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A Method to Identify the Sex of Cultured Eel (*Anguilla japonica*) using the Pectoral Fin Length: Total Body Length Ratio

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양식 뱀장어에서 전장과 가슴지느러미 비율에 따른 암수판별법

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Abstract

Previous methods for identifying the sex of eels were difficult to apply to yellow eels due to their undeveloped gonads. In this study, we investigated easy method to identify the sex of cultured eel, *Anguilla japonica*, using the ratio between the vertical and horizontal length of the pectoral fin and total body length. Females had longer total body length (TL):horizontal fin length (F*h*) and TL:vertical fin length (F*v*) ratios than males. Based on our results, we found that both the TL:F*h* and the TL:F*v* ratio were suitable for determining the sex of cultured yellow eel. We therefore suggest using the TL:F*h* ratio to determine the sex of yellow eels under natural conditions (i.e. without anesthetizing or sacrificing the eels). Specially, eels with TL:F*h* ratios below and above 31.6 should be identified as males and females. To our knowledge, this is the method to determine the sex of yellow eel.

Key words : Anguilla japonica, Identify the sex, Pectoral fin length, Body length, Japanese eel

Ⅰ. Introduction

Most of the eels migrate to fresh water upstream, and some stay in brackish water, and turn a yellowish color, a stage referred to as yellow eel (Tsukamoto et al., 1998; Tzeng et al., 2000). After five to 12 years, the yellow eels grow to reach breeding size, and migrate downstream to start their spawning journey (Tsukamoto, 2009). During the downstream migration, yellow eels become sexually mature, and, after the second metamorphosis, they turn into silver eels, and migrate back to the ocean for spawning (Davey and Jellyman, 2005; Tsukamoto, 2009).

Eel is a very important fisheries species for the East Asian countries. However, most of the eels

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are supplied by aquaculture. The aquaculture of eel is totally dependent on wild glass eels, which are caught during their upstream migration (Tsukamoto, 2009). In recent years, eel fishery has not been sustainable (FAO, 2017). Eel populations are at risk, and they may become critically endangered because very few eel remain in their natural environment, which impedes successful reproduction (FAO, 2014). Therefore, new strategies for artificially induced breeding of eels are urgently required. Such strategies may help meet the demand for glass eels, and help develop and maintain aquaculture eel production, thereby protecting the wild population of glass eels (Ohta et al., 1997; Kagawa et al., 2005). Breeding eels in captivity is the best way to avoid the problems facing wild eel populations. However, due to their complex life history, it is difficult to produce the complete life cycle of eel commercially under captive conditions. Under culturing conditions, females produce oocytes with early development stages, but males are sexually immature (Yamamoto and Yamauchi, 1974). Therefore, studying artificially induced breeding in eel is essential to solve this problem. However, sexing cultured and wild eels is a crucial problem in the eel industry, because it is extremely difficult to accurately determine the sex of eels using morphological characteristics.

There are several gonad specific genes that are currently used for fish sex determination. These include *sdY* in rainbow trout, *Oncorhynchus mykiss* (Yano et al., 2012), *DMY/dmrtbY* in Japanese rice fish, *Oryzia latipes* (Matsuda et al., 2002), *DSY/amhy* in Patagonian silverside, *Odontesthes hatcheri* (Hattori et al., 2012), *COS1* expressed in eel (Jiang et al. 2003), and *gsdf* in Luzon rice fish, *Oryzia luzonensis* (Myosho et al., 2012). Furthermore, the gene *amhr2* was isolated by Kamiya et al.(2012) from Japanese puffers, *Takifugu rubripes,* and identified as a male specific gene. *SSP120* genes may be used for sex determination in African cichlids, including *Astatotilapia burtoni*, *Pundamilia nyererei. Haplochromis sp., Melanochromis auratus, Pseudotropheus sp*., and *Pseudocrenilabrus multicolor* (Gerrard and Meyer, 2007). The *20-beta-hydroxysteroid dehydrogenase* gene has also been used for sex determination in cichlids (Baldo et al., 2011) and the aromatase (*CYP19A*) gene has been used for sex determination in olive flounder, *Paralichthys olivaceus* (Lim et al., 2013). However, the main disadvantage of these methods is that fish have to be sacrificed, which prevents them from contributing to creating next generation.

In this study, we investigated a method to identify the sex of cultured eel using the ratio between the length of the pectoral fin and total body length. We carried out gonadal histological analysis in order to confirm the accuracy of this method to identify the sex of eel.

