THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Cytogenetic Study of *Pleuronectes obscurus* (Herzenstein), *Konosirus punctatus* (Temminck et Schlegel) and *Pseudoblennius percoides* (Günther) in the Coastal Area of Jo Island, Busan, Korea

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부산 조도연안 어류, 감성가자미, *Pleuronectes obscurus* (Herzenstein), 전어, *Konosirus punctatus* (Temminck et Schlegel) 및 돌팍망둑, *Pseudoblennius percoides* (Günther)의 세포유전학적 연구

Advisor: Prof. In-Seok PARK

by

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ABSTRACT

The objective of this study was to clarify the cytogenetic features, including karyotypes, cellular DNA contents and nuclear size of erythrocytes of three species of fish, the black plaice *Pleuronectes obscurus* (Herzenstein, 1890), the dotted gizzard shad *Konosirus punctatus* (Temminck et Schlegel) and the perch sculpin *Pseudoblennius percoides* (Günther), in the coastal area of Jo Island, Busan, Korea. The karyotypes of *P. obscurus* and *K. punctatus* both had a diploid number of 2N = 48 and a fundamental number (FN) = 48, with a chromosome formula of 48T. The karyotype of *P. percoides* had a diploid number of 2N = 46 and FN = 50, with a chromosome formula of 4SM + 42T. No sex-associated heteromorphic pairs were detected for any species in this study.

The variation in the DNA values (*P. obscurus* = 1.15 pg/nucleus, *K.*

punctatus = 1.56 pg/nucleus, *P. percoides* = 1.11 pg/nucleus) was positively related to the variation in chromosome FN in fish examined.

The mean major and minor axes of erythrocyte nuclei measured 3.1 ± 0.16 μm and 2.5 ± 0.16 μm in *P. obscurus*, 4.1 ± 0.10 μm and 2.5 ± 0.07 μm in *K. punctatus*, and 3.0 ± 0.21 μm and 2.4 ± 0.16 μm in *P. percoides*, respectively. The mean surface area and volume of erythrocyte nuclei were 5.8 ± 0.12 μm^2 and 9.6 ± 0.79 μm^3, 8.1 ± 0.33 μm^2 and 13.5 ± 0.87 μm^3, and 5.9 ± 0.19 μm^2 and 9.6 ± 0.78 μm^3 in *P. obscurus*, *K. punctatus* and *P. percoides*, respectively. The ratios of major/minor axis of the nucleus were 1.64 in *K. punctatus*, 1.24 in *P. obscurus*, and 1.25 in *P. percoides*, which in turn may be responsible for the more elliptic form of the nucleus in *K. punctatus*.

Key words: DNA content, Erythrocyte size, Jo Island, Karyotype, *Konosirus punctatus, Pleuronectes obscurus, Pseudoblennius percoides*

Thesis Advisor: Prof. In-Seok PARK, Ph. D.

INTRODUCTION

Fish cytotaxonomy refers to the study of phenetic and/or phylogenetic relationships among species, based on comparisons of chromosome number and morphology, genome size, or the amount of DNA per nucleus (Gold, 1979; Kim and Park, 1990; Park, 1992; Park *et al.*, 1999). Particularly, regarding the chromosome numbers and genome sizes among diploid teleost fish, there is a highly significant, positive correlation between chromosome number and genome size (Perdersen, 1971; Hinegardner and Rosen, 1972; Gold and Amemiya, 1987).

The black plaice *Pleuronectes obscurus* (Herzenstein, 1890) in the order Pleuronectiformes, family Pleuronectidae, is widely distributed throughout the South, East, and West Seas and around Japan, in the East China Sea (Choi *et al.*, 2002). This fish generally inhabits coastal areas but has been introduced into estuaries. Its average total length is about 40 cm, and it spawns in spring; eggs are demersal-adhesive eggs (Choi *et al.*, 2002). The gizzard shad *Konosirus punctatus* (Temminck et Schlegel) in the order Clupeiformes, family Clupeidae is widely distributed in the East China Sea, Middle Sea around Japan, and South Sea of Korea. This species inhabits rivers. Its average total length is about 25 cm, and spawning mainly occurs in river estuaries from March to June; the eggs are separation-floating eggs (Choi *et al.*, 2002). The perch sculpin *Pseudoblennius percoides* (Günther) in the order Scorpaeniformes, family Cottidae, is distributed across the South Sea, including Jeju Island. This species inhabits rocky coastal regions, and its average total length is about 20 cm (Choi *et al.*, 2002).

