Thesis for the Degree of Master of Science

Morphological and genetic variation of geographic populations of the gizzard shad, *Konosirus punctatus* (Clupeidae, Pisces) from Korean waters

by

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The Graduate School

Pukyong National University

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by

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한국산 전어(*Konosirus punctatus*)의 지역 집단 간 형태 및 유전 변이

명 세 훈

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요 약

전어는 경제성 어종으로 최근 어획량이 증가하고 있어 자원붕괴를 막기 위해서는 전어 자원량을 꾸준히 모니터링 할 필요가 있다. 본 연구는 한국산 전어의 개체군 구조를 파악하기 위해 우리나라 8개 지역에서 채집된 182개체의 전어에 대해 형태 및 분자 분석을 실시하였다.

전어 182개체에 대한 mtDNA Control region 영역 896bp에 의한 분자분석결과, 높은 유전자형 다양도(0.9662-1.0000)와 낮은 염기다양도(0.0061-0.0434)를 나타냈다. Neighbor Joining tree 작성 결과 A집단과 B집단으로 나누어 졌다. A집단은 우리나라 전 해역에 걸쳐 분포하며, 동해로 갈수록 개체수가 현저히 감소하였다. B집단은 주로 동해와 남해동부에 분포하며, 남해서부와 서해쪽은 분포하지 않았다. 두 집단 간 유전거리는 5.5-6.6%이고, pairwise FST 값은 0.856($P<0.0001$)으로 매우 높은 분화정도를 나타냈다.

전어 173개체를 대상으로 5개의 계수형질 및 17개의 계측형질을 이용한 형태분석결과, 계수형질에서는 집단간 등지느러미수, 뒷지느러미수, 척추골수, 인판수에서 유의한 차이를 나타냈다. 주성분분석결과, 8개의 지역이 겹쳐져 나타났으며, 정준판별분석 결과, 제 1 판별함수 (head length)에서는 [주문진, 후포, 부산, 고성]과 [부산, 여수,

강진, 홍도, 군산]으로 크게 나누어졌으며, 제 2 판별함수 (standard length)로는 여수가 나머지 지역과 구분되는 경향을 보였다. 또한, 유전결과에 의한 리니지 별로 계수 및 계측형질을 비교한 결과, 뒷지느러미와 인판수에서 유의한 차이를 보였고, 판별분석에서 두 리니지는 계측형질에서 90.2% 구분되었다.

형태 및 분자분석결과를 종합하면, 우리나라 전어는 서해와 남해서부에 분포하는 집단과 동해와 남해동부에 분포하는 2개의 집단으로 구분해서 관리할 필요가 있다.

I. Introduction

The gizzard shad *Konosirus punctatus* in the family Clupeidae (Clupeiformes) is distributed on all coasts of Korea, China, Japan, and Taiwan. Only one species in the genus *Konosirus* is recognized in Korea, whereas two species are known in Japan (Kim et al., 2007a). The gizzard shad is one of the most commercially important fish species, and inhabits littoral and brackish areas in Korea (Choi et al., 2002). It spawns near river mouths from April to August, and the larvae become adults within two years and live for five years (Kim et al., 2007a; Lee et al, 2010). The gizzard shad catches has decreased since 2007, i.e., 9,873 tonnes in 2007 but 5,767 tonnes in 2011 (NFRDI, 2013). To prevent the collapse of the population, a monitoring and restoration program is required. For this reason, many ecological studies of gizzard shad have been conducted, e.g., examining the eggs and larval development (Kim et al., 2007a), the food items of postlarvae (Park et al., 1996), spawning season and grounds (Matsushita and Nose, 1974), reproduction (Takita, 1978a; Takita, 1978b), and age and growth (Oh et al., 2000). However, the population structure of the gizzard shad has not been investigated. Studies of the degrees of genetic exchange among fish species or populations inhabiting each coastal area allow the best management of the fisheries resources in each coastal area (Kim, 2009).

The aim of this population genetic study was to collect basic data for the conservation and management of fish stocks. A population genetic analysis is an appropriate way to evaluate genetic diversity (Crandall et al., 1999), and the most effective method involves the comparison of mitochondrial DNA (mtDNA)

sequences (Buonnacorsi et al., 2001). The mitochondrial control region (CR) is primarily used for the analysis of population genetic variation, because the CR is composed of hypervariable sites and the mutation rate is very high (Aquadro and Greenberg, 1983; Nesb et al., 1998).

Because gizzard shad lives around or near coastal or brackish waters, we assumed that the populations of the gizzard shad might differ according to their habitats. For instance, the genetic structures of the Korean and Japanese populations of *Salanx ariakensis*, which prefers to live in brackish areas such as river mouths, were shown to differ using mtDNA cytochrome b sequences (Kim et al., 2006a) and amplified fragment-length polymorphism markers (Kim et al., 2007b). *Lateolabrax japonicus*, which prefers to live near coastal areas, also shows genetic isolation with distance (Liu et al., 2006a). Therefore, our hypothesis in this study was that the Korean gizzard shad has at least two or three distinct populations, according to area (or habitat).

Meristic and morphometric characters have most often been used for population discrimination, although recently molecular characters have also been utilized (Ihssen et al., 1981; Melvin et al., 1992; Hurlbut and Clay, 1998; Murta, 2000; Turan, 2004; Kim et al., 2008). The morphological studies were conducted using the principal component analysis (PCA) and canonical discriminant analysis (CDA) (Kai and Nakabo, 2002; Kim et al., 2006a).

The present study aimed to clarify the population structure of *K. punctatus* from Korea, based on partial mtDNA CR gene sequences and morphological traits.

II. Materials and methods

1. Sampling

Samples of the gizzard shad were collected from eight localities along the Korean coasts between 2008 and 2013 (Table 1, Fig. 1): Jumunjin (Ju, n = 26) and Hupo (Hu, n = 16) in the East Sea; Busan (Bu, n = 24), Goseong (Go, n = 10), Yeosu (Yeo, n = 24) and Gangjin (Gang, n = 36) in the Korean Strait; Hongdo (Hong, n = 15) and Gunsan (Gun, n = 31) in the Yellow Sea. Samples of muscle tissue were preserved in 95% ethanol and stored frozen at –20 °C until DNA extraction; specimens of gizzard shad were preserved in 5% formalin. The specimens used in this study have been deposited at Pukyong National University.

Table 1. Collection localities and sample sizes of *Konosirus punctatus*

Locality	n	Time	Locality code
Jumunjin	26	May 2013	Ju
Hupo	16	January 2011	Hu
Busan	24	August, September 2009	Bu
Goseong	10	September, October 2008	Go
Yeosu	24	August 2013	Yeo
Gangjin	36	May, July 2009	Gang
Hongdo	15	April 2013	Hong
Gunsan	31	April 2013	Gun

Sample size (n)

