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Randall M. Claramunt

and

David F. Clapp

MICHIGAN DEPARTMENT OF NATURAL RESOURCES
FISHERIES DIVISION

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Suggested Citation Format

Claramunt, R. M., and D. F. Clapp. 2005. Image analysis procedures for aging calcified structures: an example with Lake Michigan lake whitefish. Michigan Department of Natural Resources, Fisheries Technical Report 2005-2, Ann Arbor.

Image Analysis Procedures for Aging Calcified Structures: An Example with Lake Michigan Lake Whitefish

Randall M. Claramunt and David F. Clapp

Michigan Department of Natural Resources
Charlevoix Fisheries Research Station
96 Grant Street
Charlevoix, Michigan 49720

*Abstract.−*Image analysis procedures for aging calcified structures have been recently developed for fisheries science to improve and standardize the interpretation of fish age and growth, thereby reducing reader subjectivity. Because of improvements in image analysis software, a new system, Image-Pro PLUS (Media Cybernetics®), is being implemented within the Michigan Department of Natural Resources (MDNR) Fisheries Division to provide a more systematic means of reading and archiving calcified structures electronically. This system will improve the precision of aging by creating high quality image files of the calcified structures and allow for reproducibility by saving images and the associated assignment of ages. To make image analysis systems effective, however, procedures must be standardized for the Division and implemented at each station where age and growth data are collected. This procedure has been developed using Lake Michigan whitefish scales (Whitefish Macro), but it can be easily modified for use on different calcified structures (e.g., spines, otoliths) and for other species. The techniques include aspects of physical processing, image acquisition, and image analysis that require specialized hardware and software. In addition to improving the precision in aging, implementation of an image analysis approach will provide a precise record of how the MDNR collects age and growth data, making our methods available for peer review.

Introduction

Our understanding of fish population dynamics is dependent on accurate estimates of age. In exploited fisheries, information concerning the age structure of the harvest is critical to evaluate mortality rates and to develop harvest limits (Beverton and Holt 1957). Error in estimating fish population age structures can impede our ability to manage fish populations effectively (Beamish and McFarlane 1995). Because of problems with sample preparation and subjectivity in determining fish age, standardization of aging protocols is necessary (Campana and Moksness 1991). In addition, standard procedures for aging should include a validation component for all ages and for each species to estimate the precision of determining age (Beamish and McFarlane 1983).

Fish growth is related to food quantity and quality, but influenced by physiological parameters. Daily and seasonal changes in growth are evident in calcified structures because calcium and organic deposits appear as rings (e.g., circuli on scales). Early life stages can be evaluated for age by examining daily growth whereas the age of adult fish can be estimated by examining annual growth. On a scale, fish age is determined by interpreting changes in growth through the identification of annuli. Annuli are defined as an area on the scale where circuli are incomplete and an

apparent opaque (dark) ring is evident (Casselman 1983). Opaque rings are formed where the translucent (light) zones between circuli are significantly reduced.

Age interpretation is traditionally completed by creating an impression of the scale on an acetate slide, projecting the impression via a microfiche reader, and subjectively identifying annuli (Schneider et al. 2000). Early methods to obtain more detailed and precise information from calcified structures began with simple digitizers (e.g., Frie 1982; Small and Hirschhorn 1987). Technological advances in the form of image analysis systems are now available to improve our aging results through the processing of digital images of calcified structures. For example, an image of a whitefish scale can be enhanced using an image analysis system by applying a variety of algorithms such as best-fit contrast, noise removal, sharpening, and edge detection.

Image analysis systems are being applied to reduce reader subjectivity and to automate the aging of various species (Campana 1987; Planes et al. 1991; Welleman and Storbeck 1995). Through pattern recognition, detecting alternating black and white bands on whitefish scales can be automated, but its potential is limited by image quality and pixel resolution. Image analysis systems will improve our aging effectiveness, even before an automated read system is built in, but this approach will still require experienced laboratory personnel.

System and Methods

Image analysis systems are comprised of a dissecting microscope with a digital camera that is connected to a desktop computer. The software used by the Michigan Department of Natural Resources Fisheries Division to capture and analyze the images is Image-Pro PLUS (Media Cybernetics®). The image analysis process consists of the following steps: (1) preparing the sample and acquiring an image, (2) enhancing the image, (3) assigning a read, (4) comparing and validating the reads, (5) final age determination and measurements for back-calculation, and (6) archiving the images, assigned ages, and measurement data. A macro was developed in Image-Pro Plus to complete the process (described above) in a simple, windows-based program. The macro is termed "Whitefish" and the protocol is explained below (see Stepwise Procedure for the Whitefish Macro). The Whitefish macro includes aspects of sample preparation, calibration of the optical field (see Sub 1, below), and an automated assistance program for age determination through light intensity (Sub 2.1) and through spatial frequency (Sub 2.2). The script for the Whitefish Macro is also included (Appendix 1).

Archiving the Image and Associated Data Files

With the image analysis process, several files are saved and archived for future verification. For each fish that is aged, two or more image files are saved (one of which will be a .tif file). In addition, there will be at least two files saved for independent age determination (.msr files). If back-calculation data is collected, then a file will be created to save the marks (.tag). Using this process, approximately 6 million bytes of storage space is necessary for each fish, which equates to about 1.0 gigabyte of file storage space required for every 175 fish aged (Table 1).

Conclusion and Future Potential for Image Analysis Approach

Use of the image analysis approach will improve the precision of determining fish age and should be used to validate the interpretation of age and growth data. The current technology will also allow digital images and the corresponding files for age determination to be archived and shared for continued quality control (Campana 2001). The image analysis system protocol should be adopted as a standard operating procedure, but should also include requirements for multiple reads and evaluation of reader accuracy through reference collections. As more scales are aged digitally, we will be able to test and build automated read programs to assist in determining fish age (Sub 2). It is expected that an automatic read system will further reduce subjective errors and lead to

results that are more reproducible and defensible.

Stepwise Procedure for the Whitefish Macro:

1. Open the Image Pro Plus software and click [Macro]["Whitefish"].
2. Introductory window will appear to provide a brief description and version # of the macro. Click [Continue].
3. A window will appear asking the user to either acquire an image or open an image from a file (Figure 1). Once an image is opened in the Image Pro Plus window then click [Continue]. The process for acquiring an image is described in Sub 1.
4. A window will appear telling the user that a series of filters will be applied to the image to provide a best fit contrast enhancement and convert the image to grey scale to speed up processing time (Figure 2). Click [Continue].
5. Next, the macro will ask the user if it is appropriate to apply a "sharpen image" filter (Figure 3). This filter is most useful for fish that are older (5+) and will help to detect annuli near the edge. The threshold value for the filters can be changed, but not while the macro is running. It is not critical to know the exact age. If in doubt, click [yes].
6. After the image filters are run, a window will appear asking the reader to determine the appropriate pen size for a trace object feature (Figure 4). This step will produce an image file of the scale that is traced in black and white. In most cases, this image will help in the detection of annuli. If the reader is unsure which pen size is correct, then enter [7] in the pen size box. Click [Continue].
7. The next window will ask the reader to examine the images of the scale to detect annuli. By right mouse clicking on any of the images, the reader can choose to zoom in or out to view the image for examination of annuli. Click [Continue].
8. A window will appear telling the reader to get ready to mark each annulus (Figure 5). The macro will automatically select the contrast-enhanced image for marking annuli. Click [Continue].

