anti-sulcus sides (Fig. 6).

After the second inflexion point, the opaque zones form uniformly and are more closely spaced. This is because the growth rate of Pacific bluefin tuna decreases after ca. 10 years old, but the otolith still increases in size. Opaque zones after the second inflection point should be counted on the sulcus side rather than the anti-sulcus side. Indistinct opaque zones (semi-annual opaque zones) are also formed in some otoliths even occurring in after the 10th opaque zones, but they are easily identified because they are

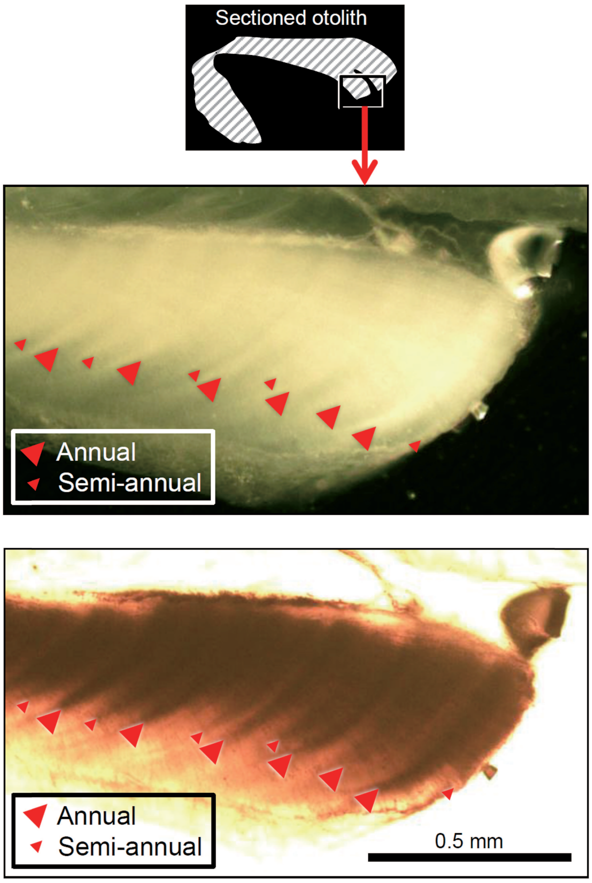

Fig. 9. Photographs of sectioned otolith near the edge of Pacific bluefin tuna *Thunnus orientalis* (No. 6236, 229 cm FL, October, Tsugaru Strait area) showing presumed annual and semi-annual (false) opaque zones. Reflected (upper) and transmitted (lower) light images are shown.

faint and less prominent than true annual opaque zones (Fig. 9). These indistinct opaque zones tend to form around December, which is not consistent with the timing of typical annual opaque zones formation which occurs during April to July (Shimose *et al*., 2009).

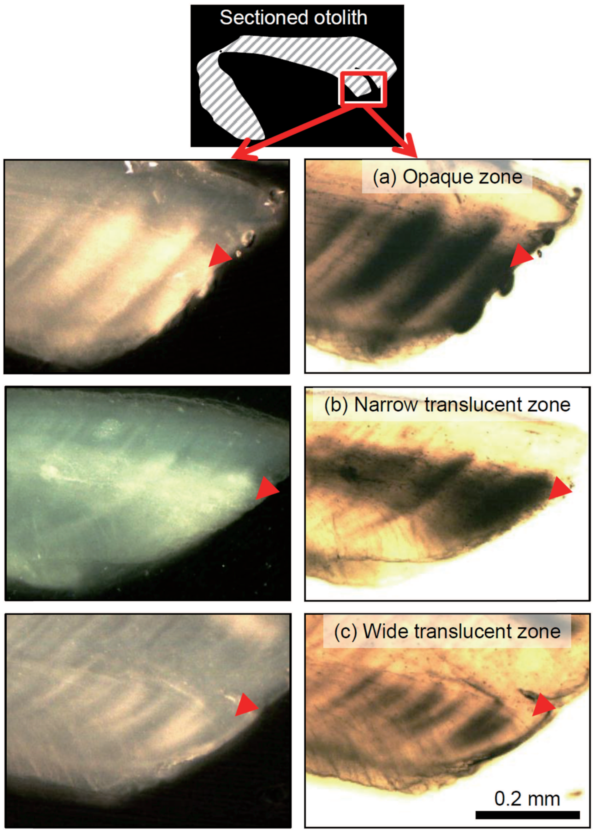

Fig. 10. Photographs of three types of edge condition on the sectioned otolith of Pacific bluefin tuna *Thunnus orientalis*. (a) Opaque zone (No. 5697, 206 cm FL, May, Nansei Islands area), (b) narrow translucent zone (No.6250, 187 cm FL, November, Tsugaru Strait area), (c) wide translucent zone (No. 5731, 200 cm FL, June, Nansei Islands area). Reflected (left) and transmitted (right) light images are shown.