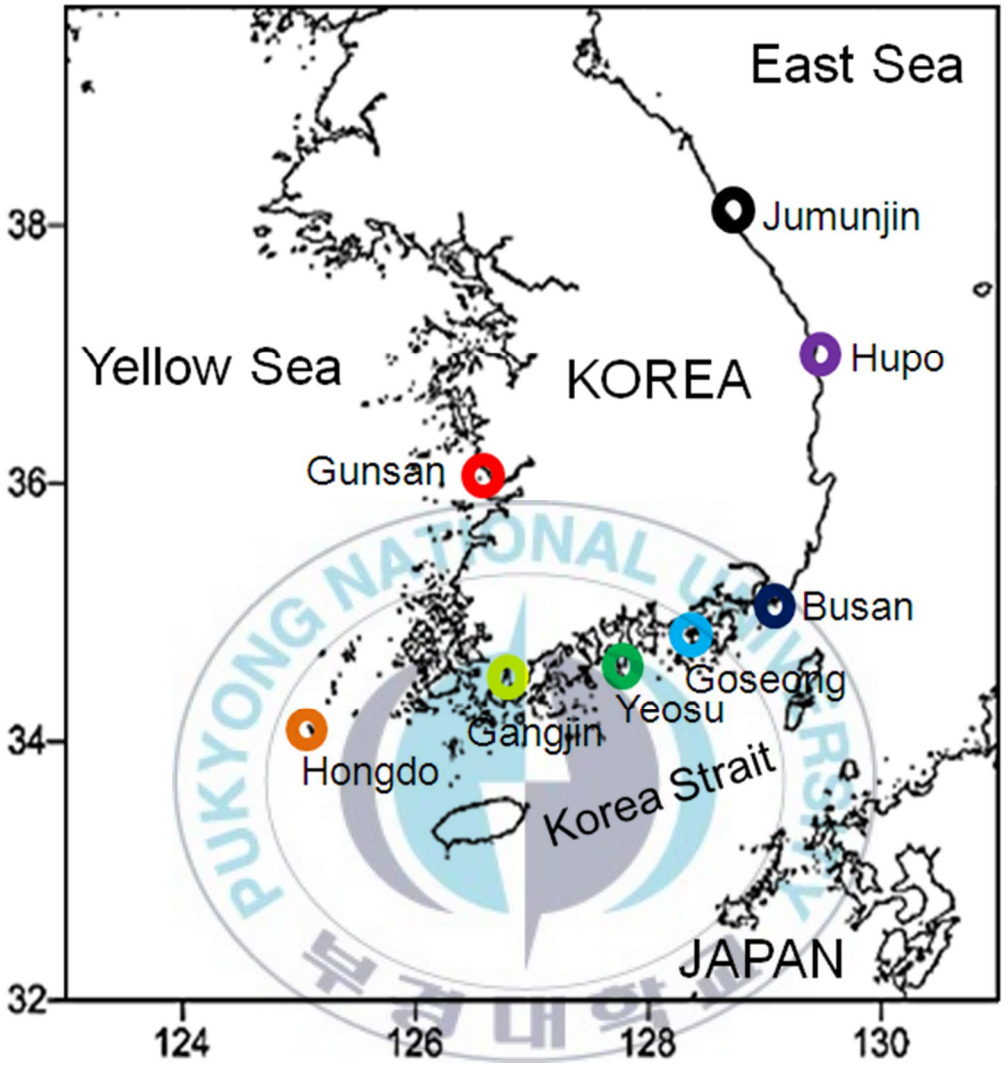

Fig. 1. Map showing the sampling area of *Konosirus punctatus* from Korea.

2. DNA extraction, PCR, and sequencing

Genomic DNA was extracted from the muscle tissues of fish using 10% Chelex 100 Resin (Bio-Rad, Hercules, CA). An 896-bp fragment of the mtDNA CR was amplified with *Konosirus*-specific primers: Kono-F (5′-ATCCTCCCTGAGGCCC AGAAAAG-3′) and Kono-R (5′-GGGGGTTTGTCGCGCGAAAACC-3′). This primer set was newly designed worked well in *K. punctatus*. PCR was performed in a 30 μl reaction containing 3 μl of 10 × PCR buffer, 1μl of each primer, 2.4 μl of dNTPs, 1 μl of genomic DNA, 0.1 μl of Ex-Taq DNA polymerase, and 22.5 μl of sterile distilled H_2O. The PCR proceeded under the following conditions: initial denaturation at 94 ℃ for 4 min, 35 cycles of denaturation at 94 ℃ for 30 s, annealing at 59 ℃ for 30 s, and extension at 72 ℃ for 5 min. The PCR products were purified with ExoSAP-IT (United States Biochemical Corporation, USA). The PCR products were sequenced with the ABI PRISM BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems Inc., USA) on an ABI 3730xl DNA Analyzer (Applied Biosystems Inc.).

3. Molecular analysis

The mtDNA CR sequences were checked and aligned with ClustalW (Thompson et al., 1994) in BioEdit ver. 7 (Hall, 1999). A neighbor-joining (NJ) tree was constructed using the Kimura two-parameter model (Kimura, 1980), and confidence was assessed with 1000 bootstrap replications. The NJ tree shows the

genetic relationships among 182 gizzard shad and one outgroup, *Clupanodon thrissa* (JX075099), downloaded from the NCBI GenBank database.

The haplotype, polymorphic sites, transitions, and transversions were estimated for each specimen using Arlequin ver. 3.5.1.2 (Excoffier et al., 2005). Nucleotide diversity (π; Nei and Li, 1979) and haplotype diversity (h; Nei, 1987) were estimated. To estimate the levels of genetic divergence among the populations of the gizzard shad, the divergence measure F_{ST} was calculated using analysis of molecular variance (AMOVA; Excoffier at al., 1992). The significance of F_{ST} was determined with 1000 nonparametric data permutations, using Arlequin ver. 3.5.1.2 (Rice, 1989). Evidence of population expansion was tested using Fu's *F*s (Fu, 1997) and neutrality tests for equilibria in mutational drift were tested with Tajima's *D* (Tajima, 1989) in Arlequin ver. 3.5.1.2. The historical demography of the gizzard shad populations was estimated from the mismatch distribution (Rogers and Harpending, 1992). Past demographic parameters were estimated, including τ (time since expansion, expressed in units of mutational time) (Li, 1997) and θ_0 and θ_1 (θ before and after population growth) (Rogers and Harpending, 1992). The values for τ were transformed to estimate the real time since expansion with the equation $\tau = 2ut$ (Rogers and Harpending, 1992), where u is the mutation rate for the whole sequence under study and t is the time since expansion. A molecular clock for the CR has not been determined with precision for marine fishes: for instance, East African cichlids, 2.2%–4.5% divergence per million years (Sato et al., 2003), Australian rainbow fish, 3% divergence per million years (Zhu et al., 1994), snooks, 3.6% divergence per million years (Donaldson and Wilson, 1999), anchovies, 5%–20% divergence per million years (Liu et al.,

2006b), and sand lance, 8% divergence per million years (Han et al., 2012). The gizzard shad has a short generation time and small body size, like the sand lance. Therefore, in the present study, a sequence divergence rate of 4%–8% divergence per million years was used to estimate its evolutionary history.

4. Morphological analysis

The morphological analysis was based on 173 specimens of *K. punctatus*. A total of 17 morphometric and 5 meristic characteristics were used: morphometric - standard length, head length, snout length, orbit length, interorbital width, postorbital length, upper jaw length, body depth, body width, caudal peduncle depth, pectoral-fin length, pelvic-fin length, dorsal-fin base length, preanal length, predorsal length, prepelvic length, anal-fin base length; meristic - dorsal-fin rays, anal-fin rays, pelvic-fin rays, vertebrae, and scutes. 17 morphometric were measurements followed Nakabo (2002) by vernier calipers after fixation in 99% ethanol. Five meristic were counted by radiograph (SOFTEX HA-100, Japan). All measurements were taken to the nearest millimeter, always by the same person. Five meristic characters were statistically analyzed by the Kruskal-Wallis nonparametic test (Zar, 1999). A principal components analysis (PCA) and canonical discriminant analysis (CDA) were conducted to identify shape-related differences among eight localities populations of *Konosirus punctatus*. All the statistical analyses were performed using SPSS version 12.01. In addition, the Kruskal-Wallis test and canonical discriminant analysis (CDA) depending on the result of molecular analysis was performed.

III. Results

1. Genetic variation

The analysis of 896 bp of the mtDNA CR in 182 gizzard shad individuals from eight localities identified 124 haplotypes. A total of 134 polymorphic sites were detected, with 88 transitions, 20 transversions, and 48 insertions/deletions. For the overall sample, the haplotype diversity (h) and nucleotide diversity (π) were 0.9662–1.0000 and 0.0061–0.0434, respectively (Table 2). The Goseong locality showed the maximum haplotype diversity (1.0000). In contrast, the haplotype diversity was lowest at the Jumunjin locality (0.9662). The Gangjin, Hongdo, and Gunsan localities showed the lowest nucleotide diversities (0.0065, 0.0061, and 0.0065, respectively).

Table 2. Genetic variability based on the mitochondrial DNA control region of *Konosirus punctatus*

Locality	n	N	*h*	π
Ju	26	18	0.9662 ± 0.0208	0.0434 ± 0.0217
Hu	16	13	0.9750 ± 0.0295	0.0353 ± 0.0189
Bu	24	21	0.9891 ± 0.0152	0.0380 ± 0.0192
Go	10	10	1.0000 ± 0.0447	0.0390 ± 0.0210
Yeo	24	23	0.9964 ± 0.0133	0.0122 ± 0.0064
Gang	36	32	0.9921 ± 0.0092	0.0065 ± 0.0035
Hong	15	13	0.9810 ± 0.0308	0.0061 ± 0.0035
Gun	31	27	0.9892 ± 0.0119	0.0065 ± 0.0036

Sample size (n), number of haplotypes (N), haplotype diversity (*h*), nucleotide diversity (π)

Ju, Jumunjin; Hu, Hupo; Bu, Busan; Go, Goseong; Yeo, Yeosu; Gang, Gangjin; Hong, Hongdo; Gun, Gunsan

2. Population structure

An NJ phylogenetic tree constructed from the complete dataset for the 124 haplotypes of the gizzard shad identified two distinct lineages (designated A and B; Fig. 2). The genetic distance between lineages A and B was 5.5%–6.6%, and the within-lineage differences for A and B were 0.0–1.1% and 0.0–2.0%, respectively. Lineage B was divided into two sublineages: sublineage b1 included fish from Jumunjin, Hupo, and Goseong; and sublineage b2 included fish from Jumunjin, Hupo, Busan, and Yeosu. The genetic distances between sublineage b1 and b2 were 1.0%–2.0% and the within-sublineage distances were 0.0%–0.5% (Fig. 2). The Gangjin, Hongdo, and Gunsan localities contained only lineage A, but Jumunjin, Hupo, Busan, Goseong, and Yeosu contained both lineages A and B. Lineage A dominated the western localities (Yellow Sea and Western Korea Strait), but its frequency declined steadily from Yeosu (95.8%) to Jumunjin (19%) (Fig. 3).

