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Original Article

# Validation of auto-counting method by NIH Image using otoliths of white-spotted char *Salvelinus leucomaenis*

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***SUMMARY:*** The ages of individual specimens of white-spotted char *Salvelinus leucomaenis*, collected from the Furuu River in Hokkaido, Japan, were determined from otoliths using NIH Image. We employed two methods to determine age. First, for a surface method (traditional method), the ages were made from examinations of otoliths under a microscope by experienced readers A, B and an unexperienced reader C. Second, for an auto-counting method, a computer program was used to detect the peaks from otolith images. We made a mathematical model to estimate the probability of a correct reading for each reader, using the results of the age determination and the per cent age agreement among readers. The ability to determine the age of white-spotted char depended on whether or not the reader had ever previously made age determinations. The ability of auto-counting method was below that of reader A (one of the experts), and the method was slightly superior to reader B (the other expert).

***KEY WORDS:*** **age determination, image analysis, NIH Image, otolith, white-spotted char.**

## INTRODUCTION

The age of individual fish provides important basic information for examining biological characteristics of fish and analyzing fish resources. Scales or otoliths are used for determination of age in many cases. Although scales are useful when a fish is short-lived and rapid-growing,[1] it is more difficult to identify the annular rings in older fish, especially when the growth is slow and the number of circuli formed each year is small. In contrast, determination of age from otoliths can be achieved by counting the number of translucent zones, which alternate with opaque zones, under a microscope or with the naked eye. Although the procedure of age determination is simple, it is labor intensive for the age-reader because the number of otolith samples is usually large. Moreover, it is well known that results of age determination from otoliths vary, dependent on the experience of the examiner.[2–8] Hence, an otolith must be examined by several readers and/or several times for age determination and the process necessarily requires extra time.

In the present study, to avoid variation of the result depending on an age-reader's experience and to reduce the labor and time consumed by age determination, we attempted to determine the age of otoliths using a computer program. We developed a method of image analysis for age determination in fish using the computer software 'NIH Image (version 1.55)' (written by Wayne Rasband at the U.S. National Institute of Health: available on the Internet by anonymous ftp from ftp://zippy.nimh.nih.gov). We also developed a mathematical model to evaluate the ability of the method, compared with the non-expert and expert age-readers. We employed four readers: two expert age-readers; one non-expert age-reader; and a computerized method (also referred to as 'reader'). Then we estimated the 'accuracy' of readings by the four readers in term of 'precision'. The 'precision' is used, in age determination studies, to describe the agreement ratio between readings by the same age-reader on two occasions or by two different age-readers. The term 'accuracy' refers to the agreement ratio between the age by a reader and the 'true' age for specimens of known age.[9] Finally, we compared the accuracies of the four readers. On the basis of our results we discuss the validity of the computerized method.

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## MATERIALS AND METHODS

### Research site

Otoliths were collected from white-spotted chars, *Salvelinus leucomaenis*, from the Furuu River (43°08.3′N, 140°26.0′E) on the Shakotan Peninsula, which is located in southwestern Hokkaido, Japan, from May to October 1992 (Fig. 1).

Parr individuals were caught in the Ogawa Stream and the Amemasunosawa Stream, tributaries of the Furuu River (Fig. 1, points A and B). Fishes were caught with a cast-net, a chasing-net and by angling. A trap to catch migrant char from the open sea was located in the lower reaches of the river (Fig. 1). The trapped fish were preserved by freezing at −20°C.

A pair of otoliths (sagittae) was removed from each fish. The pair of otoliths was stored in a small vial under dry conditions. Prior to age determination, the lateral surface of each otolith was manually ground with a whetstone. The right otolith of each pair was used for age determination. If it was lost or broken, the left one was used. The ages of 439 otoliths were determined.