Records of age determination results

The number of opaque zones and edge condition are usually recorded for individual fish in age determination study. In the case of Pacific bluefin tuna, the readability score of each otolith is also recorded for possible re-examination or exclusion of low readability otoliths. All counts should be performed without any reference to fish length, locality or collection date. Name of reader and counting date/period are also recorded in each time. The followings are recommended items to record:

1) Number of opaque zones (include if an opaque zone is forming on the otolith edge): if there are multiple possible results, all of them should be recorded. This number does not necessarily represent the age of the fish.
2) Readability score: five degrees are recommended; 1) unreadable, 2) unsure with count, 3) slightly unsure in some zones, 4) confident with count, 5) very confident with no doubt in the result.
3) Edge condition: opaque zone or translucent zone. And if possible, subcategorized narrow or wide translucent zones (Fig. 10). Observation of the edge may be better using reflected light with black background and

higher magnification. Information about edge type is used for adjusting the individual age assignment (see below). Although the edge condition is difficult to identify in young individuals, it should be decided by the distance from the last counted opaque zone to otolith edge.

4) Note: other observations about sample condition or pattern characteristics; e.g. section broken, surface damaged, abnormal shape, indistinct (regarded as semi-annual) opaque zones found etc.

In the case of southern bluefin tuna, three otolith readings are recommended for each individual (Anonymous, 2002). If age determination is performed by a single reader, then two independent readings are recommended with an interval of at least 2 weeks between readings. If the counts differ, then a third reading is conducted to resolve to the final count. If there are two readers, the first and second counts are made by the two readers independently, and the final counts are conducted by both readers with access to their previous counts.

If possible, counted opaque zones are marked on the digital image of sectioned otolith to compare other counts and/or future reference. Currently, "Inkscape" software (http://www.inkscape.org/en/) is used to mark opaque zones in National Research Institute of Far Seas Fisheries.

Evaluation of precision on counts

Precision is a measure of the repeatability between multiple ageing results, and implies consistency (Campana, 2001; Anonymous, 2002). Precision of multiple counts is commonly evaluated by average percent error (APE; Beamish and Founier, 1981) and coefficient of variance (CV; Campana, 2001) in which a smaller values indicates better precision. These indices can be applied for multiple counts by a single reader or for multiple readers. The only available information on these values for Pacific bluefin tuna is 4.51 for mean APE and 6.38 for mean CV (within reader; Shimose *et al*., 2009). These values are generally lower in larger individuals (Shimose *et al*., 2009) because the opaque zones are more distinct in the older ages, reducing the proportion of miss-counts in the older fish. Current authors compared their counts between two readers for 100 specimens, and mean APE and CV were 4.88 and 6.90, respectively. Age-bias plots are also useful to compare counts between and among readers to determine if (and which age classes) bias is occurring (Campana, 2001).

Validation of otolith opaque zone formation

For southern bluefin tuna, the annual formation of opaque zones was validated directly and/or indirectly for both young and old individuals. Fifty nine southern bluefin tuna injected with strontium that were released and recaptured show that the 1st to 6th opaque zones are formed annually (Clear *et al*., 2000). Bomb radiocarbon dating analysis showed that otolith core δ^{14}C values in old individuals was synchronized with the known values in seawater in the years when the southern bluefin tuna were hatched (as estimated by opaque zone counts) (Kalish *et al*., 1996). This bomb radiocarbon dating analysis was also successfully applied to Atlantic bluefin tuna (Neilson and Campana, 2008).

Annual opaque zone formation is not validated for all age classes of Pacific bluefin tuna. Otolith edge condition was only identifiable in larger individuals for Pacific bluefin tuna (0.0 % for 46–99 cm FL, 18.2% for 100–149 cm, 55.8% for 150–199 cm, 94.1% for 200–260cm; Shimose *et al*., 2009). Using edge type analysis, the formation of opaque zones in larger fish was validated to be annual (Shimose *et al*., 2009). Opaque zones formed in smaller/younger fish are less distinct and have not been validated as annual yet. However, the early growth curve (<5 years) estimated using sectioned otolith (47–260 cm FL; Shimose *et al*., 2009) is similar to the growth rates estimated by other reliable techniques (Fig. 11); e.g. tag-recapture analysis (Bayliff *et al*., 1991) and otolith daily growth increment analysis (Foreman, 1996). Therefore, the age determination method using counts of opaque zones in sectioned otoliths is assumed to be possible for both younger and older individuals. Further direct validation studies are needed to obtain reliable length-at-age data.