AMOVA showed strong structuring (F_{ST} = 0.856; P < 0.0001) between lineages A and B. The greatest differentiation index was observed between the individuals from Jumunjin and those from Gangjin (F_{ST} = 0.663; P < 0.0001), and the smallest was observed between the individuals of Yeosu and Hongdo (F_{ST} = 0.025; P = 0.045) (Table 3).

Table 3. Pairwise F_{ST} values (below the diagonal) and Pairwise F_{ST} *P* values (above the diagonal) for the mitochondrial DNA control region among eight populations of *Konosirus punctatus*

	Ju	Hu	Bu	Go	Yeo	Gang	Hong	Gun
Ju		0.928	0.000	0.027	0.000	0.000	0.000	0.000
Hu	-0.041		0.063	0.072	0.000	0.000	0.000	0.000
Bu	0.167**	0.099		0.748	0.000	0.000	0.009	0.000
Go	0.163*	0.086	-0.043		0.009	0.000	0.000	0.000
Yeo	0.576**	0.542**	0.230**	0.234*		0.396	0.045	0.153
Gang	0.663**	0.651**	0.335**	0.378**	0.001		0.045	0.081
Hong	0.589**	0.559**	0.258*	0.279**	0.025*	0.047*		0.595
Gun	0.649**	0.634**	0.319**	0.363**	0.010	0.019	-0.007	

*P<0.05; ** P<0.001

Ju, Jumunjin; Hu, Hupo; Bu, Busan; Go, Goseong; Yeo, Yeosu; Gang, Gangjin; Hong, Hongdo; Gun, Gunsan

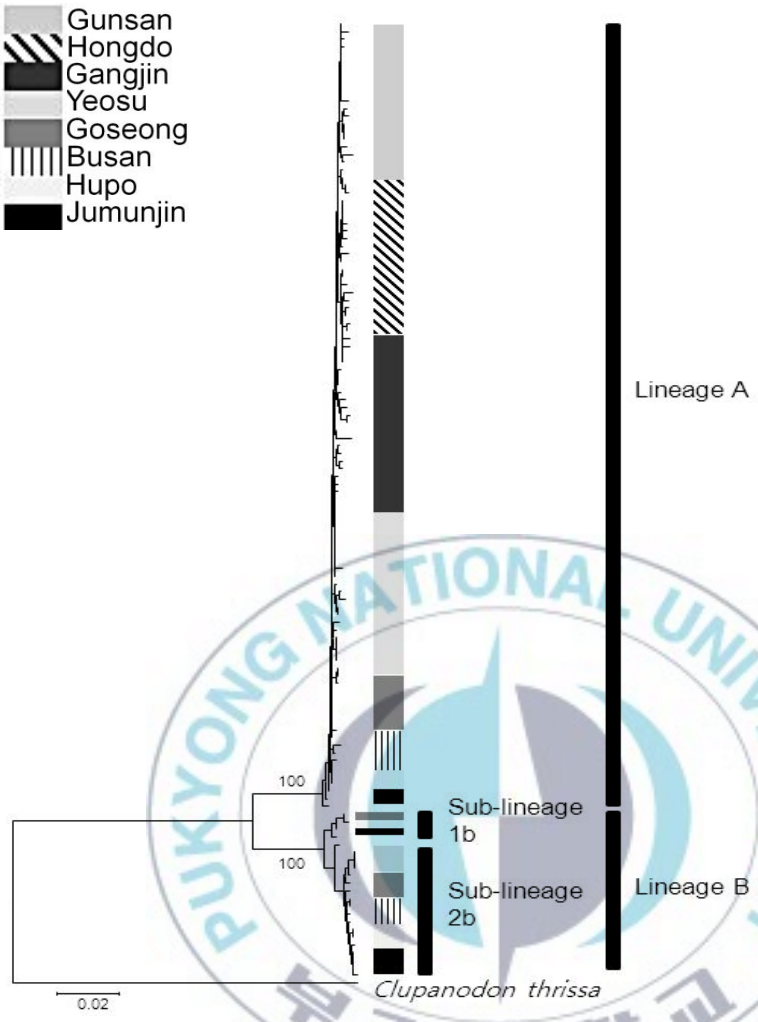

Fig. 2. Neighbor-joining (NJ) tree showing the relationships between 124 haplotypes of *Konosirus punctatus* and *Clupanodon thrissa* (outgroup). Numbers at branches indicate bootstrap probabilities in 1000 bootstrap replications. Bar indicates 0.02 of Kimura's (1980) genetic distance. Shaded bars indicate proportional representation of individuals from each station group, as identified by the embedded key to the figure.

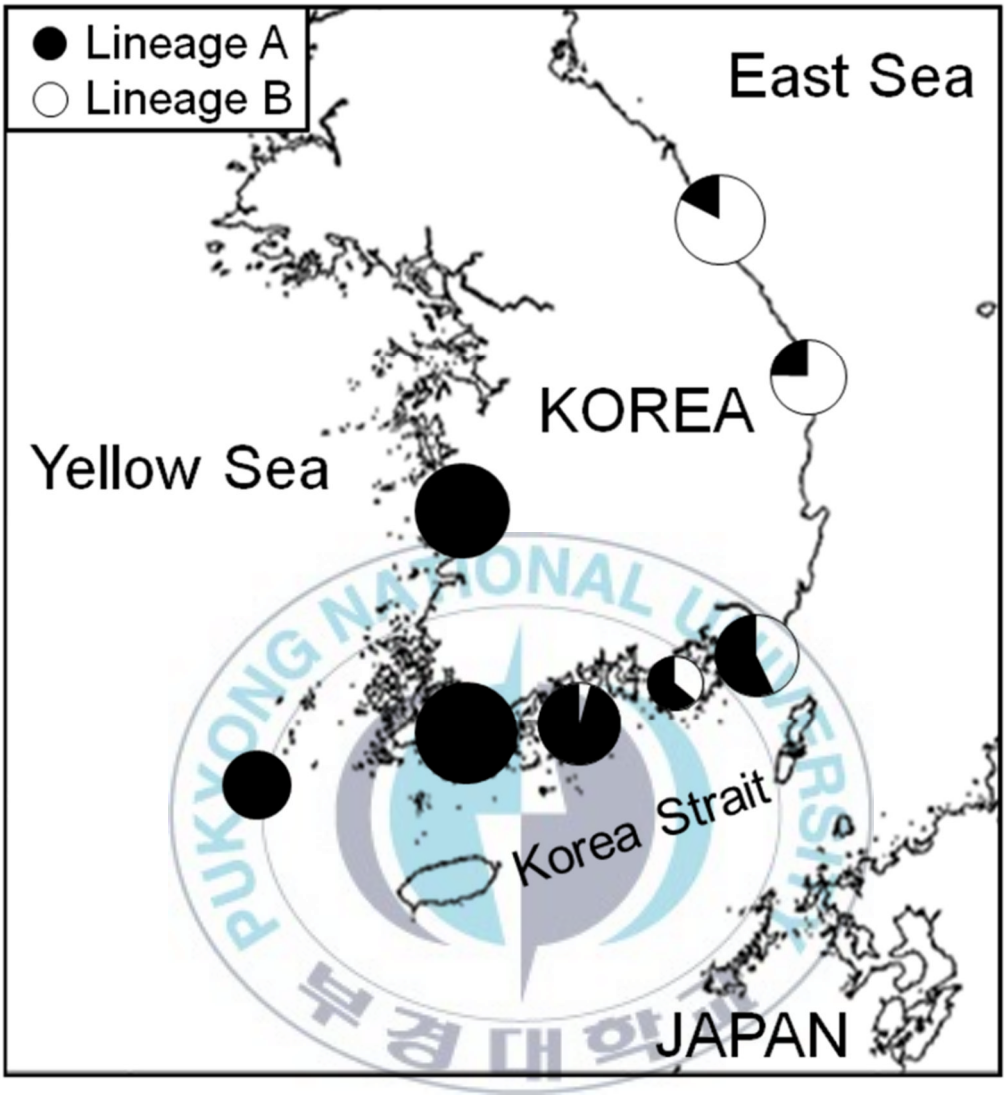

Fig. 3. Haplotype frequencies for *Konosirus punctatus* populations. The area of circle is proportional to sample size.