The coastal areas of Busan Yeongdo, Korea, are affected by the open sea and have abundant aquatic organisms. Nonetheless, studies about the distribution of useful aquatic organisms and other resources around coastal Yeongdo are rare. Recently, several articles have investigated seasonal variation in the species composition of fish in the coastal waters of Yeongdo, as well as the distribution of useful aquatic organisms and resources (Park, 2005). In addition, cytogenetic studies of fish from coastal areas around Jo Island have been limited to *Parapercis sexfasciata* (Temminck et Schlegel), *Sebastiscus marmoratus* (Cuvier), and *Pleuronectes yokohamae* (Günther) (Park and Lee, 2005).

In this study, we sought to clarify the cytogenetic aspects of *P. obscurus*, *K. punctatus*, and *P. percoides* collected off the coast of Jo Island, Busan, Korea. Details of their karyological features, flow cytometry, and erythrocyte nucleus size are described.

MATERIALS AND METHODS

Ⅰ. Sampling and species identification

The black plaice *Pleuronectes obscurus* (Herzenstein, 1890), the dotted gizzard shad *Konosirus punctatus* (Temminck et Schlegel), and perch sculpin *Pseudoblennius percoides* (Günther) were collected using traps and nets nearshore around Korea Maritime University (KMU) on Jo Island, Busan, Korea, where this study was conducted from September 2005 to January 2006. The fish were transported to the Fishery Genetics and Breeding Laboratory, KMU, where they remained alive until they were analyzed.

The current classifications of the three fish species examined were based on Choi *et al.* (2002). After being anesthetized with 200 ppm lidocaine-HCl/1,000 ppm $NaHCO_3$ at 22℃, the body length of each specimen was measured to the nearest 0.1 cm using digital Vernier calipers (CD-20CP, Japan). External morphology was recorded with a digital camera using a photocopy stand.

Ⅱ. Chromosome analysis

Ten specimens of each species (five females and five males) were subjected to chromosome number and karyological analyses. The sex of each genotype was determined by gonadal inspection after killing the specimen. Fish were intraperitoneally injected with 0.02% colchicine (1 ml per 100 g body weight) and left in a well aerated aquarium for 3 h, and then killed with an overdose of 200 ppm lidocaine-HCl/1,000 ppm

$NaHCO_3$. The kidneys were removed and minced in hypotonic 0.075 M KCl solution until a good cell suspension was obtained, and then allowed to hypotonize for 20 min at 37℃ (Almeida-Toledo *et al.*, 1995). The kidney cells were fixed with methanol/acetic acid (3:1, v/v) and shaken gently. The suspension was replaced three times on ice for 15 min and then centrifuged.

Chromosome slides were made by means of a conventional air-drying technique. Detailed procedures for the preparation are provided by Im *et al.* (2001) and Park *et al.* (2003). Briefly, the final suspension was dropped on well cleaned dry slides and then placed on a 60℃ slide warmer. Chromosome preparations were stained with 10% Giemsa (Gurr's R66) for conventional analysis, and at least 20 countable metaphase spreads were obtained per fish.

Well spread chromosomes at metaphase were selected and photographed. Chromosome morphology was determined on the basis of arm ratio, as proposed by Levan *et al.* (1964), and chromosomes were grouped into four categories (metacentric, submetacentric, acrocentric, and telocentric) and arranged in decreasing order of size.

Ⅲ. Flowcytometry

Flow cytometric analysis was performed to estimate the average cellular DNA content of ten individuals from each species as described by Park and Lee (2005). After anesthetizing the fish with 200 ppm lidocaine-HCl/1,000 ppm $NaHCO_3$, a 0.5~1.0 mL sample of whole blood was collected from the caudal veins of each of ten individuals. Blood cells were fixed in 10 mL cold 70% ethanol and filtered through a 30 μm filter.

The cell solution was stored at 4℃.