### Age determination method

We usually determine a fish's age from its otolith by counting the number of translucent zones that alternate with opaque zones. It is generally recognized[10] that an opaque zone is formed under good conditions and that a translucent zone is formed under poor conditions. It has been demonstrated that the translucent zones of otoliths of white-spotted char are formed from the end of May through August.[11]

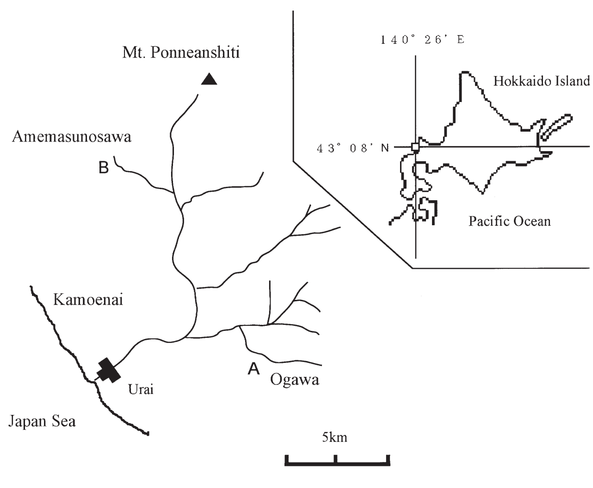


**Fig. 1** Map showing the location of the Furuu River in Hokkaido, Japan. Sites A (Ogawa Stream) and B (Amemasunosawa Stream) are sites at which parr of white-spotted char were collected. Migrant white-spotted char were collected in a trap at Urai.

Two methods were used to determine the ages from 439 otoliths: a direct method and an auto-counting method. The determination of age by the direct method was made by three different readers. We carried out age determination four times on 439 otoliths.

### Direct method

The otoliths were placed in ethanol and were examined under reflected light with a binocular dissecting microscope at magnifications ×20 to ×60.

Three different readers were represented as RA, RB and RC. Both RB and RC determined the age of each otolith only once, while RA did so twice. The second determination by RA was made more than 3 months after the first determination. While both RA and RB had experience in determining the age of white-spotted char from otoliths, RC had no experience with the age determination of this species of fish. RA was more experienced in the age determination method than RB.

### Auto-counting method

Image analysis of otoliths was performed by computer (Macintosh Quadra 840AV, CA, USA) using the public domain software NIH Image (Version 1.55), as follows:
(1) An image of the otolith was fed into the computer via a CCD camera (TI-23A; NEC, Tokyo, Japan) (Fig. 2a).
(2) The 'smooth' command was executed from four to six times to smooth the image of the otolith. The function of this command was to remove fine fluctuations in the image (Fig. 2b).
(3) A line was selected from the nucleus of the otolith to the end margin in a lateral direction, and the 'Plot Profile' command was executed along this line. This command generated a density profile, namely, a graph of monochrome color density values, along the selected line (Fig. 3). The density was divided into 256 levels, from 0 to 255. Level zero represented white and the color approached black as the density increased. The horizontal axis in the graph represented the distance from the nucleus of the otolith and vertical axis represented color density at each point.

The resultant graph had several peaks in every case (Fig. 3). Translucent zones were represented by the peaks on the graph, and the number of peaks represented the age of each individual since the age corresponds to the number of translucent zones. The number of peaks on the graph was counted with the Macro program (a computer program by Makino,[12] adapted by the authors) that detected the peaks by Savitzky-Golay's method.[13] The program involved identification of points at which the differential value (slope) of the density curve changed from positive to negative. Figure 4 was obtained with this