Ⅱ. Material and methods

1. Experimental animals, sampling, and morphometric measurements

A total of 254 cultured eel were purchased from the Eel Fisheries Cooperative Farm in Younggwang, Jeollanamdo Province, Republic of Korea. Prior to the experiment, the eels were transferred to the Marine Science Institute of Jeju National University, Republic of Korea. The eels were kept in an indoor plastic tank (5 metric tons) with running fresh water for one week. The fish were not fed until the sampling day. After collection, the sampling procedure involved anesthetization using

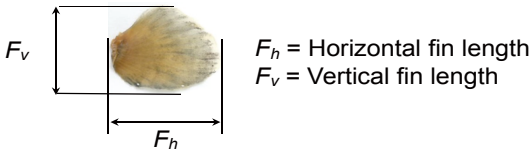
2-phenoxyethanol (Junsei Chemical Co., Ltd., Chuo-Ku, Tokyo, Japan) and decapitation, in accordance with the guidelines of the Animal Care Welfare Committee of Jeju National University. Body weight (BW), and total body length (TL) were measured in all individuals before they were sacrificed. To identify the sex of the eels, the gonads were isolated and fixed in Bouin's solution until histological analysis. Pectoral fins were isolated from the left side of all eels, and fixed in Bouin's solution prior to the fin measurements.

2. Pectoral fin fixation and measurement

The left pectoral fin of each eel was isolated and spread on a spreadsheet using pins. Subsequently, both sides of the fins were painted with 4% chloroform. This step was repeated five or six times, until fins became fully dry. Then, the pins were removed and fin measurements were taken. Horizontal fin length (F*h*) was measured from the base of the fin horizontally, and vertical fin length (F*v*) was measured as the maximum length of the fin. Measurements were taken using a digital Vernier caliper ([Fig. 1]). Based on these variables, TL:F*h* and TL:F*v* ratios were calculated as follow:

TL:F*h*= TL(cm)/ F*h* (mm) x 10

TL:F*v*= TL(cm)/ F*v* (mm) x 10



[Fig. 1] Accurate fin measuring technique using Vernier calipers. We measured the pectoral fin length from the base of the fin to the maximum vertical (F*v*) and horizontal (F*h*) length.

3. Histological analysis of gonads

Histological analysis was carried out to confirm the sex of the sacrificed eels. The gonad, which were fixed in Bouin's solution, were consecutively dehydrated. Subsequently, dehydrated gonad tissues were embedded in paraffin wax. The sections were cut at 7 μm thickness and mounted on glass slides. Sectioned tissues were stained with Mayer`s hematoxylin and eosin, to determine the sex of each individuals under a light microscope (data not shown).

4. Statistical analyses

Statistical analyses were done using GraphPad Prism 8.0.2 Software. Length and ratio between male and female group were performed by the Unparied *t*-test. In this study, P <0.05 was accepted as statistically significant. Differences among mean values were examined by ANCOVA for the data of total length and pectoral fin length, with a criteria of significance fo P <0.01.

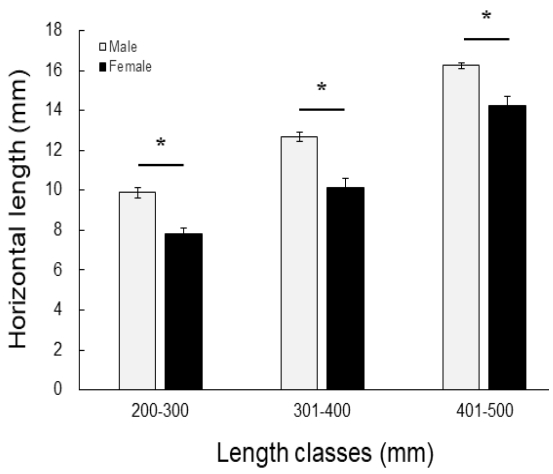
Ⅲ. Results

The total length of cultured eel was 424.2 ±47.4 (n= 214) in male and 334.1 ±64.9 cm (n= 40) in female. The F*h* of male were significantly longer than that female ([Fig. 2] and <Table 1>, male; 15.4 ±2.6 mm, female; 10.6 ±2.9 mm, unpaired *t*-test; P <0.05, ANCOVA; P >0.01). However, no significant differences were found between male and female of F*v* ([Fig. 3] and <Table 2>, male; 13.3 ±2.5 mm, female; 9.2 ±3.1 mm). The TL:F*h* ratios of female were significantly longer than that male ([Fig. 4] and <Table 3>; female; 32.2 ±4.1; male; 28.0 ± 3.2, unpaired t test, P <0.05). The