9. A window will appear asking the user to draw a reference line for aging. The recommended line will start at the center and run at a 45° angle to the vertical axis. Once the reference line is drawn then click [Continue].
10. A window will appear asking the user to age the scale by marking each annulus (Figure 6). Simply click on each annulus and a point feature icon ($\oplus$) will appear.
11. A window will recommend that you save your measurements (not as a template) by clicking on the [Input/Output] tab in the Measurement Window (Figure 7). Use a naming format to match the measurements with the image (See Sub 1. #8). The file name can be the same as the image name since it will be given a different extension (.msr).
12. The next window will recommend that the reader load any previous reads for the image (Figure 8). If this is the first read, the user can exit the macro at this point. Once other reads are loaded, or if the user would like to continue, click [Continue].
13. A window will then appear asking the user to examine all the reads for final age determination (Figure 9). At this point, the user could be directed to access the auto-read program as described in Sub 2. Once the final age is determined, the user will be asked to take the measurements for back-calculation (Figure 9). Click [Continue].
14. The next window will ask the user to click on the center, edge, and each annulus from the [Tag Points] window box (Figure 10). When the [Tag Points] window is opened, a drop-down menu will appear that allows the user to select which type of mark will be made (e.g., center). Once the features are marked then click [OK] and the data will appear. If the data window is correct click [Continue].
15. A window will appear asking the user to save the points using the [File] [Save Points] feature (Figure 10). Save the points using the same naming format as the image (.tif) and measurement (.msr) files and click [Continue].
16. Once the points are saved, the macro will automatically download the back-calculation data to an Excel spreadsheet and ask the user

to review the data by clicking on the Excel spreadsheet and enter a fish or scale identification code (Figure 11). When this step is completed then click [Continue].

17. The last window will direct the user to close the image file and rerun the macro if another scale is going to be processed (Figure 12). Continuing to process images will append the back-calculation data to the current Excel spreadsheet.

18. After processing the desired number of images, the Excel spreadsheet should be saved.

Sub 1. Acquiring a Digital Image of a Calcified Structure

1. Open the Image Pro software and power up the scope and digital camera.

2. Adjust the microscope and room settings to standardize light conditions. Light sources that interfere with image quality should be eliminated.

3. Place the whitefish scale (cleaned) on the microscope to determine the appropriate calibration.

4. Calibrate the Image Pro System to the microscope focus setting by clicking on [Measure] [Calibration] [Spatial Calibration] and selecting the matching number from the drop down menu. For most of the scales, readers will use a 1x-2x setting (1mm=270.8 pixels).

5. Click on the Scan Icon (white icon that looks like a flatbed scanner)
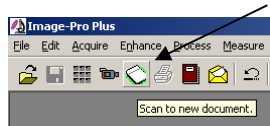
or click on [Acquire] [Scan]. The Spot Camera Window will then appear.

6. Click on Image Setup at the bottom of the Spot Camera Window and select the WhitefishScale setup. The WhitefishScale setup should be using a <Brightfield-transmitted light> setting, with white balance at 1.35, 1.0, and 2.3 (or reset using [whitebalance]).

7. Next click on the [Live] icon to view the image. Make adjustments to the image in the Live Image-Running window by adjusting the focus and centering the scale on the scope. Image quality should be adjusted in the Image Setup/Spot Camera Window. Click on [Snap] or [Acquire][Transfer] to capture the image and then close or minimize the Live Image and Spot Camera Windows.

8. Save the image by clicking on [File] [Save As] and use a descriptive filename for each image. For example: "Sample ID-Fish#-Scale letter-Year- Your Initials.tif".

9. It is recommended that images are captured from at least two scales per fish and this can be accomplished simply by returning to the Spot Camera Window. Check to make sure the image light and calibration settings are appropriate.

Run the Whitefish Macro. Note: The aging macro will allow the user to either capture the image as part of the initial steps or to open an image file that has already been saved. It is recommended, though, that the images from a given sample or lot be acquired before the aging macro is run. This will limit calibration errors and variation in image quality.

Sub 2. Automated Assistance Program for Age Determination

Two methods can be used to automatically assist in the detection of annuli on a whitefish scale. The first method evaluates ring intensity (Sub 2.1; Figure 13) and the second method evaluates ring spacing (Sub 2.2; Figure 14). Both programs apply algorithms to the data and are able to 'learn' as more scales are read (Figure 15). Analysis rules will have to be developed to address (1) the characteristics that can be used in identifying annuli (intensity, width, center), and (2) the values to determine what qualifies as annuli.

Data should be exported from the Whitefish Macro in Image Pro Plus using the "DDE to" function (text form). The data will be placed in a column of numbers starting in Row #1. Each row corresponds to a pixel and the column of pixels is arranged in a line starting from the center of the scale to the edge of the scale. Most scales will have approximately 700-1000 pixels in a line from the center to the edge and each

pixel is a fixed, known distance. For Sub 2.2, the Macro will run a best-fit contrast filter and convert the image to grey scale so that it can be traced.

Sub 2.1-Examination of Ring Intensity through Line Profile and Bitmap Analysis

For this analysis, peak heights and valleys in light intensity will be counted and evaluated using the [Line Profile] feature. The [Line Profile] feature analyzes light intensity from the center to the edge of the scale (Figure 13). A base line or threshold value can be set to evaluate possible annuli. For example, a value below 175 (luminescence) indicates a possible annulus (Figure 13).