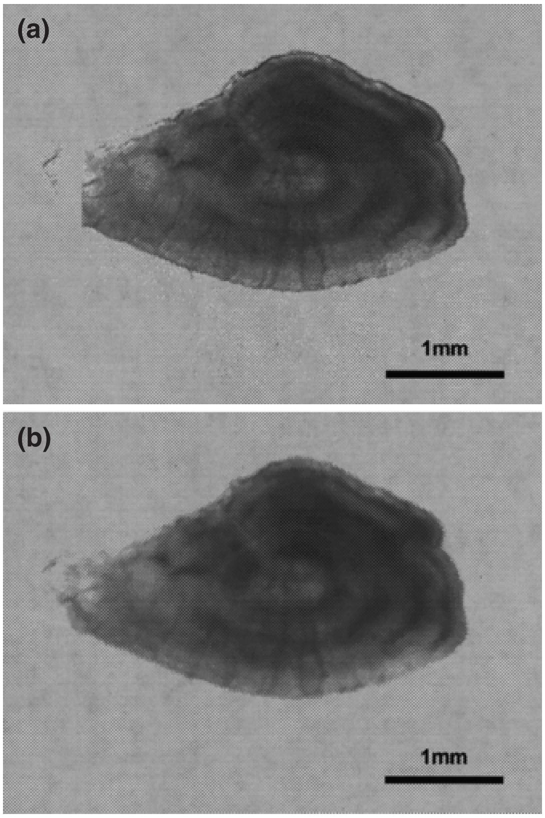


**Fig. 2** Photographs of an otolith (a) without and (b) after smoothing.

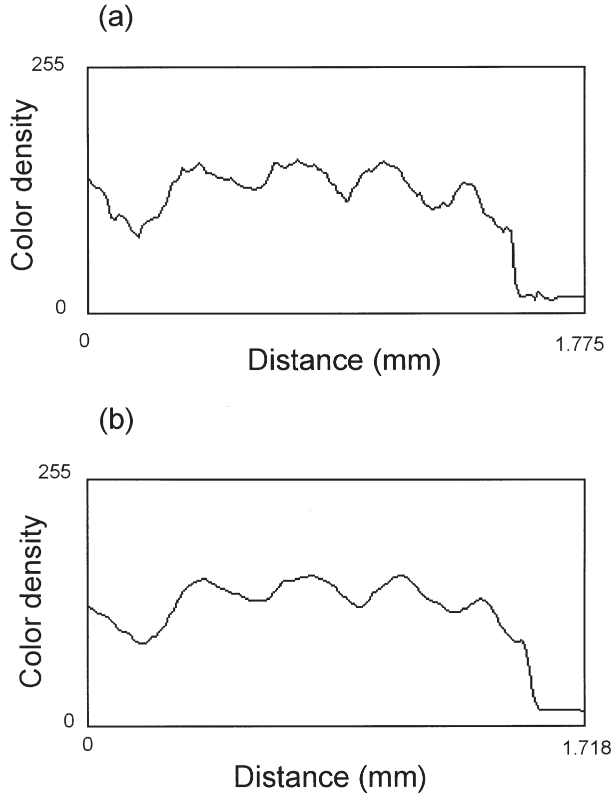


**Fig. 3** Color density curves for the otolith in Fig. 2a and Fig. 2b. The $x$-axis represents the distance from the center of the otolith. The $y$-axis represents color density. The color density was divided into 256 levels, ranging from 0 to 255. Zero on the $y$-axis represents white and the color approaches black as the value increases.

program. The vertical lines that cross the density curve were drawn by the program at the peaks.

### Comparison of accuracy among readers

Although Beamish and Fournier (1981)[14] proposed a way to evaluate the precision of readers rather than the accuracy,[15,16] the true ages of specimens were necessary information when we compared the abilities (or the accuracy of readings) of the age-readers to determine ages correctly. Unfortunately, we mostly did not know the true ages of fish. Therefore, we constructed a simple model which yields the probability that a reader determined the age of an otolith correctly from the per cent age agreement we defined below.

The per cent age agreement ($P_{ij}$) between Reader $i$ and $j$, can be represented as follows:

$$P_{ij} = r_i \cdot r_j + \frac{(1-r_i)}{2} \cdot \frac{(1-r_j)}{2} + \frac{(1-r_i)}{2} \cdot \frac{(1-r_j)}{2} \qquad (1)$$

where, $r_i$ is the probability that the age determined by reader $i$ is correct. We consider $r_i$ as accuracy of reader $i$. Then, the probability of a mistake is $(1-r_i)$. We assume that the probability of reader $i$ reading the age as 1 year older than the correct age is $(1-r_i)/2$, and that the prob-

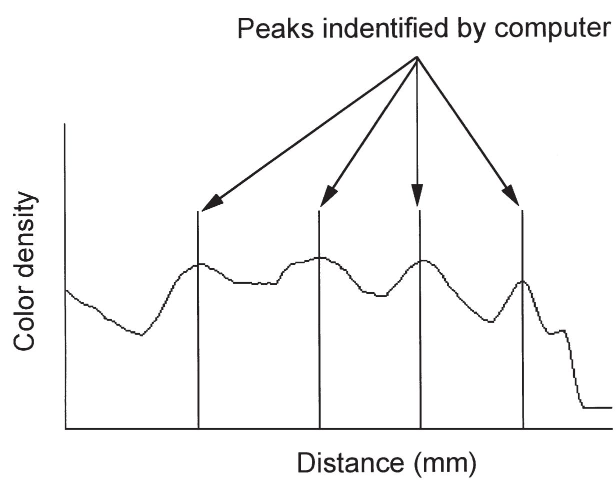


**Fig. 4** Results obtained by the auto-counting method. Solid arrows indicate the peaks of hyaline zones identified by the computer program.