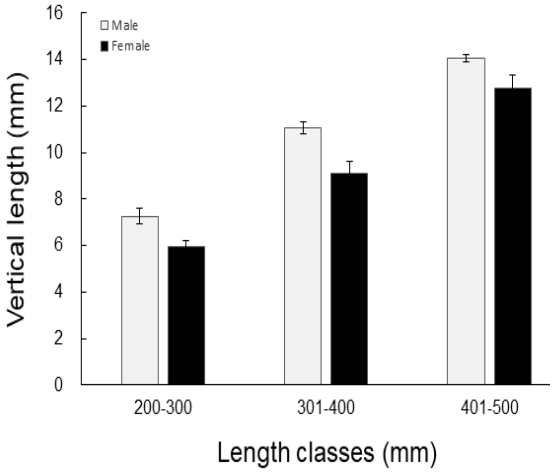
female of TL:F*v* ratios were also significantly longer than that male ([Fig. 5] and <Table 4>; female; 38.1 ±6.8; male; 32.65 ±4.7, unpaired t test; P <0.05, ANCOVA; P >0.01).

<Table 1> Results of sequential analysis of covariance (ANCOVA) for significant variation in the horizontal length (F*h*) of the left pectoral fins between female and male in cultured eel.

Source	Sum of Squares	df	Mean Square	F-value	P-value
Intercept	43.914	1	43.914	17.564	0.000
TL (mm)	1173.465	1	1173.465	469.340	0.000
Sex	19.555	1	19.555	7.821	0.006
Error	627.561	251	2.500		
Total	56997.051	254			



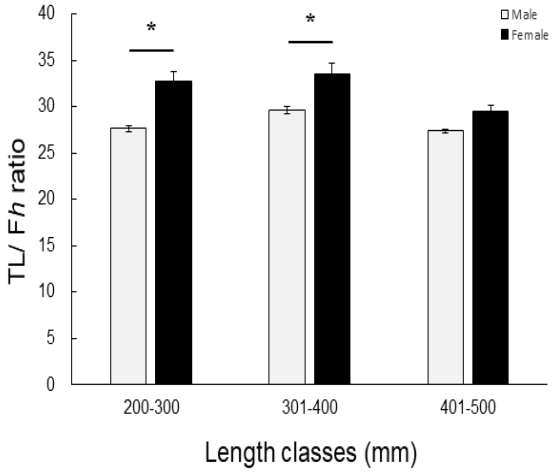
[Fig. 2] The horizontal length of the left pectoral fins (F*h*) of male and female cultured eels (200–300 length group; male, n = 5, female, n = 12, 301–400 length group; male, n = 58, female, n = 21, 401–500 length group; male, n = 151, female, n = 7) in different length classes. The horizontal length of pectoral fins (F*h*) was not significantly different (P <0.05) between males and females in the relevant length classes. Data are shown as mean ± standard error (SE).



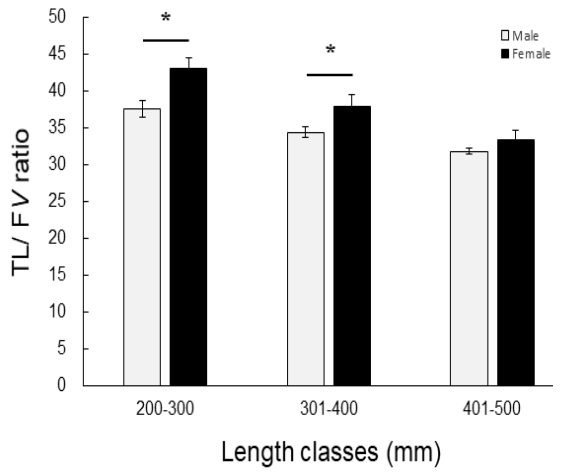
[Fig. 3] The vertical length of the left pectoral fins (F*v*) of male and female cultured eels (200–300 length group; male, n = 5, female, n = 12, 301–400 length group; male, n = 58, female, n = 21, 401–500 length group; male, n = 151, female, n = 7) in different length classes. The vertical length of pectoral fins (F*v*) was not significantly different (P <0.05) between males and females in the relevant length classes. Data are shown as mean ± standard error (SE).