Sub 2.2 Use of Two-Dimensional Contrast to Determine Spatial Frequency of Rings

The Whitefish Macro will run a best-fit contrast filter and convert the image to grey scale so that it can be traced. By using the trace feature, each pixel is transformed to either white or black (Figure 14). To interpret the pixels, they are assigned a value (0=white; 1=black). The data will include groups of 0s or 1s that correspond to a space (0s) or a circuli (1s). An annulus occurs on the scale when growth is slow and several circuli are formed close together. Spaces (groups of 0s) between circuli will tend to decrease near annuli. In addition, the circuli (groups of 1s) may increase in width in annuli. However, the reduction in spaces between circuli is probably more pronounced than the increase in circuli width (Figure 15).
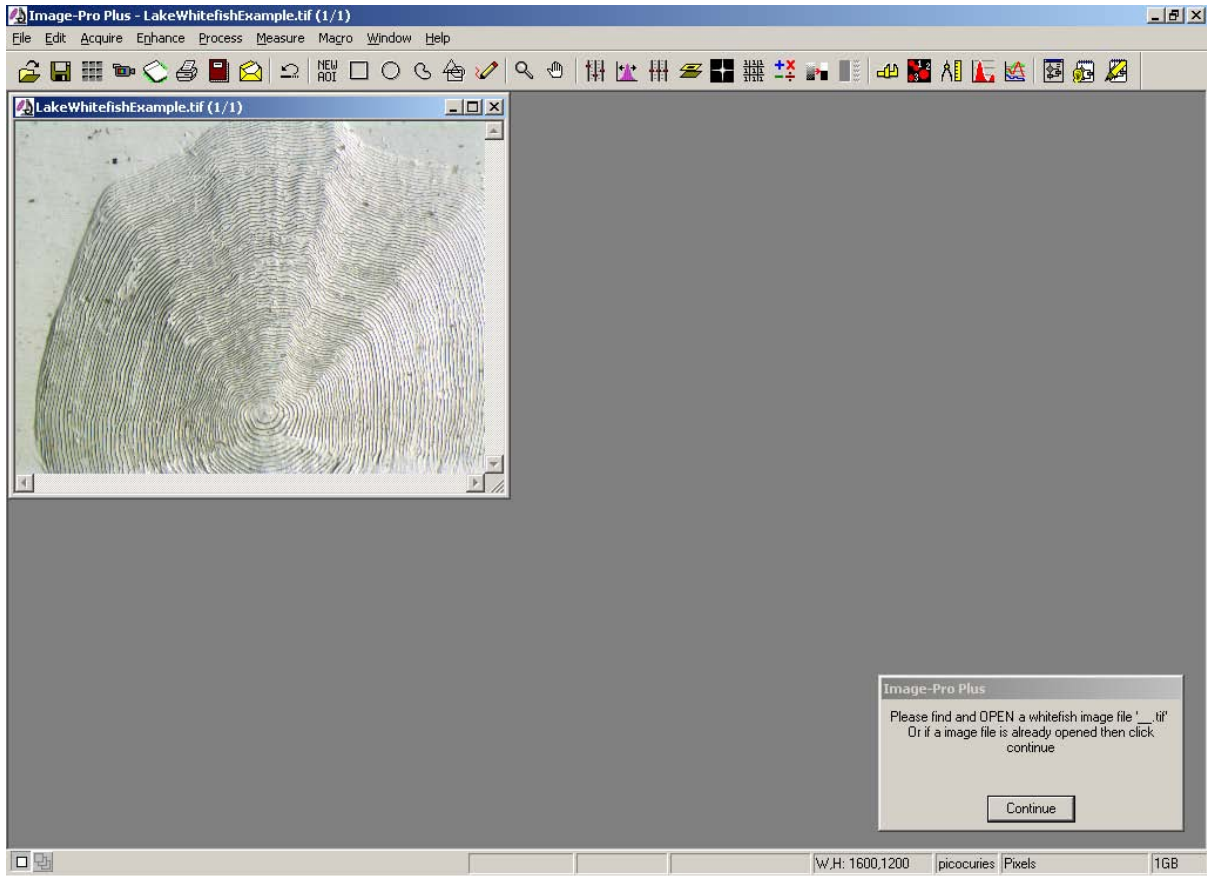
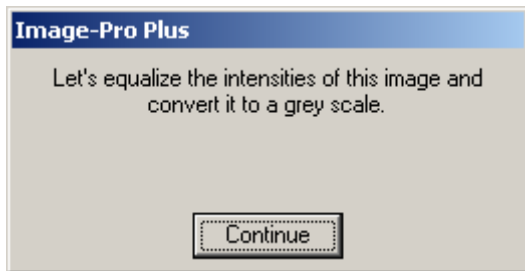Figure 1.–Whitefish Macro - Step 3
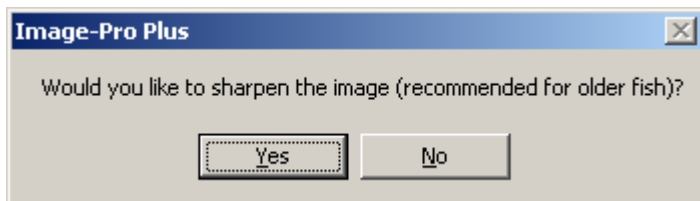