ability reader $i$ reads it as 1 year younger is $(1-r_i)/2$. Therefore, the first term on the right-hand side of Eqn 1 is the probability that both reader $i$ and $j$ read the age correctly. The second term is the probability that both reader $i$ and $j$ read the age incorrectly, adding 1 year. The third term is the probability that both reader $i$ and $j$ read the age incorrectly, subtracting 1 year. Therefore:

$$P_{RA,RA} = a^2 + \frac{(1-a)^2}{2} \tag{2a}$$

$$P_{RA,RB} = a \cdot b + \frac{(1-a)\cdot(1-b)}{2} \tag{2b}$$

$$P_{RA,RC} = a \cdot c + \frac{(1-a)\cdot(1-c)}{2} \tag{2c}$$

$$P_{RA,RD} = a \cdot d + \frac{(1-a)\cdot(1-d)}{2} \tag{2d}$$

where $a$, $b$, $c$ and $d$ are the probabilities of a correct reading by RA, RB, RC and RD, respectively. RD represents an auto-counting method.

If we can obtain the values on the left-hand sides of these equations ($P_{RA,RB}$ to $P_{RA,RD}$), we can solve these equations simultaneously to obtain the probability of a correct reading by each reader ($a$ to $d$). $P_{RA,RB}$ to $P_{RA,RD}$ are the per cent age agreement obtained from the age determination data (Table 1).

## RESULTS

### Per cent age agreement

We obtained the results of the age determination for 439 individuals by four readers (Table 1). Each column represents the results for the indicated reader. Each row represents the results for each otolith. For example, the result of RB agrees with that of RC for the first individual. Picking up the rows in which RA and RB give the same age, we can calculate the proportion of the agreement between RA and RB. The proportion is the per cent age agreement between RA and RB. The per cent age agreements between RA and other readers are shown in Table 2. $P_{RA,RA}$ represents the per cent age agreement between repeated age determinations made by RA. The per cent age agreement between RA and RA is the highest (74.9%), while that between RA and RC is the lowest (28.7%). The values of $P_{RA,RB}$, $PR_{A,RD}$ and $P_{RA,RC}$ imply that the ability of RC is not high and that the experienced RA and RB are better at the age determination than the non-experienced RC.

### Probability of correct determination

We obtained values of probability of correct determination from the per cent age agreements in Table 2. Substituting 74.9% ($P_{RA,RA}$) into Eqn (2a) and solving the equation, we found that the probability for RA was 85.9%. The results obtained from the other equations are shown in Table 3. Therefore, we concluded that the rank order of the abilities of the four readers was:

$$RA > RD \fallingdotseq RB > RC$$

Although the ability of RD was below that of RA (one of the experts), RD was slightly superior to RB, who was the other expert.

**Table 1** The results of age determination

| Fish (no.) | $RA_1$ | RB | RC | RD |
|---|---|---|---|---|
| 1 | 3 yr | 4 yr | 4 yr | 5 yr |
| 2 | 4 | 3 | 3 | 3 |
| 3 | 3 | 3 | 5 | 3 |
| 4 | 3 | 4 | 4 | 3 |
| 5 | 2 | 2 | 3 | 2 |
| 6 | 4 | 3 | 5 | 4 |
| 7 | 3 | 3 | 4 | 2 |
| 8 | 2 | 2 | 3 | 2 |
| 9 | 3 | 3 | 3 | 2 |
| 10 | 4 | 4 | 5 | 4 |
| • | | | | |
| • | | | | |
| • | | | | |
| 437 | 5 | 5 | 6 | 5 |
| 438 | 6 | 5 | 6 | 5 |
| 439 | 6 | 6 | 6 | 6 |

Each column represents the results for the indicated reader. Each row represents results for each otolith. $RA_1$ is the result of the first age determination.

**Table 2** The per cent age agreement among readers

| Readers | $n$ | Per cent age agreement |
|---|---|---|
| $A_1$, $A_2$ | 329 | 74.9 |
| $A_1$, B | 265 | 60.4 |
| $A_1$, C | 126 | 28.7 |
| $A_1$, D | 267 | 60.8 |

The second column represents the number of times two readers agree on the ages of 439 individuals. The last row represents the number of times the results of three readers agree.