One million cells were collected and stained using a High Resolution DNA Staining Kit (Partec GmbH, Münster, Germany) under dark conditions at room temperature for 15 min. Stained samples were analyzed on a Partec PA-Ⅱ flow cytometer (Partec GmbH) to determine relative DNA content. The red blood cells (2.8 pg DNA/nucleus) of Chinese muddy loach, *Misgurnus mizolepis* (Günther, 1888), were used as a standard reference (Park *et al.*, 1999).

To improve the standard DNA contents of *M. mizolepis*, we attempted to culture human white blood cells (WBC). We obtained 10 mL of whole blood from a human male. Isolation of WBC was achieved by the stirring method from 10 mL of whole blood diluted with 5 mL of serum-free culture medium in a clear culture tube. After centrifugation, the buffy coat on the surface of the erythrocytes was floated in plasma by gentle stirring with a pipette along the inside wall of the tube, and the lymphocyte-rich plasma was then collected in a culture tube (Abe *et al.*, 2001). Plasma was harvested after incubation for 3 to 4 days at 37℃. Cells from human leukocytes were fixed with 70% ethanol. The fixed cells were treated by the CyStain DNA 2-step Kit method (Partec GmbH).

Whole blood was sampled from *M. mizolepis* and then fixed with 70% ethanol. The fixed cells were treated by the CyStain DNA 2-step Kit method (Partec GmbH). The DNA content of *M. mizolepis* was confirmed as for WBC using standards. The DNA content of control human WBCs was 7.0 pg/nucleus, while *M. mizolepis* contained 2.8 pg/nucleus.

Ⅳ. Erythrocytes nucleus size

Erythrocytes from ten fish of each species were used to evaluate erythrocyte nuclear size. After anesthetizing the fish with 200 ppm lidocaine-HCl/1,000 ppm $NaHCO_3$, heparinized peripheral blood was collected from the caudal vein of each specimen. The blood from each sample was extracted with a 1 mL syringe and smeared on a glass slide, then fixed with 95% ethanol and stained with Giemsa or May-Grünbaldt-Giemsa solution (Park *et al.*, 1994, 2004; Kim *et al.*, 2001).

The major and minor axes (μm) of 100 erythrocyte nuclei were measured using an eyepiece micrometer under 1,000 fold magnification. The mean values of the major axis (a) and minor axis (b) characteristics of each subject were calculated. The surface areas (S, μm^2), $S = 1/4ab\pi$ (Sezaki and Kobayashi, 1978), and volumes (V, μm^3), $V = 4/3\pi(a/2)(b/2)^2$ (Lemoine and Smith, 1980), of the erythrocyte nuclei were also calculated.

RESULTS

Figure 1 shows the external morphology of fish used in this experiment. The mean total body length of black placice *Pleuronectes obscurus* (Herzenstein, 1890), the dotted gizzard shad *Konosirus punctatus*, and perch sculpin *Pseudoblennius percoides* was 25.7 ± 1.29 cm, 22.5 ± 1.69 cm, and 16.2 ± 1.23 cm, respectively.

The modal chromosome number of *P. obscurus* was 2N = 48 with a mode of chromosome distribution frequency of 83% (Table 1), consisting of 24 pairs of telocentrics. The fundamental number (FN) of *P. obscurus* was 48 (Table 1, Fig. 2*a*). The modal chromosome number of *K. punctatus* was 2N = 48 with the mode of chromosome distribution frequency of 86% (Table 1), consisting of 24 pairs of telocentrics. The FN of *K. punctatus* was 48 (Table 1, Fig. 2*b*). The modal chromosome number of *P. percoides* was 2N = 46 with the mode of chromosome distribution frequency of 86% (Table 1), consisting of two pairs of submetacentrics and 21 pairs of telocentrics. The FN of *P. percoides* was 46 (Table 1, Fig. 2c). There was no evidence of polymorphism including aneuploidy or sex-related heteromorphic chromosomes in any species examined (Fig. 2).

The mean values of the DNA contents of each species examined using mud loach as a standard reference are shown in Table 2. The mean value of DNA content of *P. obscurus* was 1.15 ± 0.103 pg/nucleus. The mean values of the DNA contents of *K. punctatus* and *P. percoides* were 1.56 ± 0.132 pg/nucleus and 1.11 ± 0.160 pg/nucleus, respectively.