Figure 2.–Whitefish Macro - Step 4

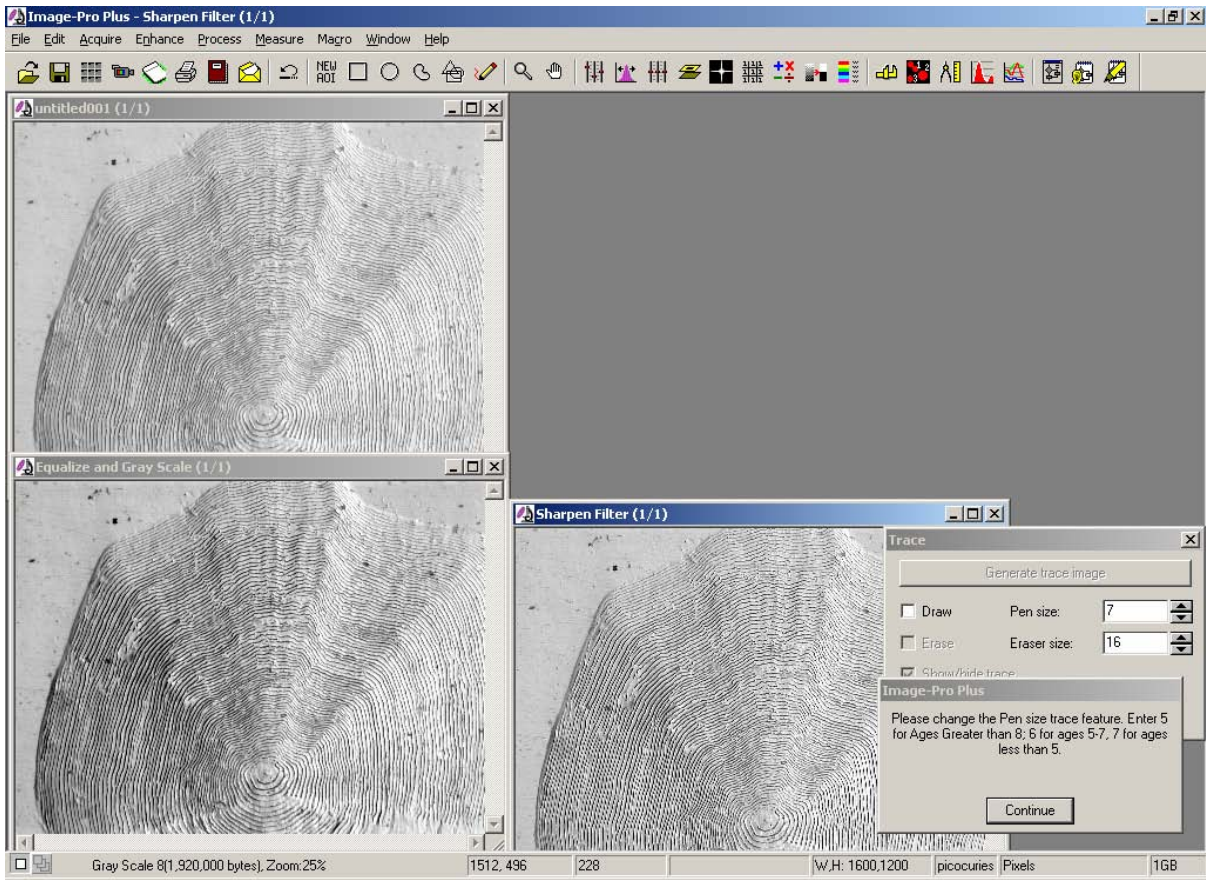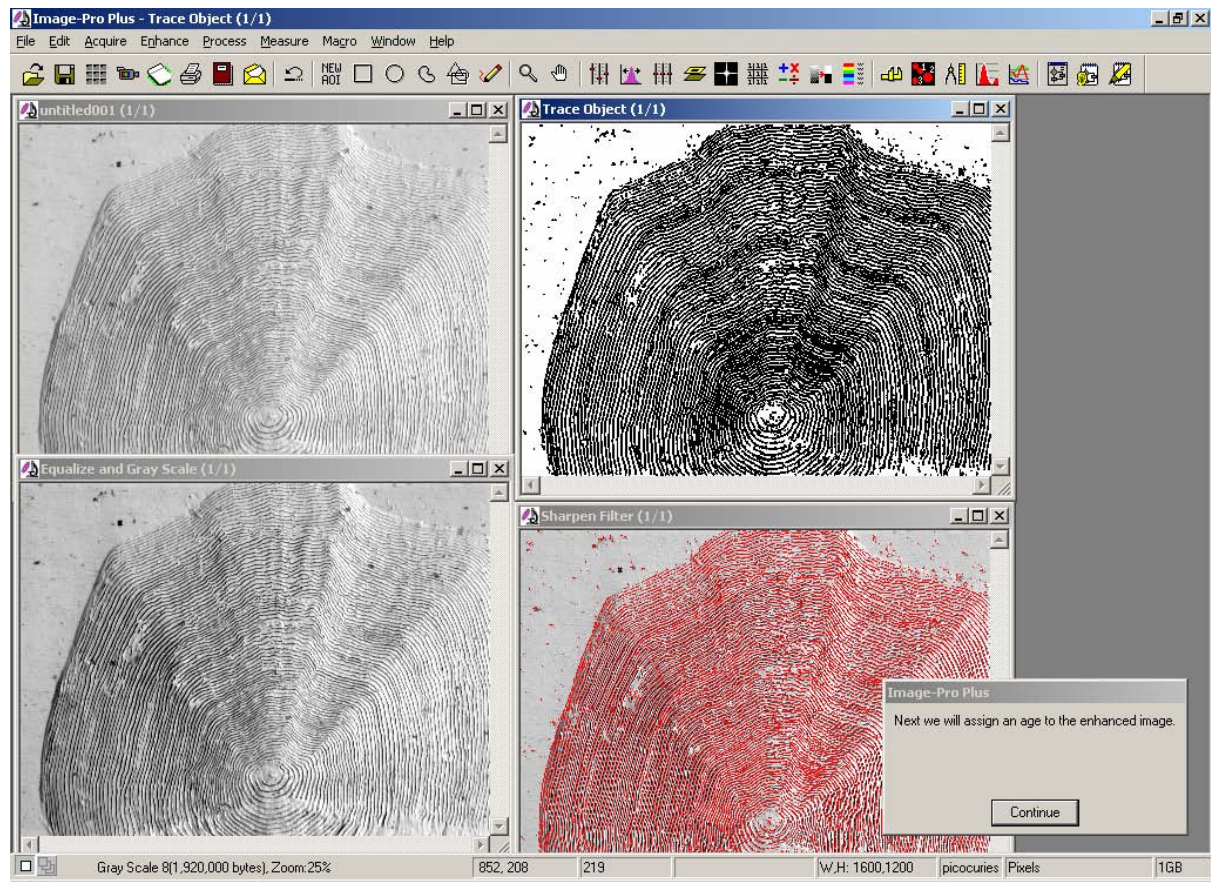Figure 3.–Whitefish Macro - Step 5