3. Demographic history

The mismatch distribution of lineage A was unimodal in shape, and closely fitted the expected distribution under the sudden expansion model (Fig. 4). Fu's *F*s and Tajima's *D* of lineage A agreed well with the mismatch analysis. Fu's *F*s was negative (–24.886) and highly significant ($P = 0.00$). Tajima's *D* was also negative (–1.535) and statistically significant ($P = 0.025$) (Table 4). In contrast, the mismatch distribution of lineage B was bimodal in shape. Fu's *F*s (*F*s = 2.39, $P < 0.821$) and Tajima's *D* ($D = 1.882$, $P < 0.981$) were not statistically significant. The observed values for the age expansion parameter (τ) were 5.736 and 2.514 of the mutational time for lineages A and B, respectively. Based on the method of Rogers and Harpending (1992), the expansion time was calculated to be about 89,000 years ago for lineage A. However, the expansion time for lineage B was not calculated because lineage B had two marked modes at five and 49 substitutions (Fig. 4).

Table 4. Summary of molecular diversity for two lineages of *Konosirus punctatus*

Lineage	n	N	*h*	π
A	134	103	0.992±0.003	0.007±0.004
B	48	21	0.943±0.016	0.023±0.011

	Tajima's *D*		Fu's *Fs*		Mismatch distribution		
Lineage	*D*	*P*	*Fs*	*P*	τ	θ_0	θ_1
A	-1.535	0.025	-24.886	0.000	5.736	0.972	109.531
B	1.882	0.981	2.39	0.821	2.514	6.692	18.756

Sample size (n), number of haplotypes (N), haplotype diversity (*h*), nucleotide diversity (π), Tajima's *D* and Fu's *Fs*, corresponding *P* value, and mismatch distribution parameter estimates for each lineage were indicated

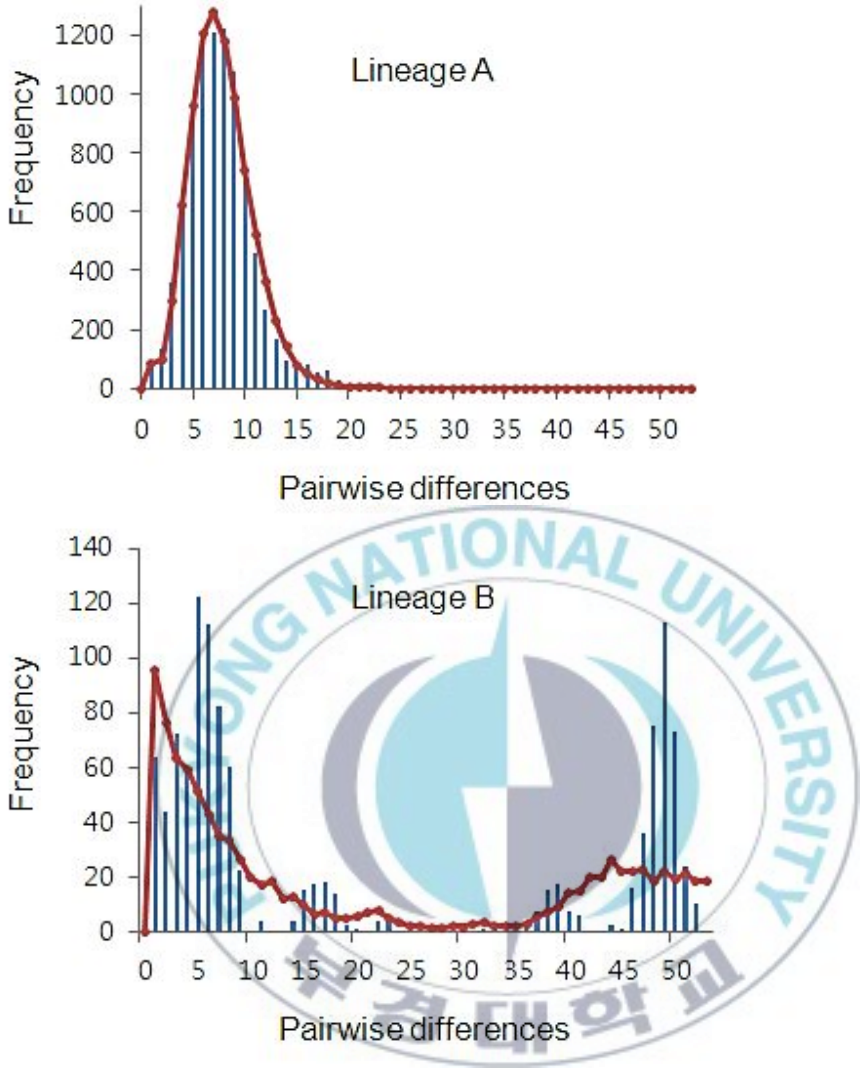

Fig. 4. The observed pairwise differences (bars), and the expected mismatch distributions under the sudden expansion model (solid line) for two lineages of *Konosirus punctatus*.

4. Morphological analysis

Meristic and morphometric characters of the eight localities populations of gizzard shad are shown in Table 5. Frequency distributions in meristic characters are shown in Table 6. Although each meristic characters overlapped in the frequency distributions, the Kruskal-Wallis test showed significant differences in the number of dorsal fin rays, anal fin rays, vertebrae, and scutes ($P<0.05$) (Table 7). The mean number of anal fin ray was most differences between in the Jumunjin (22.4) and Gangjin (23.6) (Fig. 5).

Principal components analysis (PCA) based on 17 morphometric characters indicates an eigenvalue of PC1 as 15.754, accounting for 92.67% of the total variation. The second and third components explained 1.81% and 1.18% respectively (Table 8). The Standard length (0.9919) and Body depth (0.2451) being largest in PC1 and PC2, respectively (Table 8). Plotting PC1 and PC2 showed that eight localities populations overlapped each other (Fig. 6).

In canonical discriminant analysis (CDA), seven discriminant functions were produced and the first CAN1 accounted for 58.9% and the CAN2 accounted for 20.1% of the between group variability among populations (Table 9). The head length (1.273) and body width (1.749) being largest in CAN1 and CAN2, respectively (Table 9). Only 33 specimens were misclassified from among the 173 specimens (19.1%). The lowest percentage of misclassification was obtained for the Yeosu (0%) and highest for the Gunsan (30%) (Table 10). This is divided into two groups according to CAN1 (Fig.7). One is polpulations of Jumunjin, Hupo, Busan, and Goseong, and the other includes Busan, Yeosu, Gangjin, Hongdo, and Gunsan. Busan population has both group and show same trend with genetic

analysis (Fig. 8). Yeosu was separated in other populations according to CAN2 (Fig. 7). Figure 9 is a scatter diagram of the body width of gizzard shad showing the difference between Yeosu population and other 7 populations. The Yeosu population wider body differentiates it from others. To compare the body width between Yeosu and others, 67 specimens (Hupo, 1; Busan, 5; Goseong, 1; Yeosu, 23; Gangjin, 12; Hongdo, 13; Gunsan, 12) are selected. Those 67 specimens reach a standard length of between 130mm and 160mm, the standard length of Yeosu population. As a result, most of the Yeosu population (20 specimens) is distinguished from other populations by wider body, except for two specimens of Gangjin (1) and Hupo (1) (Fig. 9).

Molecular analysis of gizzard shad in Korea were formed two lineages. Meristic and morphometric characters of the two lineages are shown in Table 11. The Kruskal-Wallis test showed significant differences in the number of anal fin rays and scutes ($P<0.05$). The mean number of anal fin rays and scutes in the lineage B (23.5 and 34.3, respectively) were higher than lineage A (22.8 and 33.6, respectively). Result of canonical discriminant analysis (CDA), one canonical discriminant function was calculated and the eigenvalue was 1.235. The postorbital length (0.856) and head length (0.845) being largest in CAN1. Lineage B has a bigger postorbital length (about 17% SL) and head length (about 28% SL) than lineage A (about 16% SL and about 26% SL, respectively) (Fig. 10).