Table 3 and Figure 3 show the mean values of erythrocyte nucleus

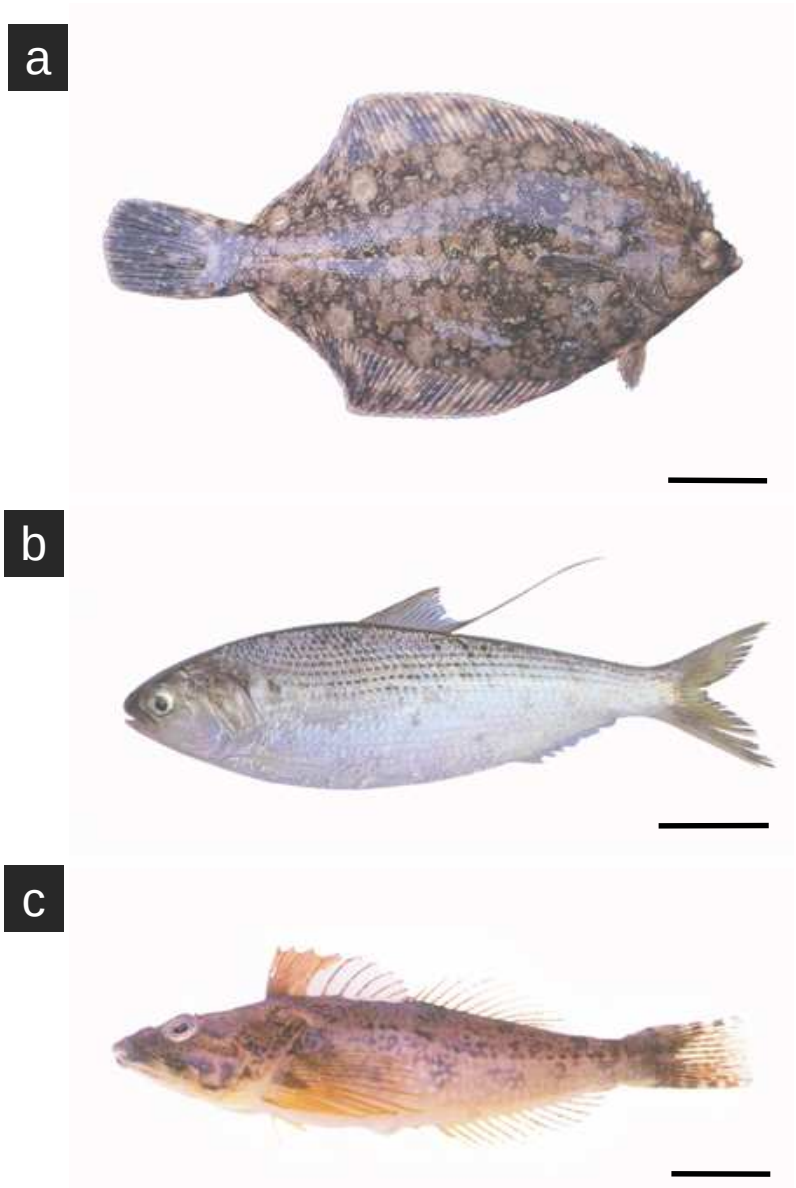

Fig. 1. External morphology of fish used in this experiemnt; (*a*) *Pleuronectes obscurus* (*bar* = 4 cm), (*b*) *Konosirus punctatus* (*bar* = 4 cm), (c) *Pseudoblennius percoides* (*bar* = 3 cm).