Figure 4.–Whitefish Macro - Step 6

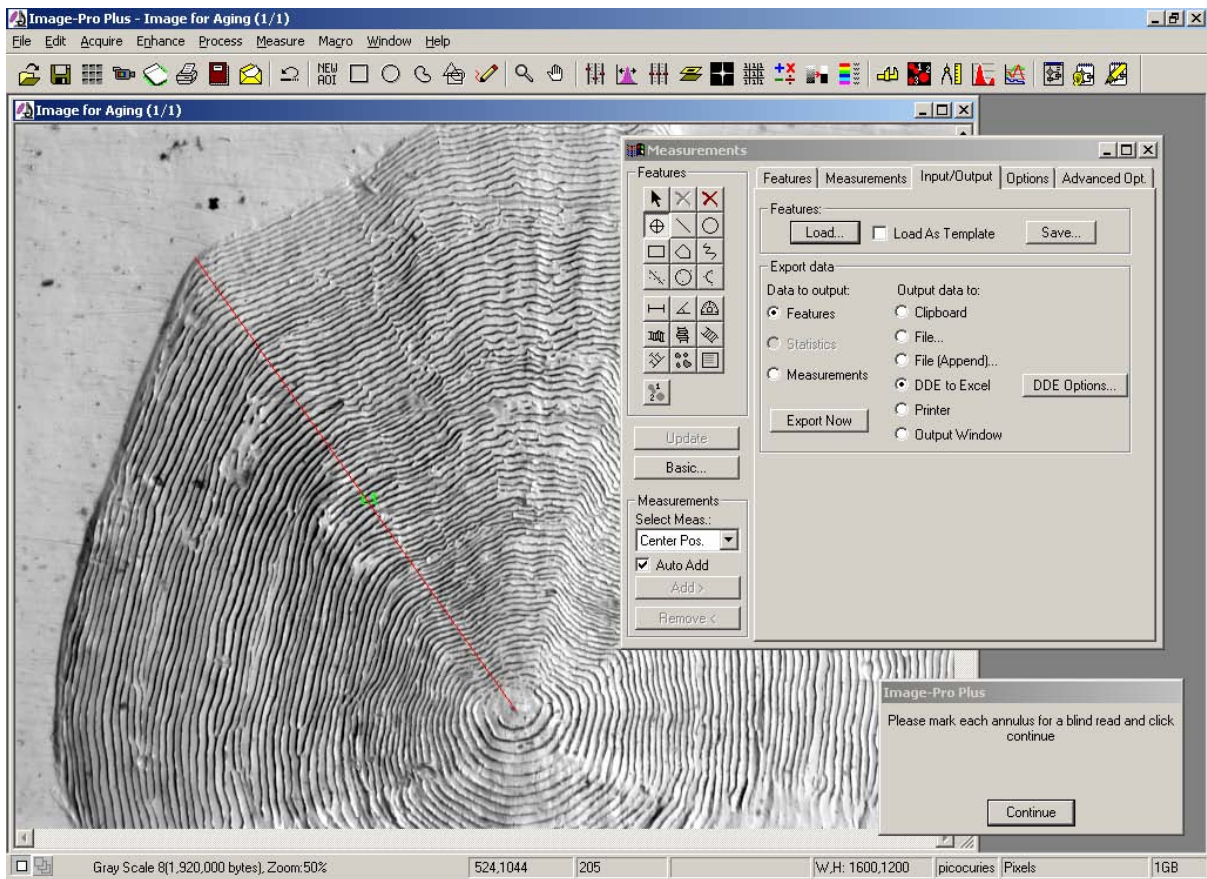Figure 5.–Whitefish Macro - Step 8

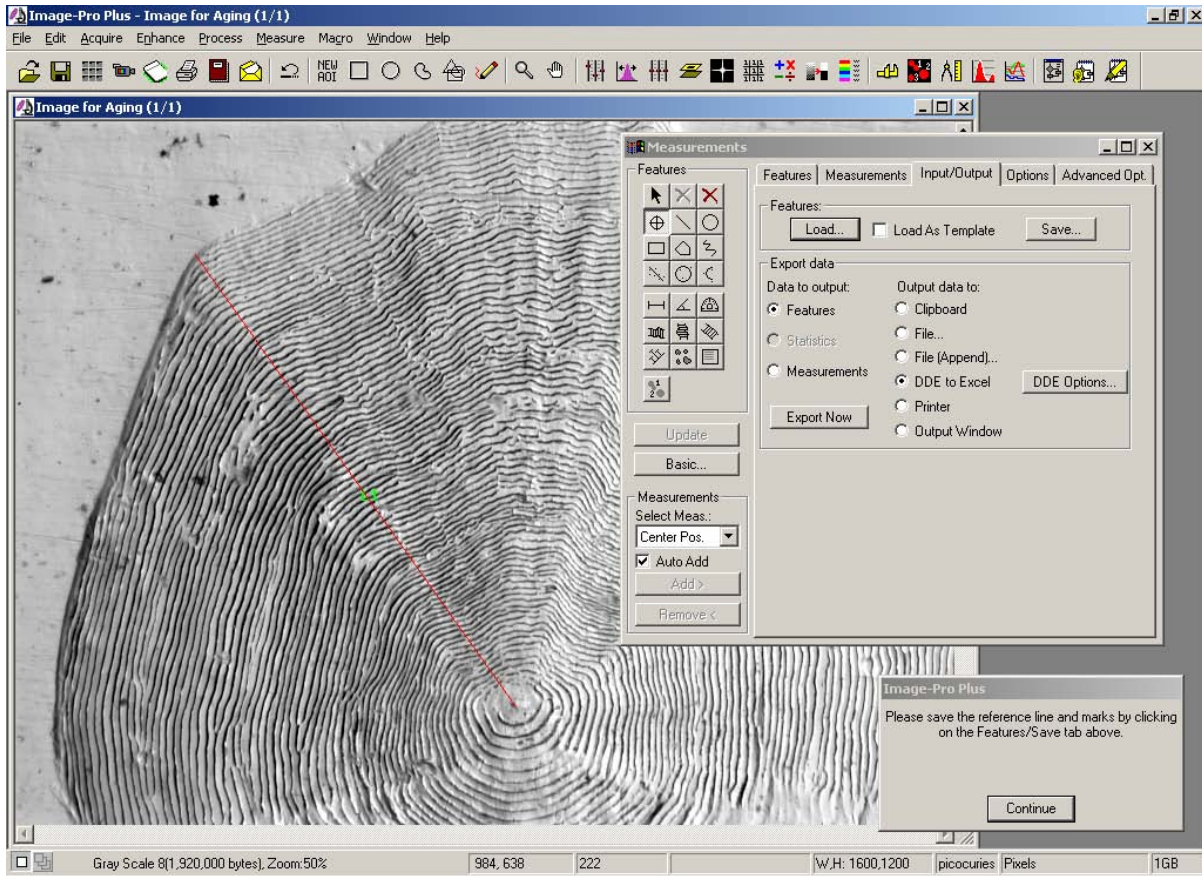Figure 6.–Whitefish Macro - Step 10
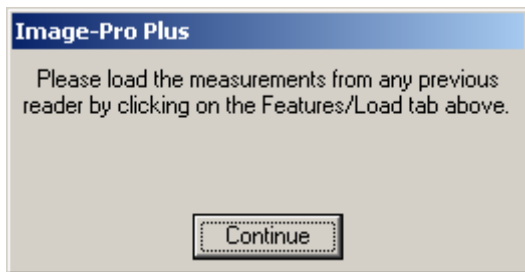
Figure 7.–Whitefish Macro - Step 11
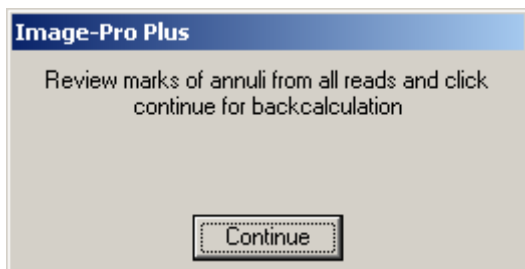