Only 17 specimens were misclassified from among the 173 specimens (9.8%). The highest percentage of misclassification was obtained for the lineage A (12%) and lowest for the lineage B (2%) (Table 12).

Table 5. meristic and morphometric characters of eight localities of *Konosirus punctatus*

	Jumunjin (Ju)	Hupo (Hu)	Busan (Bu)	Goseong (Go)
Number of specimens	25	14	23	9
Standard length (mm)	169.6-226.7 (188.8)	108.5-221.4 (187.3)	101.6-180.1 (152.3)	154.7-196.4 (173.4)
Counts				
Dorsal-fin rays	17-19 (18.1)	17-20 (18.6)	17-19 (18.2)	17-19 (18.0)
Anal-fin rays	19-25 (22.4)	22-25 (23.1)	22-26 (23.4)	22-24 (22.7)
Pelvic-fin rays	8 (8.0)	8 (8.0)	8 (8.0)	8 (8.0)
Vertebrae	47-49 (48.1)	48-50 (48.8)	47-50 (48.2)	47-49 (48.1)
Scutes	32-35 (33.6)	33-34 (33.8)	29-35 (33.6)	32-35 (33.7)
In % of SL				
Head length	26.2-29.8 (28.4)	26.5-30.6 (28.4)	26.7-31.0 (28.3)	26.3-28.5 (27.4)
Snout length	5.8-7.1 (6.5)	5.1-7.3 (6.6)	5.6-7.2 (6.3)	5.7-6.7 (6.2)
Orbit length	4.1-6.1 (5.2)	4.7-6.5 (5.3)	5.0-6.9 (5.7)	5.1-5.6 (5.3)
Interorbital width	6.0-7.3 (6.7)	5.9-7.1 (6.5)	5.9-8.2 (6.9)	6.2-7.1 (6.7)
Postorbital length	16.1-18.5 (17.4)	16.2-18.5 (17.2)	15.7-19.5 (16.9)	15.8-17.1 (16.6)
Upper jaw length	8.2-10.0 (9.1)	7.8-10.2 (9.0)	8.1-10.2 (9.0)	8.1-9.5 (8.7)
Body depth	27.0-32.1 (29.6)	27.6-31.7 (29.3)	20.8-34.8 (31.2)	27.9-31.0 (29.7)
Body width	10.6-14.2 (12.7)	6.8-15.4 (12.6)	8.6-12.5 (11.3)	11.8-13.9 (12.9)
Caudal peduncle depth	8.2-9.8 (9.0)	7.6-9.0 (8.6)	8.4-10.8 (9.5)	8.3-9.3 (8.9)
Pectoral-fin length	16.1-20.4 (18.6)	17.5-20.5 (18.7)	17.6-21.4 (19.3)	17.3-19.5 (18.3)
Pelvic-fin length	9.6-11.8 (10.9)	9.6-11.8 (10.8)	10.2-12.2 (11.2)	9.4-10.7 (10.2)
Dorsal-fin base length	15.8-18.2 (17.1)	16.4-18.9 (17.8)	17.1-19.6 (18.0)	15.9-18.5 (17.1)
Preanal length	69.9-75.3 (72.1)	69.6-74.7 (72.0)	69.7-75.1 (71.7)	69.6-73.6 (71.5)
Predorsal length	44.2-48.8 (46.1)	43.8-48.6 (46.3)	44.5-47.7 (46.0)	42.6-47.1 (45.0)
Prepelvic length	47.0-52.4 (50.0)	47.3-51.7 (49.3)	46.6-50.8 (49.0)	47.9-51.3 (49.0)
Anal-fin base length	17.1-19.9 (18.3)	16.9-20.6 (19.0)	17.6-21.9 (20.2)	17.9-20.9 (19.5)

	Yeosu (Yeo)	Gangjin (Gang)	Hongdo (Ho
Number of specimens	23	35	15
Standard length (mm)	137.4-156.9 (146.1)	105.7-195.8 (141.4)	142.1-166.8 (
Counts			
Dorsal-fin rays	17-19 (17.9)	17-19 (17.8)	17-19 (18.
Anal-fin rays	20-24 (22.9)	20-27 (23.6)	23-27 (24.
Pelvic-fin rays	8 (8.0)	8 (8.0)	8 (8.0)
Vertebrae	48-49 (48.3)	48-50 (48.6)	48-49 (48.
Scutes	33-36 (34.5)	33-37 (34.6)	33-35 (34.
In % of SL			
Head length	25.0-27.4 (26.1)	25.2-30.0 (27.2)	25.6-28.1 (2
Snout length	5.6-6.6 (6.0)	5.5-7.0 (6.4)	5.8-6.7 (6.
Orbit length	5.2-5.9 (5.6)	4.6-6.8 (5.6)	5.0-5.7 (5.
Interorbital width	5.8-6.9 (6.5)	5.9-6.7 (6.4)	5.6-6.4 (6.
Postorbital length	13.9-16.3 (15.1)	14.6-17.2 (15.7)	14.9-16.6 (1
Upper jaw length	8.0-9.5 (8.7)	6.8-10.0 (8.9)	8.2-9.3 (8.
Body depth	13.2-35.8 (31.4)	26.9-33.6 (29.8)	28.3-32.1 (3
Body width	11.5-13.8 (12.7)	7.9-13.2 (10.1)	9.4-11.9 (1
Caudal peduncle depth	8.6-10.3 (9.5)	8.4-10.1 (9.2)	8.1-9.6 (8.
Pectoral-fin length	17.9-21.2 (19.5)	17.5-21.5 (19.9)	18.0-20.6 (1
Pelvic-fin length	9.3-11.6 (10.4)	9.0-12.6 (11.0)	10.0-11.6 (1
Dorsal-fin base length	16.8-19.6 (18.0)	16.7-18.9 (17.7)	16.5-19.6 (1
Preanal length	69.0-73.2 (71.0)	69.4-73.7 (71.1)	67.9-71.6 (6
Predorsal length	44.1-47.7 (46.1)	43.6-47.5 (46.0)	43.9-47.4 (4
Prepelvic length	33.0-49.9 (47.2)	46.1-51.2 (48.6)	46.9-49.8 (4
Anal-fin base length	17.1-20.9 (19.7)	17.8-21.9 (20.3)	18.7-22.6 (2

Table 6. Frequency distributions of meristic characters of eight localities o

Locality	n	Dorsal-fin rays				Anal-fin rays						
		17	18	19	20	19	20	21	22	23	24	25
Ju	25	4	14	7	-	1	-	4	10	6	2	2
Hu	14	1	8	2	3	-	-	-	4	6	3	1
Bu	23	3	12	8	-	-	-	-	7	5	7	2
Go	9	3	3	3	-	-	-	-	4	4	1	-
Yeo	23	5	16	2	-	-	1	1	5	8	8	-
Gang	35	11	19	5	-	-	1	2	3	9	12	6
Hong	15	1	7	7	-	-	-	-	-	4	6	3
Gun	30	3	21	6	-	-	-	2	3	10	7	4

Locality	n	Vertebrae									Sc
		45	46	47	48	49	50	29	30	31	32
Ju	25	-	-	3	17	5	-	-	-	-	2
Hu	14	-	-	-	4	9	1	-	-	-	-
Bu	23	-	-	6	8	8	1	1	-	-	3
Go	9	-	-	1	6	2	-	-	-	-	1
Yeo	23	-	-	-	15	8	-	-	-	-	-
Gang	35	-	-	-	16	18	1	-	-	-	-
Hong	15	-	-	-	11	4	-	-	-	-	-
Gun	30	1	-	1	20	7	1	-	-	-	1

Sample size (n)

Ju, Jumunjin; Hu, Hupo; Bu, Busan; Go, Goseong; Yeo, Yeosu; Gang, Gangjin

Gunsan samples

Table 7. Result of Kruskal-Wallis test for meristic characters of eight localities of *Konosirus punctatus*

Locality	Dorsal-fin rays	Anal-fin rays	Pelvic-fin rays	Vertebrae	Scutes
Ju	18.1	22.4^{a}	8	48.1^{a}	33.6^{a}
Hu	18.6^{a}	23.1^{ab}	8	48.8^{ab}	33.8^{b}
Bu	18.2^{b}	23.4^{ac}	8	48.2^{b}	33.6^{c}
Go	18.0	22.7^{d}	8	48.1^{b}	33.7^{d}
Yeo	17.9^{ac}	22.9^{e}	8	48.3^{b}	34.5^{abcd}
Gang	17.8^{ab}	23.6^{ade}	8	48.6^{ac}	34.6^{abcde}
Hong	18.4^{cd}	24.3^{abcde}	8	48.3^{b}	34.5^{abcd}
Gun	18.1^{d}	23.7^{ad}	8	48.2^{bc}	34.1^{e}

Note: Values and different superscript letters indicate means and significant differenced ($P<0.05$), respectively. If superscripts and the same, there is significant difference, but if the superscripts are different, there is no significant difference in the level of 95%.