Table 1. Karyotypes and frequency distribution (%) of diploid chromosome numbers of material fish examined. M, Metacentrics; A, acrocentrics; T, telocentrics

Species	Frequency distribution (%)							2N	Karyotype			FN	Total cell count
	21	22	23	24	25	26	27		M	SM	T		
Pleuronectes obscurus	1	3	5	83	6	3	2	48	0	0	48	48	400
Konosirus punctatus	0	1	3	86	6	2	0	48	0	0	48	48	360
Pseudoblennius percoides	1	4	88	3	3	2	1	46	0	4	42	50	380

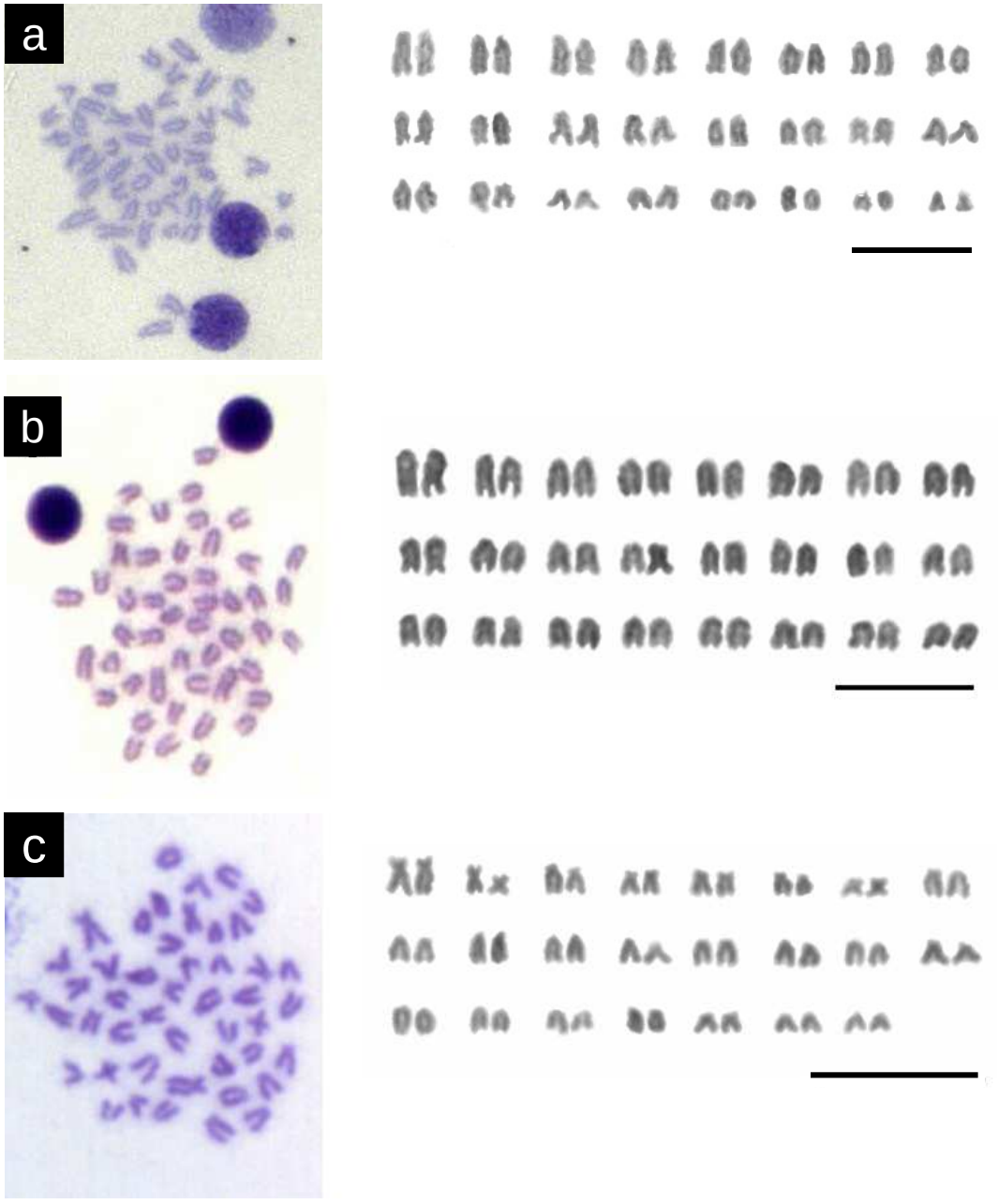

Fig. 2. Methaphases and Idiograms of (*a*) *Pleuronectes obscurus,* (*b*) *Konosirus punctatus,* (c) *Pseudoblennius percoides.* Bars are 10 ㎛.