Figure 8.–Whitefish Macro - Step 12

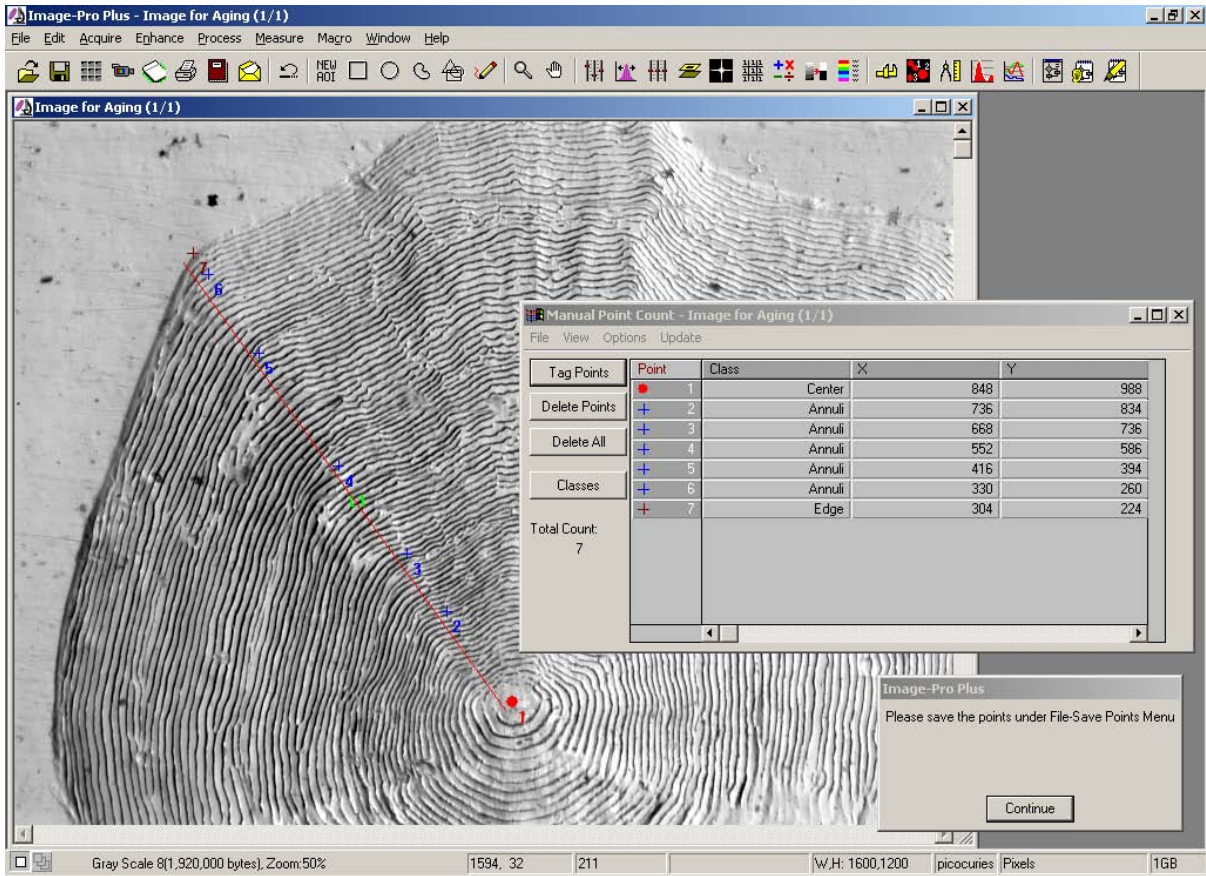Figure 9.–Whitefish Macro - Step 13

Figure 10.–Whitefish Macro - Steps 14 and 15
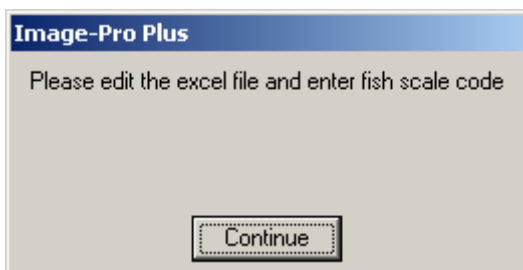
Figure 11.–Whitefish Macro- Step 16
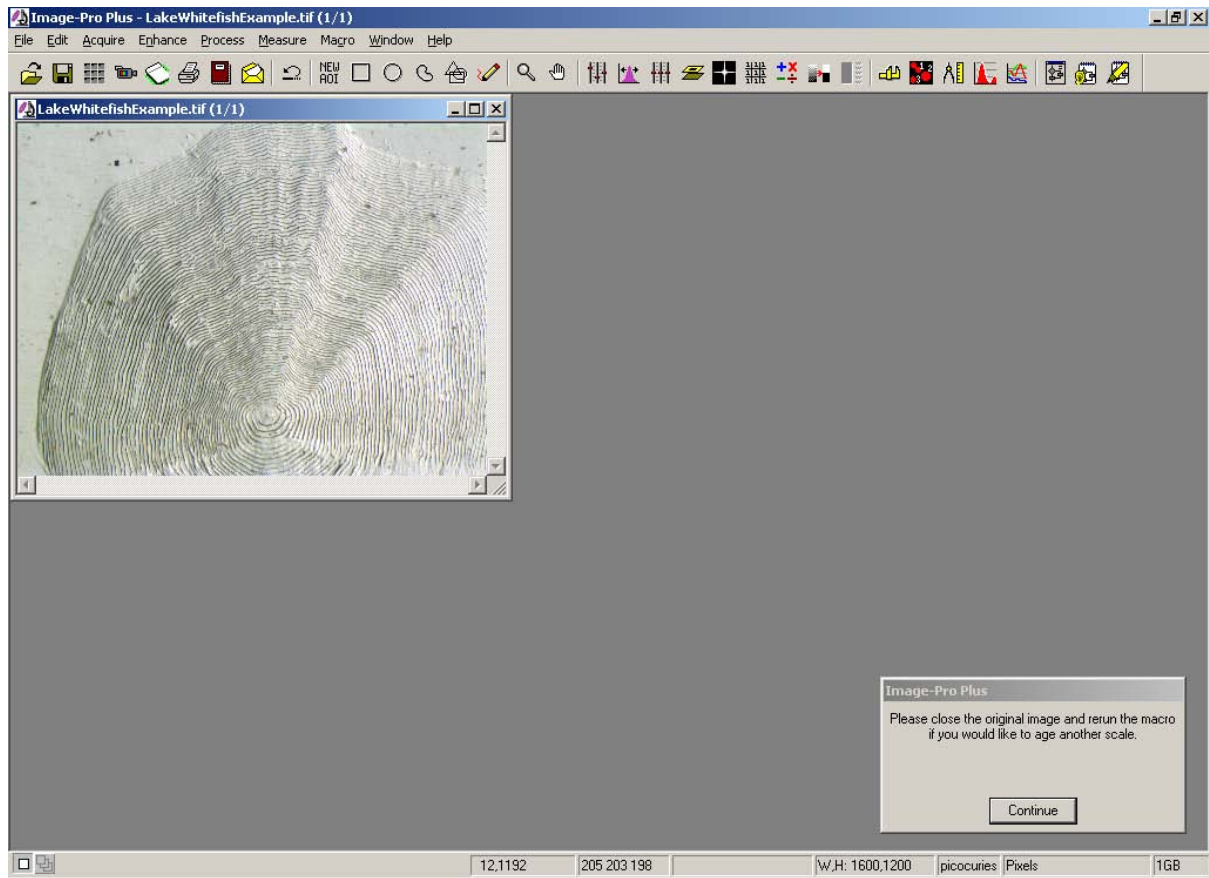
Figure 12.–Whitefish Macro - Step 17