Table 8. Eigenvectors for the first three principal components (PC) based on 17 morphometric characters of *Konosirus punctatus*

Character	PC1	PC2	PC3
Standard length	0.9919	0.0260	-0.0326
Head length	0.9821	-0.1564	0.0034
Snout length	0.9487	-0.1968	-0.0352
Orbit length	0.9221	-0.1100	0.0285
Interorbital width	0.9638	-0.0282	0.0879
Postorbital length	0.9763	-0.1590	0.0261
Upper jaw length	0.9634	-0.1805	0.0536
Body depth	0.9481	0.2451	0.0782
Body width	0.9336	0.1308	0.2795
Caudal peduncle depth	0.9540	0.2062	0.0531
Pectoral-fin length	0.9637	0.0760	-0.1006
Pelvic-fin length	0.9600	-0.0555	-0.0959
Dorsal-fin base length	0.9695	0.1086	-0.0645
Preanal length	0.9902	0.0019	0.0106
Predorsal length	0.9907	0.0221	-0.0033
Prepelvic length	0.9756	-0.0800	-0.0126
Anal-fin base length	0.9276	0.1616	-0.2748
Eigenvalue	15.7538	0.3080	0.2007
Proportion	0.9267	0.0181	0.0118
Cumulative	0.9267	0.9448	0.9566

Table 9. Standardized canonical (CAN) coefficients based on 17 morphometric characters of *Konosirus punctatus*

Character	CAN 1	CAN 2	CAN 3
Standard length (mm)	-0.737	1.554	1.221
Head length	1.273	-1.442	0.338
Snout length	0.240	0.143	0.835
Orbit length	0.196	0.854	-0.166
Interorbital width	-0.492	0.277	-1.521
Postorbital length	0.622	-0.407	-1.864
Upper jaw length	0.335	-0.128	0.173
Body depth	-0.280	0.237	-0.602
Body width	0.802	1.749	0.447
Caudal peduncle depth	-0.667	-0.046	-1.320
Pectoral-fin length	-0.488	0.247	0.900
Pelvic-fin length	0.145	-0.946	-0.207
Dorsal-fin base length	-0.288	0.105	1.103
Preanal length	0.541	-0.265	-0.989
Predorsal length	-0.127	-0.637	1.832
Prepelvic length	0.206	-0.388	0.127
Anal-fin base length	-0.698	-0.634	-0.297
Eigenvalue	5.172	1.763	0.888
Proportion	0.589	0.201	0.101
Cumulative	0.589	0.789	0.891

Table 10. Number (and percentage) of individuals classified correctly into their original group for the morphometric data. Rows are the original sample group and columns the reclassification group.

	Ju	Hu	Bu	Go	Yeo	Gang	Hong	Gun	Total
Ju	21 (84%)	3 (12%)	0 (0%)	1 (4%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	25
Hu	1 (8%)	12 (92%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	13
Bu	2 (9%)	0 (0%)	17 (74%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	4 (17%)	23
Go	0 (0%)	1 (11%)	0 (0%)	8 (89%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	9
Yeo	0 (0%)	0 (0%)	0 (0%)	0 (0%)	23 (100%)	0 (0%)	0 (0%)	0 (0%)	23
Gang	0 (0%)	0 (0%)	1 (3%)	0 (0%)	1 (3%)	25 (71%)	4 (11%)	4 (11%)	35
Hong	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (13%)	13 (87%)	0 (0%)	15
Gun	0 (0%)	0 (0%)	2 (7%)	0 (0%)	0 (0%)	5 (17%)	2 (7%)	21 (70%)	30

Ju, Jumunjin; Hu, Hupo; Bu, Busan; Go, Goseong; Yeo, Yeosu; Gang, Gangjin; Hong, Hongdo; Gun, Gunsan samples

Table 11. Comparison of meristic and proportional measurements of *Konosirus punctatus* by lineage

	Lineage A	Lineage B
Number of specimens	129	44
Standard length (mm)	101.6-221.3 (145.7)	153.6-226.7 (184.1)
Counts		
Dorsal-fin rays	17-20 (18.2)	17-19 (18.0)
Anal-fin rays	19-25 (22.8)	20-27 (23.5)
Pelvic-fin rays	8 (8.0)	8 (8.0)
Vertebrae	47-50 (48.2)	45-50 (48.3)
Scutes	32-35 (33.6)	29-37 (34.3)
Measurements (in SL)		
Head length	25.0-31.0 (27.2)	26.2-30.3 (28.2)
Snout length	5.5-7.3 (6.3)	5.1-7.3 (6.5)
Orbit length	4.1-6.9 (5.6)	4.2-6.1 (5.2)
Interorbital width	5.6-7.9 (6.4)	5.9-8.2 (6.7)
Postorbital length	13.9-19.5 (15.8)	15.8-18.5 (17.2)
Upper jaw length	6.8-10.2 (8.9)	7.8-10.2 (9.0)
Body depth	26.2-35.8 (30.2)	20.8-33.7 (29.9)
Body width	6.8-14.2 (11.0)	10.3-15.4 (12.6)
Caudal peduncle depth	7.9-10.8 (9.2)	7.6-10.0 (9.0)
Pectoral-fin length	16.6-21.5 (19.4)	16.1-20.6 (18.7)
Pelvic-fin length	9.0-12.6 (10.8)	9.6-11.8 (10.9)
Dorsal-fin base length	15.8-19.6 (17.8)	16.0-18.9 (17.5)
Preanal length	67.9-75.1 (71.0)	69.6-75.3 (72.0)
Predorsal length	42.6-48.4 (45.9)	43.8-48.8 (46.1)
Prepelvic length	33.0-51.4 (48.4)	46.6-52.4 (49.6)
Anal-fin base length	16.9-23.2 (20.0)	17.3-21.3 (19.1)

Table 12. Number (and percentage) of individuals classified correctly into their original group for the morphometric data. Rows are the original sample group and columns the reclassification group.

	Re- classification group		
Original group	Lineage A	Lineage B	Total
Lineage A	113 (88%)	16 (12%)	129
Lineage B	1 (2%)	43 (98%)	44

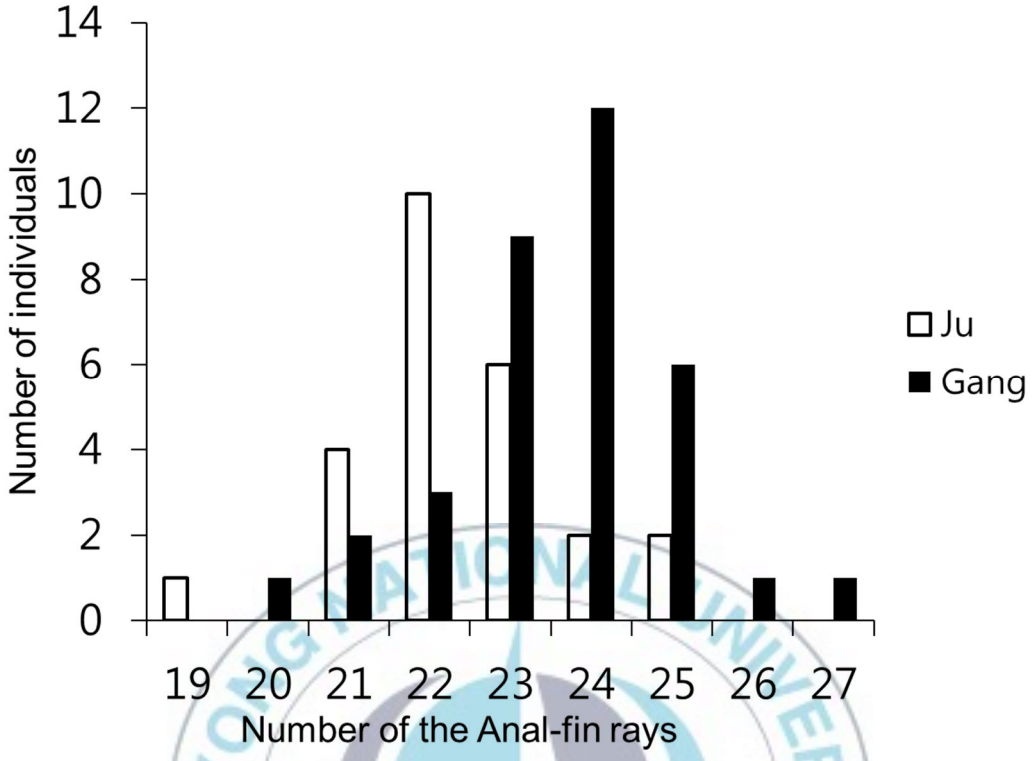

Fig. 5. Frequency distributions of total anal-fin rays among population of *Konosirus punctatus*. Open and closed bars indicate the Jumunjin (Ju) and Gangjin (Gang), respectively.