Table 2. Nuclear DNA content of material fish in this experiment

Species	DNA content (pg/nucleus)*
Pleuronectes obscurus	1.15 ± 0.103
Konosirus punctatus	1.56 ± 0.132
Pseudoblennius percoides	1.11 ± 0.160
Standard	
*Misgurnus mizolepis***	2.80

* Values are means ± SD (n = 10).

** *Misgurnus mizolepis* (from Park *et al.*, 1999).

Table 3. Comparison of erythrocyte nucleus size measured from *Pleuronectes obscurus*, *Konosirus punctatus* and *Pseudoblennius percoides*[*]

	Pleuronectes obscurus	*Konosirus punctatus*	*Pseudoblennius percoides*
Major axis (㎛)	3.1±0.16	4.1±0.10	3.0±0.21
Minor axis (㎛)	2.5±0.16	2.5±0.07	2.4±0.16
Surface area (㎛^{2})	5.8±0.12	8.1±0.33	5.9±0.19
Volume (㎛^{3})	9.6±0.79	13.5±0.87	9.6±0.78

[*] Values are means ± SE (n = 100).

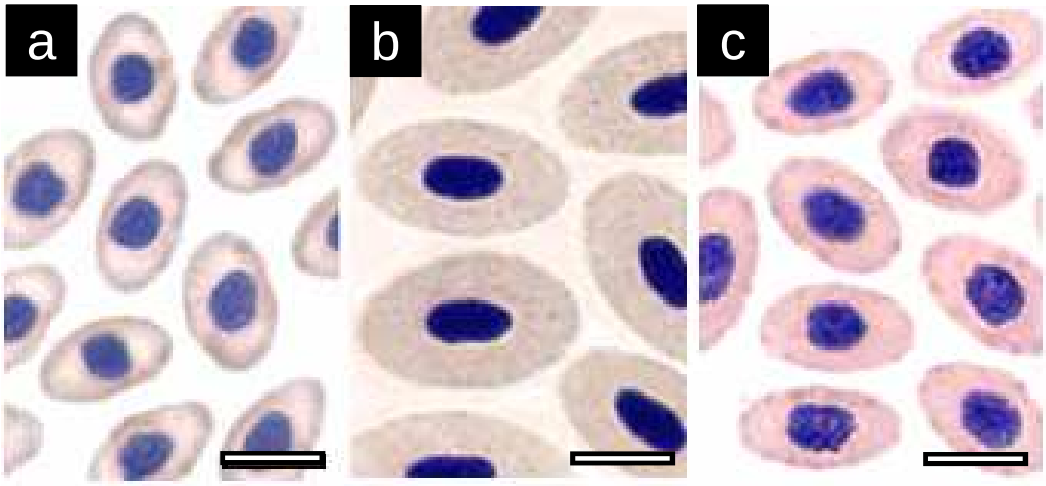

Fig. 3. Microphotographs of erythrocyte in (*a*) *Pleuronectes obscurus,* (*b*) *Konosirus punctatus,* (c) *Pseudoblennius percoides.* Bars are 5 ㎛.

sizes and their morphology. The major and minor axes of an erythrocyte nucleus of *P. obscurus* were 3.1 ± 0.16 μm and 2.5 ± 0.16 μm, and the surface area and volume were 5.8 ± 0.12 μm^2 and 2.5 ± 0.16 μm^3, respectively. The major and minor axes of *K. punctatus* were 4.1 ± 0.10 μm and 2.5 ± 0.07 μm, and the surface area and volume were 8.1 ± 0.33 μm^2 and 13.5 ± 0.87 μm^3, respectively. The major and minor axes of *P. percoides* were 3.0 ± 0.21 μm and 2.4 ± 0.16 μm, and the surface area and volume were 5.9 ± 0.19 μm^2 and 9.6 ± 0.78 μm^3, respectively. We found no significant differences in erythrocyte measurements between females and males.

As shown in Figure 3, the ratios of major/minor axis of the nucleus were 1.64 in *K. punctatus*, 1.24 in *P. obscurus*, and 1.25 in *P. percoides*, which in turn may be responsible for the more elliptic form of the nucleus in *K. punctatus*.