12
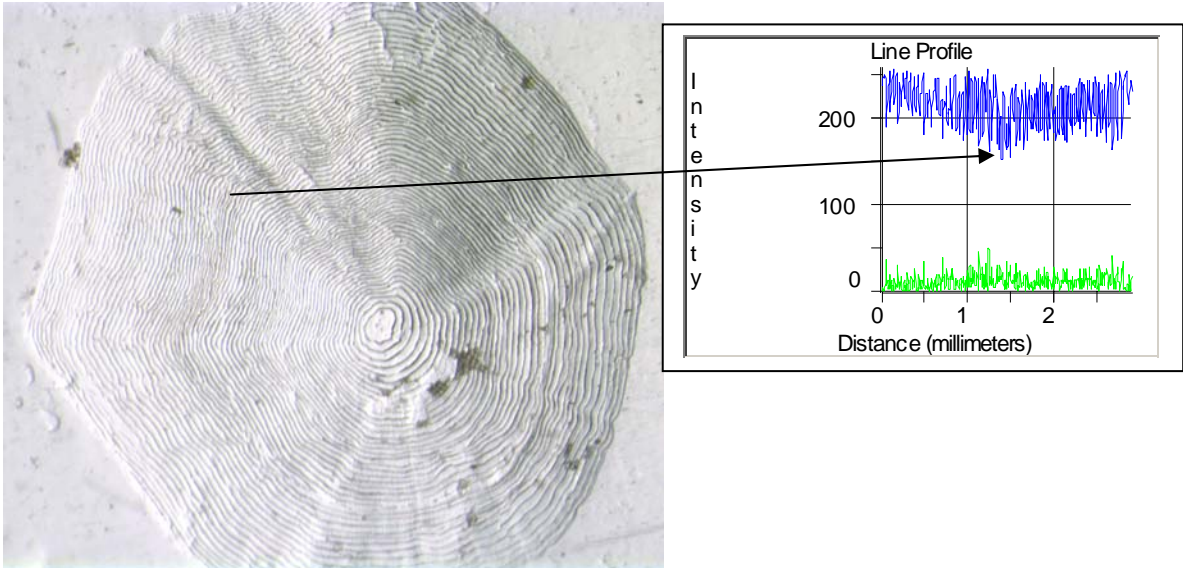
Figure 13.–An example analysis of light intensity (luminescence) indicating a possible annulus.

00000111110000001111110011111000111111101111110001111110000001111110000011111000001111100000011111

# of 0s	5		6		2		3		1		3		6		5		5		6	
# of 1s		5		6		5		7		6		6		6		5		5		5

start------center of annuli---end
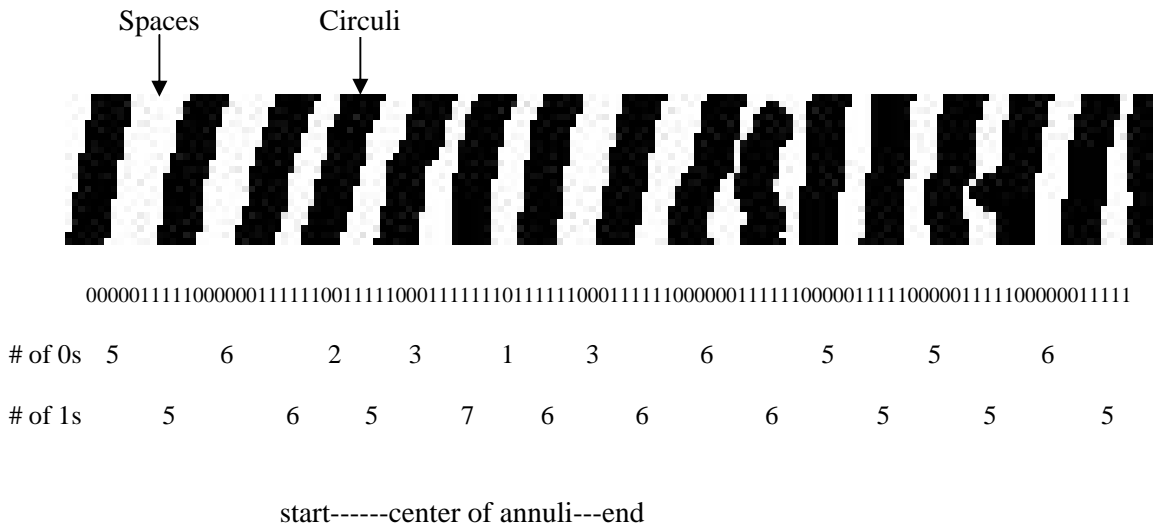
Figure 14.–Example of two-dimensional contrast to determine spatial frequency from a section of the traced image of a whitefish scale. Pixels in the traced image are assigned a value (0=white; 1=Black) and groups of pixels are defined (groups of 0s=Space between circuli; groups of 1s=Circuli).
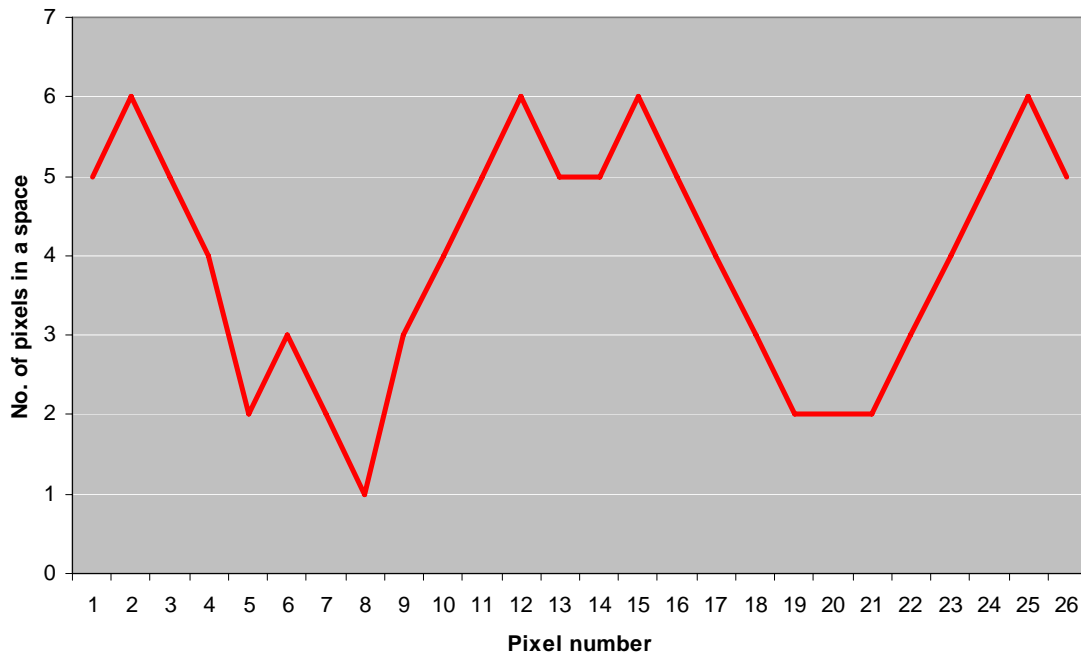
Figure 15.–Number of pixels in the spaces between circuli (classified as 0s) on a whitefish scale. As the spaces between circuli get smaller, an algorithm evaluates the "valley" to determine if it qualifies as an annulus.