<Table 2> Results of sequential analysis of covariance (ANCOVA) for significant variation in the vertical length (F*v*) of the left pectoral fins between female and male in cultured eel.

Source	Sum of Squares	df	Mean Square	F-value	P-value
Intercept	46.905	1	46.905	15.115	0.000
TL (㎜)	948.410	1	948.410	305.627	0.000
Sex	8.241	1	8.241	2.656	0.104
Error	778.894	251	3.103		
Total	42955.487	254			



[Fig. 4] The ratio of total length to horizontal length of the pectoral fin (F*h*) in cultured eels (200–300 length group; male, *n* = 5, female, *n* = 12, 301–400 length group; male, *n* = 58, female, *n* = 21, 401–500 length group; male, *n* = 151, female, *n* = 7) in different length classes. The ratio of total length to F*h* differed significantly between male and female eels in the 200–300 mm and 301–400 mm length groups, while there was no significant difference between the ratio of total length to F*h* of male and female eels in the 401–500 mm length group. Data are shown as mean ± standard error (SE). **P* <0.05



[Fig. 5] The ratio of total length to vertical length of the pectoral fin (F*v*) in cultured eels (200–300 length group; male, *n* = 5, female, *n* = 12, 301–400 length group; male, *n* = 58, female, *n* = 21, 401–500 length group; male, *n* = 151, female, *n* = 7) in different length classes. The ratio of total length to F*v* differed significantly between male and female eels in the 200–300 mm and 301–400 mm length groups, while there was no significant difference between the ratio of total length to F*v* of male and female eels in the 401–500 mm length group. Data are shown as mean ± standard error (SE). **P* <0.05

<Table 3> Results of sequential analysis of covariance (ANCOVA) for significant variation in the ratio of total length (TL) to horizontal length (F*h*) of the left pectoral fins between female and male in cultured eel.

Source	Sum of Squares	df	Mean Square	F-value	P-value
Intercept	5845.347	1	5845.347	573.295	0.000
TL (㎜)	196.754	1	196.754	19.297	0.000
Sex	168.906	1	168.906	16.566	0.000
Error	2559.209	251	10.196		
Total	211835.278	254			

<Table 4> Results of sequential analysis of covariance (ANCOVA) for significant variation in the ratio of total length (TL) to vertical length (F*v*) of the left pectoral fins between female and male in cultured eel.

Source	Sum of Squares	df	Mean Square	F-value	P-value
Intercept	9467.038	1	9467.038	396.414	0.000
TL (mm)	580.350	1	580.350	24.301	0.000
Sex	175.725	1	175.725	7.358	0.007
Error	5994.305	251	23.882		
Total	292638.547	254			

Ⅳ. Discussion

In recent years, eel populations have drastically declined due to the mass exploitation of glass eels, yellow eels in East Asia area (MAFF in Japan: http://www.jfa.maff.go.jp/j/press/sigen/attach/pdf/180713-5.pdf accessed December 2018). Artificially induced breeding and reintroduction of yellow eels to natural waterbodies are the main successful methods to maintain wild populations of glass eels (Haenen et al., 2009). Sexing of cultured eels is one of the main problems that needs to be overcome in artificial eel breeding. In general, a higher percentage of cultured eels are male (Davey and Jellyman, 2005). In European and Japanese eel, males may make up 75 - 90% of the cultured population (Egusa, 1979). Therefore, it is important to have several available sex determination methods to be able to determine the sex of eels. During the silver stage, female eels generally are longer in size than males (Tesch, 2003). Hence, in the past several decades, length variation was used to determine the sex of migrating eels in the eel industry. Several studies have been conducted to determine the sex of eels using the total length of migrating eels (e.g. eel, Tzeng et al., 2000;

European eel, Colombo et al., 1984), American eel, *Anguilla rostrata* (Barbin and McCleave, 1997), short-finned eel, *Anguilla australis* (Todd, 1980), New Zealand longfin eel, *Anguilla dieffenbachii* (Todd, 1980), and speckled longfin eel, *Anguilla reinhardtii* (Walsh et al., 2003). However, to our knowledge, no studies have been previously carried out that use this method to determine the sex of eels during the yellow eel stages (before migrating stages).