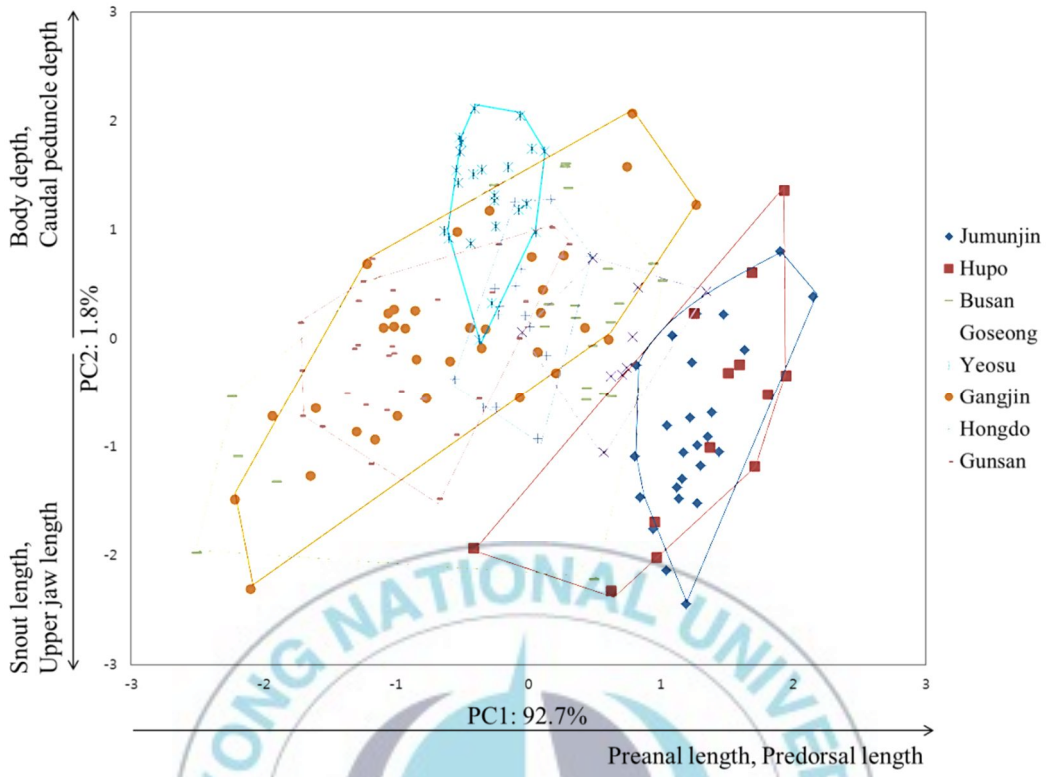

Fig 6. Plots of the first two principal component (PC) scores based on 17 morphometric characters of *Konosirus punctatus*.

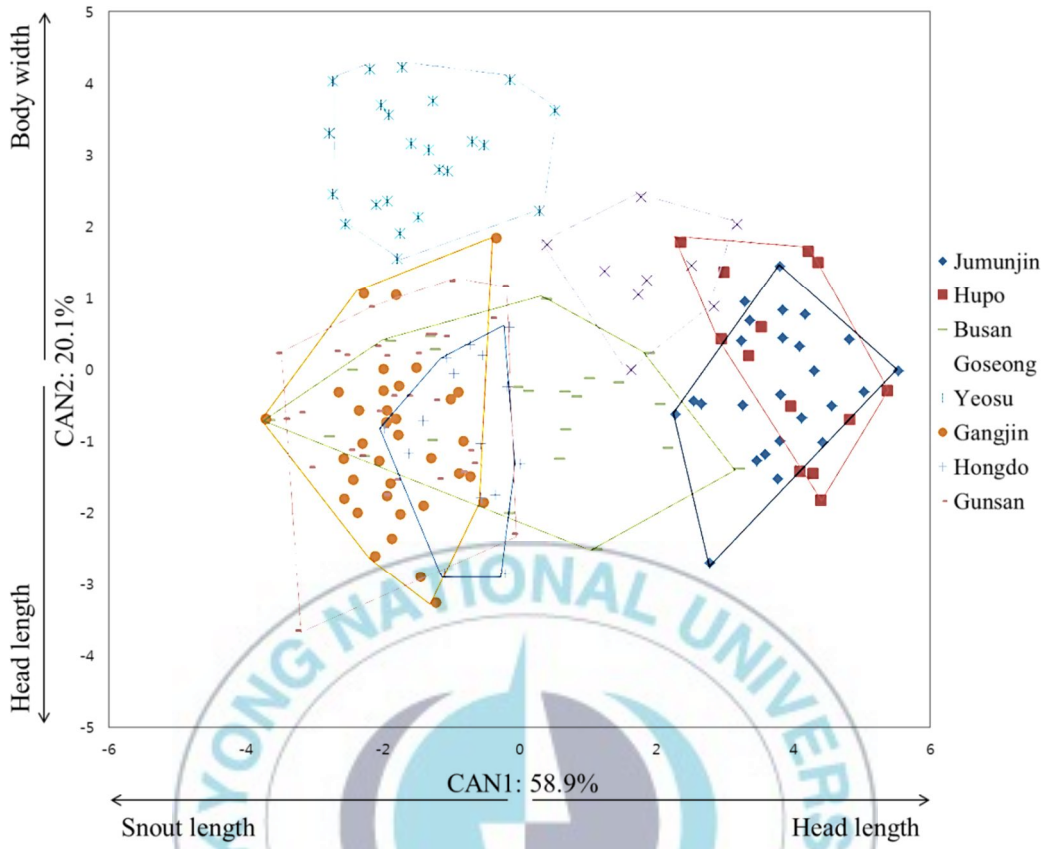

Fig 7. Plots of discriminant scores on the first and second canonical (CAN) axes based on 17 morphometric characters of *Konosirus punctatus*.