Table 1.–Image analysis files and estimated file size.

File	Type	Estimated Size (bytes)
Image	.tif	5,500,000
Image	.jpg	800,000
Read 1	.msr	4,000
Read 2	.msr	4,000
Back-calculation	.tag	4,000
Total bytes per fish aged		6,312,000

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Welleman, H. C., and F. Storbeck. 1995. Automatic aging of plaice (*Pleuronectes platessa L.*) otoliths by means of image analysis. Pages 271-282 *in* D. H. Secor, J.M. Dean, and S.E. Campana, editors. Recent developments in fish otolith research. University of South Carolina Press, Columbia.

Appendix 1.–Script for Lake Whitefish Macro.

Sub Whitefish()

```
' Aging and Back-calculation. February 5, 2003.  R. Claramunt.
        Dim str1 As String, str2 As String
        Dim r1 As Integer
        Dim textpt As POINTAPI

        str1 = "This macro designed to assist in aging and back-calculating for lake
                whitefish scales. "
        str2 = "Please note any changes to this macro or its development in the macro
                script. MDNR Survey Manual Version 1.1."
        ret = IpMacroStop("Please find and OPEN a whitefish image file '__.tif'  Or if a

                image file is already opened then click continue",0)
        ret = IpMacroStop("Let's equalize the intensities of this image and convert it to a
                grey scale.",
                MS_MODAL)
        ret = IpWsConvertImage(IMC_GRAY, CONV_SCALE, 0, 0, 0, 0)
        ret = IpWsDuplicate()
        ret = IpWsChangeDescription(INF_NAME,"Equalize and Gray Scale")
        ret = IpHstEqualize(EQ_BESTFIT)
        ret = IpDocMove(1, 305)
        ret = IpMacroStop("Would you like to sharpen the image (recommended for older
                fish)?",MS_MODAL+MS_YESNO)
        If ret = 0 Then 'get out
                GoTo end_separate
                If ret = 1 Then

                ret = IpWsDuplicate()
                ret = IpFltShow(1)
                ret = IpFltSharpen(5, 9, 2)
                ret = IpFltShow(0)
                ret = IpAppSelectDoc(3)
                ' The image signature for the image is: 19E8-90B5-FB6D-3947-CA12-
                        F1DC-99CB-63BA
                ret = IpDocMove(427, 345)
        End If

        ret = IpTraceShow(1)

        ret = IpMacroStop("Please change the Pen size trace feature. Enter 5 for Ages
                Greater than 8; 6 for
                ages 5-7, 7 for ages less than 5.",0)
        ret = IpTraceDo(TR_AUTO)
        ret = IpTraceDo(TR_IMAGE)
        ret = IpTraceShow(0)
        ret = IpDocMove(428, -1)
        ret = IpWsChangeDescription(INF_NAME,"Trace Object")

        ret = IpMacroStop("Use the Zoom feature to examine annuli on any of the
                images.",0)
        ret = IpMacroStop("Next we will assign an age to the enhanced image.",0)
        ret = IpDocClose()
```

Appendix 1.–Continued.

```
ret = IpDocClose()
'ret = IpMacroStop("This next step will redo the enhance and contrast of the

        image",
        MS_MODAL)
'ret = IpWsDuplicate()
ret = IpWsConvertImage(IMC_GRAY, CONV_SCALE, 0, 0, 0, 0)
ret = IpHstEqualize(EQ_BESTFIT)
ret = IpWsZoom(50)
ret = IpWsChangeDescription(INF_NAME,"Image for Aging")
ret = IpDocMaximize()
ret = IpDocSize(829, 646)
ret = IpMeasShow(MEAS_SHOWADVANCED)
ret = IpMeasTool(MEAS_LENGTH)
ret = IpMeasAttr(MEAS_DISPCOLOR, 0)
ret = IpMeasTool(MEAS_LENGTH)
ret = IpMacroStop("Please draw a reference line at a 45 degree angle for
        backcalculation and click continue",0)
ret = IpMeasShow(MEAS_SHOWADVANCED)
ret = IpMeasTool(MEAS_POINT)
ret = IpMacroStop("Please mark each annulus for a blind read and click
        continue",0)
ret = IpMacroStop("Please save the reference line and marks by clicking on the
        Features/Save tab above.",0)
ret = IpMacroStop("Please load the measurements from any previous reader by
        clicking on the Features/Load tab above.",0)
ret = IpMeasShow(MEAS_HIDE)
ret = IpMacroStop("Review marks of annuli from all reads and click continue for
        backcalculation", MS_MODAL)
ret = IpTagShow(1)
ret = IpTagLoadEnv("F:\WhitefishAging\whitefish.tag")
ret = IpMacroStop("Please mark the center, edge, and each annulus using the 'Tag
        Points' feature",0)
ret = IpMacroStop("Please save the points under File/Save Points Menu",0)
ret = IpTagSaveData("", S_DDE+S_HEADER+S_X_AXIS)
ret = IpMacroStop("Please edit the excel file and enter fish scale code",0)
ret = IpTagShow(0)
ret = IpMacroStop("Please close the original image and rerun the macro if you
```

End Sub