As is the case for mammals, ultrasonography methods are used in modern aquaculture industries to determine the sex of fish species such as yellowtail flounder, *Pleuronectes ferruginea* (Martin-Robichaud and Rommens, 2001), Atlantic halibut, *Hippoglossus hippoglossus* (Martin-Robichaud and Rommens, 2001), haddock, *Melanogrammus aeglefinus* (Martin-Robichaud and Rommens, 2001), small spotted catshark, *Scyliorhinus canicula* (Whittamore et al., 2010), thornback ray, *Raja clavata* (Whittamore et al., 2010), and striped bass, *Morone saxatilis* (Jennings et al., 2005). In a recent study, du Colombier et al.(2015) applied ultrasonography methods to determine the sex and maturation of silver stage European eels. However, this method is difficult to apply to yellow eels due to their undeveloped gonads before seaward migration. In addition, the equipment used in this method is comparatively expensive, and is generally designed for medical purposes. Therefore, the ultrasonography equipment is not user friendly for use in the field on aquatic organisms.

Sex determining gene expression is also widely used in aquaculture industry to confirm the sex of fish. To our knowledge, no studies have been conducted to determine the sex of eels using gene expression technology. This may be due to the complex life history strategies of eels, and the modes of gonadal differentiation, which vary in different eel species and sexes (Davey and Jellyman, 2005).

Sex manipulation by environmental modification and hormone administration are sex conversion methods commonly used in the aquaculture industry. At high density culturing conditions, the male conversion rate is high, whereas at low densities, the number of females will increase (Tesch, 2003). In addition, there are several sex steroid hormones that have been used to increase the female population in eel industry. Generally, diethylstilbestrol is commonly used to increase the number of female eel (Satoh et al., 1992), phytoestrogen, estradiol and 17α-ethynylestradiol are commonly used to increase the number of female carp (Tzchori et al., 2004), and 17ß-estradiol and phytoestrogens are commonly used to increase the number of female European eels (Colombo and Grandi, 1995; Grandi et al., 2000; Tzchori et al., 2004). However, these methods cannot be used to determine the sex of eels using morphometrics.

The aim of the current study was to investigate a suitable method for determining the sex of cultured yellow eels. We measured different morphometric parameters to investigate the sex of eels. To do so, we separated all experimental yellow eels into three total length classes, i.e. 200–300 mm, 301–400 mm, and 401–500 mm, and calculated the TL:F*h* and TL:F*v* ratios in cultured eel.

Both TL:F*h* and TL:F*v* ratios in female were significantly longer than male in the 200–300 mm and 301–400 mm (but not 401–500 mm) length classes ([Fig 4] and [Fig. 5]). As shown in [Fig. 4] and [Fig. 5], female eels had longer TL:F*h* and TL:F*v* ratios than male eels. However, although the TL:F*h* and TL:F*v* ratios of female and male eels

were significantly different in the 200–300 mm and 301–400 mm total length groups, there were no significant differences in male and female TL:F*h* and TL:F*v* ratios in the 401–500 mm total length group ([Fig. 4] and [Fig. 5]). Therefore, based on these results, we conclude that it is possible to use TL:F*h* and TL:F*v* ratios to determine the sex of yellow eels that have total lengths between 200 and 400 mm. In the 200–300 mm group, eels with a TL:F*h* ratio below 27.6 were found to be male, and eels with a TL:F*h* ratio above 32.7 were found to be female.

Based on the TL:F*v* ratio, the threshold values to determine sex in female cultured eels are 40.3 and 36.2 for the 200–300 mm and 301–400 mm length classes, respectively. TL:F*v* ratios that exceed the 40.3 threshold value indicate the eels are female, and TL:F*v* ratios that are below the 36.2 threshold value indicate the eels are male. Accordingly, the ratio of TL:F*v* can be used to determine the sex of the cultured eels. Based on these results, both ratios (TL:F*h* and TL:F*v*) can be used to determine the sex of cultured eels. However, in the field, it is comparatively difficult to measure the vertical length of eel fins due to their forceful retraction. Therefore, we strongly suggest using the TL:F*h* ratio to determine the sex of eel under natural conditions (without sacrificing). Hence, to our knowledge, the TL:F*h* ratio is the best and easiest method for determining the sex of yellow stage cultured eel.

In conclusion, sex determination of eels is a difficult task due to the body shape and color of eels. In the current study, we investigated a method to determine the sex of yellow stage cultured eel by using the ratio between horizontal pectoral fin length and total length.

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