DISCUSSION

Chromosome numbers and variability in chromosome number distinguish certain major taxonomic groupings of fish (Gold, 1979; Park *et al*, 1995, 1999, 2000; Park and Lee, 1996). Chromosome numbers and karyotypes have unique numerical forms according to species, and provide useful data to identify species through cytogenetic analyses (Gold, 1979). To our knowledge, this is the first report on the karyotype of the black plaice *Pleuronectes obscurus* (Herzenstein, 1890), the dotted gizzard shad *Konosirus punctatus* (Temminck et Schlegel), and perch sculpin *Pseudoblennius percoides* (Günther).

In this study, the modal chromosome number of *P. obscurus* was 2N = 48 (83%), consisting of 24 pairs of telocentrics (FN = 48). That of *K. punctatus* was 2N = 48 (86%), consisting of 24 pairs of telocentrics (FN = 48), and that of *P. percoides* was 2N = 46 (88%), consisting of two pairs of submetacentrics and 21 pairs of telocentrics (FN = 50). There was no evidence of polymorphism including aneuploidy or sex-related heteromorphic chromosomes in any species examined.

Pseudoblennius percoides and *P. cottoides* (Richardson) are both in the same order (Scorpaeniformes) and family (Cottidae), and are similar species in terms of appearance, life history, and habitat use. In this study, the karyotype of *P. percoides* was 2N = 46, 4SM + 42T, FN = 50, although the karyotype of *P. cottoides* reported by Arai and Fujiki (1978) was 2N = 46, 4M + 8SM + 34ST, FN = 58. The FN of chromosomes is very important to compare species within a genus, and indicates the progenitor type from

which the acrocentric chromosome number increases (Ohno, 1974; Kim *et al.*, 2004). The FN increases with further differentiation of species (Arai, 1983). Therefore, in this comparison of the karyotype of *P. percoides* and the karyotype of *P. cottoides*, the chromosome configuration of *P. cottoides* was more variable than that of *P. percoides*, and the FN value of *P. cottoides* was higher than that of *P. percoides* therefore, *P. cottoides* is considered to have differentiated from *P. percoides*. However, our knowledge of cytogenetic traits remains insufficient, especially with regard to their comparative karyotypes (Park *et al.*, 1999). Accordingly, further detailed molecular studies are needed.

The DNA nuclear content, which is a species-specific value, may be correlated with the morphological basis for the definition of species, i.e., it may be related to the karyotype regarded as its total dimension or as values correlated to the latter, such as the area or total length of chromosomes (Chiarelli and Capanna, 1973). The direct measurement of the genome size by flow cytometry analyzes the unique genetic material of each specimen using a large amount of nuclear material within a short time, which is a great advantage of using this method (Lovett *et al.*, 1980; Thorgaard *et al.*, 1982; Wolters *et al.*, 1982; Park *et al.*, 1999; Park, 2004).

The results of flow cytometric analysis in our study of the DNA content per nucleus of the tested fish resulted in 1.15 pg/nucleus in *P. obscurus*, 1.56 pg/nucleus in *K. punctatus*, and 1.11 pg/nucleus in *P. percoides*. No difference in DNA content was found between the sexes within species.

The use of flow cytometryto analyze the DNA content of a given species has been considered to be the most effective method in terms of

non-perturbance to the study animal and success in identifying individuals (Kim *et al.*, 2004). Moreover, it is a convenient way to analyze species cytogenetically (Kim *et al.*, 2004). Evidence has indicated that DNA nuclear content is statistically constant in all cells exhibiting identical ploidy in various tissues from the same animals of the same species, whereas it is significantly divergent among different species (Chiarelli and Capanna, 1973; Park and Lee, 2005). In addition, DNA content analysis in species classification of similar species provides a basis to study phylogeny between species (Vendrely and Vendrely, 1948). According to this knowledge of the nuclear DNA amount and the karyotype, in a given individual, this correlation ensures the number and shape of the chromosome characteristics of the species (Lovett *et al.*, 1980; Park *et al.*, 1999, 2000, 2003).

We found that the mean sizes of erythrocyte nuclei were similar between *P. obscurus* and *P. percoides*, while that of *K. punctatus* was larger. The ratios of the major/minor axis of the nucleus were 1.64 in *K. punctatus*, 1.24 in *P. obscurus*, and 1.25 in *P. percoides*, which in turn may be responsible for the more elliptic form of the nucleus in *K. punctatus*.