**Table 3** The accuracies of each reader

| Reader | Probability of a correct reading (%) |
|---|---|
| A | 85.9 |
| B | 67.6 |
| C | 27.5 |
| D | 68.1 |

RA has the highest ability, while RC (non-expert reader) had the lowest ability. RD is computer counted.

## DISCUSSION

### Effects of the reader's experience on age determination

When we need to know the life history or population size of fishes, the ages and growth rate of individual fishes provide essential information. Accordingly, it is necessary to establish a very accurate method for age determination. The age is often determined from otoliths. Translucent zones of otoliths are mainly counted by the direct method. However, since recognition of the translucent zone depends on the condition of the otolith, it is expected that errors can easily arise in efforts at the age determination.

Obtaining the per cent age agreement between age-readers with and without experience, Kimura and Lyons[9] demonstrated significant between-reader bias in the case of older specimens of walleye pollock (*Theragra chalcogramma*) and, moreover, that the coefficient of variation of results from an experienced reader is smaller than that from an inexperienced reader. We obtained a similar result: the accuracy in determining the age of white-spotted char depends on the experience of the age-readers. The order of the accuracies for the age determination by three readers was RA > RB > RC (Table 2). RA and RB were experts in reading the age of white-spotted char, while RC was a beginner. RA was more experienced than RB. The accuracy of RC was very low, 27.5%. The difference of accuracies between experienced readers (RA and RB) was 18.3%. This result implies that accuracy depends on the amount of experience and that it increases with increasing experience.

### Evaluation of auto-counting methods

Image analysis has been recently employed for detecting the annular and daily rings of otoliths.[15,17,18] However, the image analysis was used as a supplementary tool in age determination, not as a tool to read automatically. In the present study, we attempted to use image analysis software (NIH Image) as an auto-counting tool of age to develop a method of image analysis which had nearly equal accuracy to that of an experienced age-reader. The accuracy of our auto-counting method was nearly equal to that of an experienced reader (RB), who is inferior to RA, an expert of age determination of white-spotted char.

Why is our auto-counting method inferior to an expert of age determination (RA)? Since the computer algorithm in our auto-counting method is too simple, it probably detected small peaks that did not represent age peaks but, rather, random noise on the color density curve. On the contrary, RA skillfully ignored the small peaks when counting the peaks. Furthermore, an error by an otolith image is possible. For example, a shadow that was present at the edge of an otolith may be judged with one age, and hence overestimated age. Therefore, the accuracy of the auto-counting method was expected to be lower than that of RA. However, it reduces the time required for the analysis and the result is independent of the experience of the age-readers. The age determination with NIH image could be applied to both the 'break and burn method' and the 'cross-section method'. Furthermore, it could be applied to the analysis of daily rings in otoliths. If the computer algorithm used in the auto-counting method could be improved, it might be more useful in age determination studies.

### Assumptions of the model

In this study, the model that estimated the accuracy of the age determination from the per cent age agreement was based on two assumptions. One is that the results of the first and second age determination by the same age-reader are independent. To avoid dependency of the first and second results, the second age determination was made more than 3 months after the first, and the age determination was carried out selecting the otoliths randomly.

The other is that the age-reader mistakes age of fishes only by –1 or +1 year. We think that this assumption is plausible in this set of otolith samples of white-spotted char for two reasons: (i) the range of the results of the age determination—when we consider the result of the age determination that RA, RB and RC agreed as true age, the proportion of the ages involved in the range ±1 age was high (*i.e.* 95.2%); (ii) the result of computer simulation in the process of calculation—in the simulation we set three ideal age-readers and they read the ages with the accuracies 80%, 60% and 40%, respectively. We obtained the results of the age determination on a different assumption from the model (Eqn 1) where the ideal age-readers read the biased age from –2 to +2 years. From the result of the age determination, we obtained the per cent age agreement between a pair of three ideal age-readers. Then we estimated the accuracies of the readers using the model (Eqn 1). As a result, we found there was no difference between the accuracies set in the computer simulation and estimated with the model. Therefore, the model in this study has no difficulty in determining the age of white-spotted char.

In order to increase the accuracy of the auto-count method for various fishes, the computer algorithm could be improved. The model that estimated accuracy from the per cent age agreement should be verified using specimens of known age and/or with extended simulation. The validity of the model with known age otoliths is currently being investigated by the authors.

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