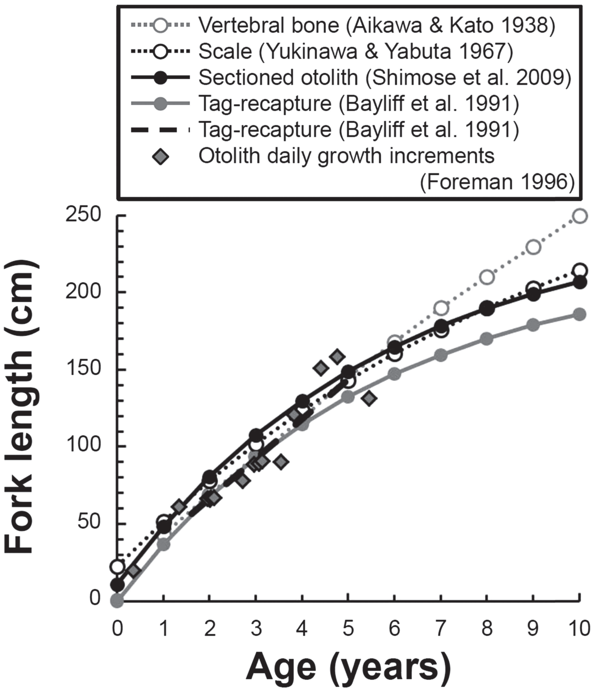

Fig. 11. Comparison in growth of Pacific bluefin tuna *Thunnus orientalis* estimated by various methods.

Assignment of age

Individual ages are estimated using the count of opaque zones, otolith edge condition, catch date, assumed spawning month, and timing of opaque zone formation (Campana, 2001). Spawning of Pacific bluefin tuna is known to occur from May to June in the southwestern North Pacific (Chen *et al*., 2006; Itoh, 2009; Okochi, unpublished data) and from July to August in the Sea of Japan (Tanaka *et al*., 2007; Itoh, 2009; Okochi, unpublished data). The former spawning group is thought to contribute more to the catch of age-0 Pacific bluefin tuna (Itoh, 2009), and the second quarter of the year (April to June) is assumed to be main spawning season (15 May, if the middle date is used). Otolith opaque zones of Pacific bluefin tuna form mainly during April to July corresponding to their spawning season at least for older individuals (Shimose *et al*., 2009). As the spawning and opaque zone formation months are similar, the number of otolith opaque zones is almost equal to their annual age. However, true hatch (spawned) date is unknown and the opaque zone formation month may also different among individuals.

The following two adjustments are useful to correct for these variations;

1) Specimens with an opaque zone on otolith edge collected in the first quarter of the year (January–March) are aged as number of opaque zones −1 year. This is because they have a new opaque zone forming on the otolith edge before they truly reach the birth months (= the second quarter). This assumption is based on the information that main spawning season of Pacific bluefin tuna is May–June (nearly equal to the second quarter of the year, April–June).
2) Specimens with a wide translucent edge collected in the second quarter (April–June) are aged as number of opaque zones +1 year. This is because their new opaque zone is not formed yet despite they actually reach the birth months.

Although more adjustment may be needed, these two major adjustments are reasonably applicable and thought to be sufficient at this stage. Ages may be assigned as 0.25 year interval to produce a smoother growth curve (Shimose *et al*., 2009); i.e. +0.25 years if caught in July to September, +0.50 years if caught in October to December, and +0.75 years if caught in January to March. Furthermore, birth date and/or recruitment month can be adjusted and fixed to fit the stock assessment model.

Reference collection

Reference collection images of sectioned otolith of Pacific bluefin tuna are prepared. There are two types of images; simple images of ventral arm area of sectioned otolith photographed using transmitted light, and the same image with marks showing opaque zones counted by authors. This collection consists of 90 individuals measured 50 to 260 cm FL from various fishing areas. Images of this collection will be available by request to authors (email: T.S.; shimose@affrc.go.jp or T.I.; ishiha@affrc.go.jp).

Conclusion

Age determination of Pacific bluefin tuna is improved by the development of the sectioned

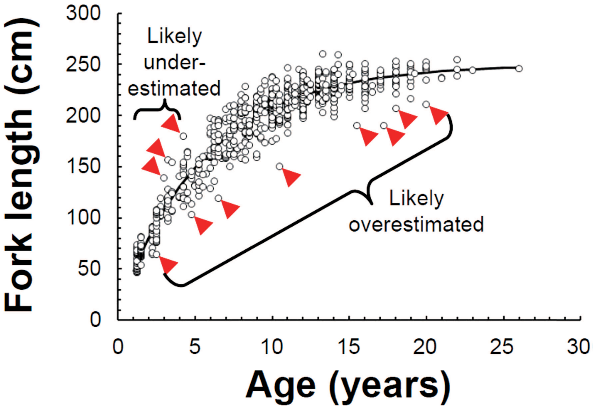

Fig. 12. Length at age and von Bertalanffy growth curve of Pacific bluefin tuna *Thunnus orientalis*. Modified from Shimose *et al.* (2009). Arrows show outlier individuals which may indicate possibly misinterpretation of ages.

otolith method, although the method appears to include some incorrect results (Fig. 12). Collecting more samples and accumulating data are necessary for increasing reliability of the current growth curve for Pacific bluefin tuna. Sex-specific growth and area specific age compositions are also needed and will require further studies.

The age determination manual for southern bluefin tuna "A manual for age determination of southern bluefin tuna *Thunnus maccoyii* (Anonymous, 2002)" may be useful when trying to determine the age of any other tuna species.

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