The major and minor diameters of erythrocytes can be used to identify individuals among collected specimens without damaging the animals, even when the nuclear size cannot be determined by chromosome observations (Sezaki and Kobayashi, 1978; Park *et al.*, 1996, 1997, 2004; Park and Kim, 2000). In particular, the standard of judgment for ploidy and hybrid cytogenetic-specific analysis can be useful (Benfey *et al.*, 1984; Park *et al.*, 2004).

Large numbers of *P. obscurus* are captured in Korea in the winter

with other flatfish, and the commercial value of this catch is high (Park, 2005). *Konosirus punctatus* is frequently captured in the fall in coastal areas of Jo Island (Park, 2005), and its commercial value is also high. No studies have attempted to conduct cytogenetic research on *K. punctatus*. Therefore, the results of this cytogenetic study of *K. punctatus* may provide useful basic data for the aquaculture industry. A previous cytogenetic study of Jo Islandfish targeted the saddled weever *Parapercis sexfasciata* (Temmick et Schlegel), the marbled rockfish *Sebastiscus marmoratus* (Cuvier), and the marbled sole *Pleuronectes yokohamae* (Günther) (Park, 2005). This previous report and our current investigation can be used as a basis for continuous cytogenetics studies of coastal fish of Jo Island to accomplish species classification or maintenance, as well as for basic data construction to be used in the commercial production of new species.

KOREAN ABSTRACT (국문요약)

이학석사 학위논문

부산 조도연안 어류, 감성가자미, *Pleuronectes obscurus* (Herzenstein), 전어, *Konosirus punctatus* (Temminck et Schlegel) 및 돌팍망둑, *Pseudoblennius percoides* (Günther)의 세포유전학적 연구

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2006년 8월

한국의 부산 조도 연안에 서식하는 감성가자미, *Pleuronectes obscurus* (Herzenstein, 1890), 전어, *Konosirus punctatus* (Temminck et Schlegel) 및 돌팍망둑, *Pseudoblennius percoides* (Günther) 3종의 핵형, DNA 함량 및 적혈구 핵 크기에 관한 세포유전학적인 연구를 수행하였다. 감성가자미의 핵형은 2N = 48 으로 48T, FN = 48 이었다. 전어의 핵형은 2N = 48 으로, 48T, FN = 48 이었다. 돌팍망둑의 핵형은 2N = 46 으로, 4SM + 42T, FN = 50 이었다. 본 연구의 모든 종에서 성과 연관

된 이형의 염색체 쌍은 발견되지 않았다.

감성가자미, 전어 및 돌팍망둑의 DNA 함량 (감성가자미 = 1.15 ± 0.103 *p*g/nucl*eus*; 전어 = 1.56 ± 0.132 *p*g/nucl*eus*; 돌팍망둑 = 1.11 ± 0.160 *p*g/nucl*eus*) 은 이들의 염색체 FN 변이와 양성적 상관관계를 보였다.

감성가자미의 적혈구 핵의 장·단축은 각각 3.1 ± 0.16 ㎛, 2.5 ± 0.16 ㎛ 이었으며, 표면적과 부피는 각각 5.8 ± 0.12 $㎛^2$, 9.6 ± 0.79 $㎛^3$ 이었다. 전어의 적혈구 핵의 장·단축은 각각 4.1 ± 0.10 ㎛, 2.5 ± 0.07 ㎛ 이었고, 표면적과 부피는 각각 8.1 ± 0.33 $㎛^2$, 13.5 ± 0.87 $㎛^3$ 이었다. 돌팍망둑의 적혈구 핵의 장·단축은 각각 3.0 ± 0.21 ㎛, 2.4 ± 0.16 ㎛ 로 표면적과 부피는 각각 5.9 ± 0.19 $㎛^2$, 9.6 ± 0.78 $㎛^3$ 이었다. 적혈구 핵의 장축/단축 비에서 전어는 1.64, 감성가자미는 1.24, 돌팍망둑은 1.25를 나타내어, 전어가 적혈구 핵 크기에서 가장 장방형을 보였다.

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