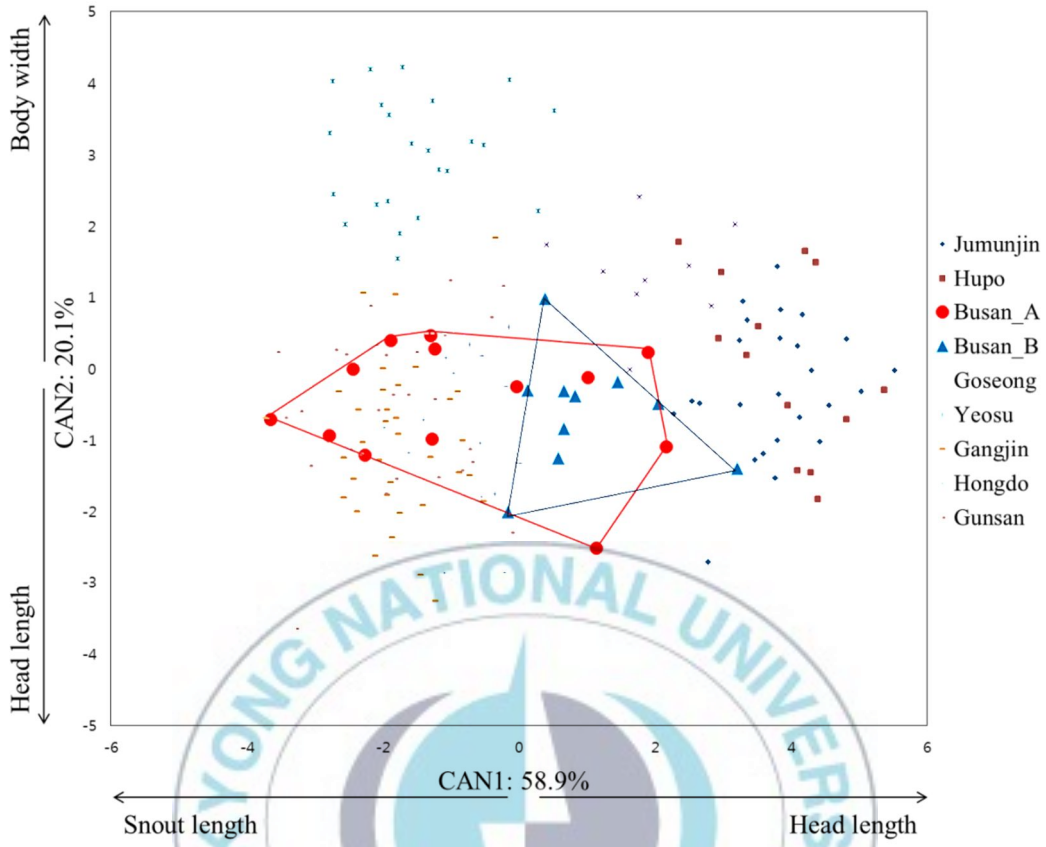

Fig 8. Plots of discriminant scores on the first and second canonical (CAN) axes based on 17 morphometric characters of *Konosirus punctatus*. Circle and triangle indicate the lineage A in Busan (Busan_A) and lineage B in Busan (Busan_B), respectively.

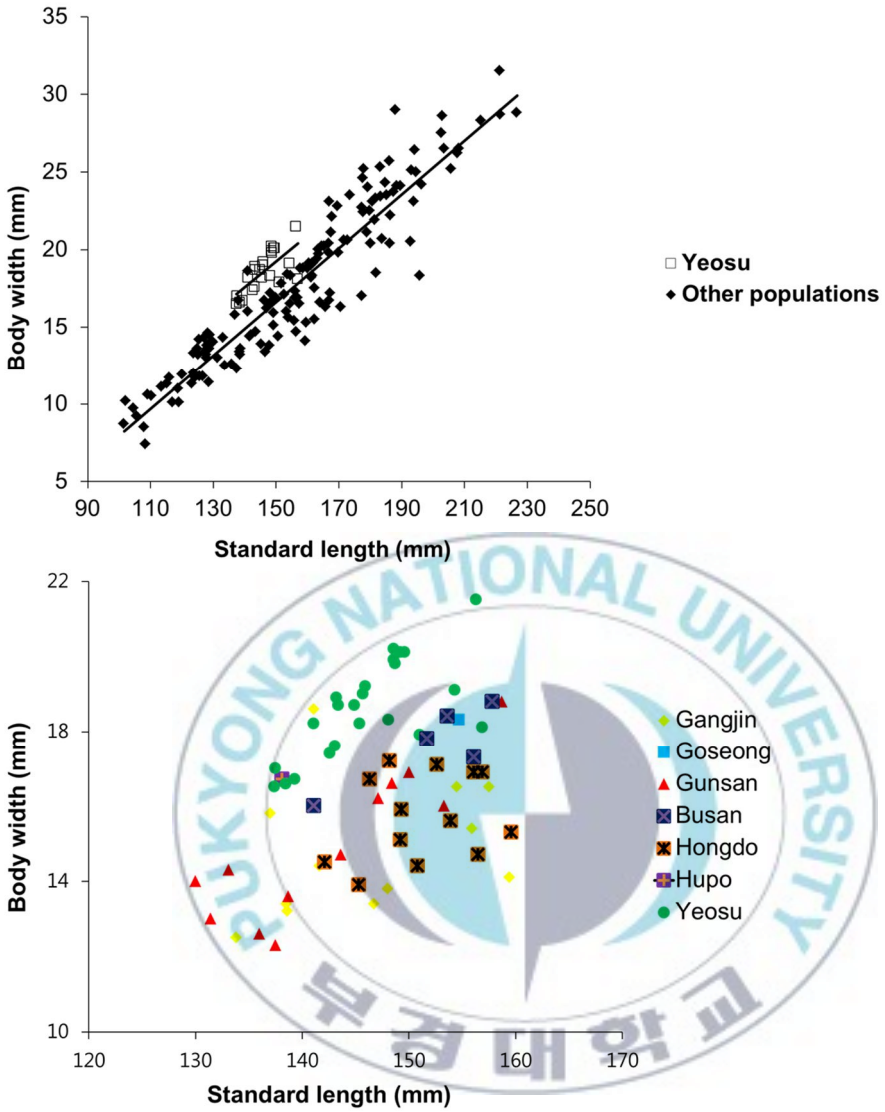

Fig 9. Relationship between body width and standard length among population of *Konosirus punctatus*. All population (up) and standard length of between 130mm and 160mm (down)

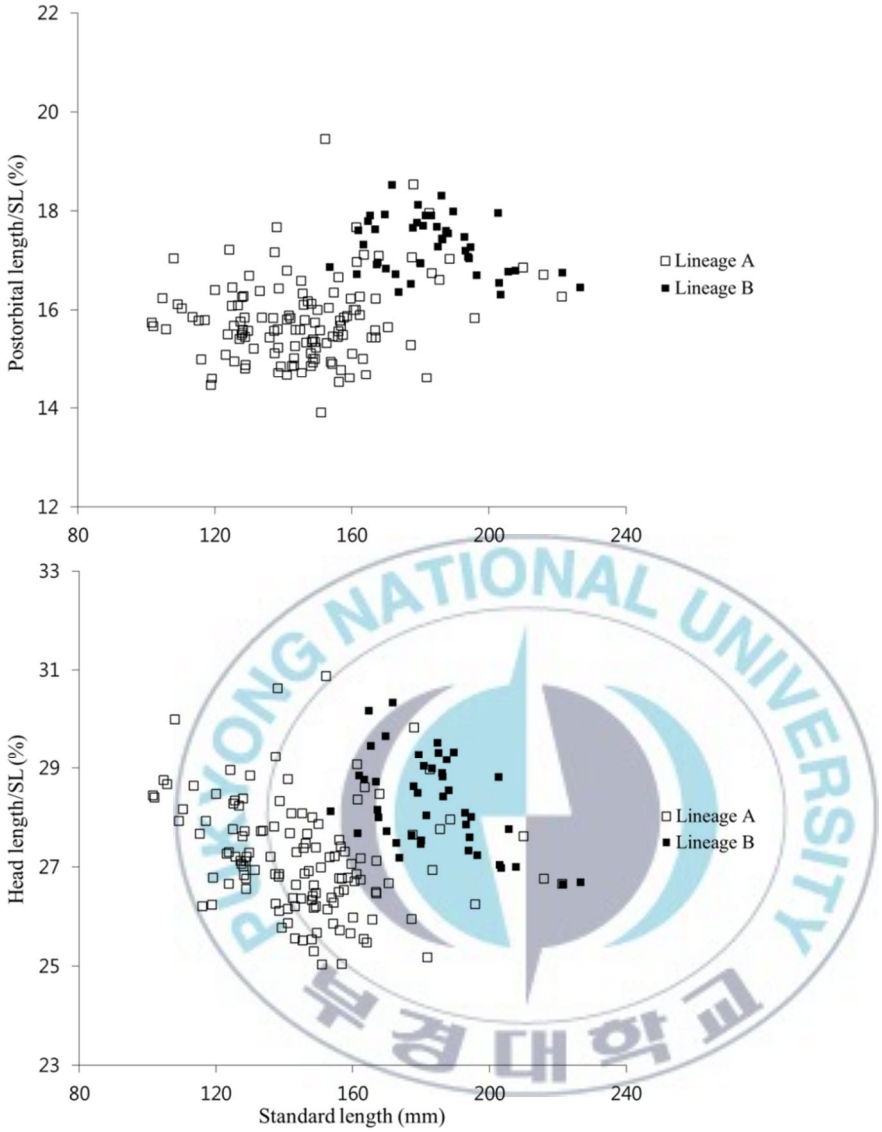

Fig 10. Relationship between postorbital length/SL and standard length (up) and between head length/SL and standard length (down) in lineage A and lineage B of *Konosirus punctatus*.

IV. Discussion

1. Genetic diversity and relationships

We analyzed 896 bp of the mtDNA CR sequence in 182 individuals of the gizzard shad collected from eight localities of Korea. As a result, the Korean gizzard shad was divided into two distinct lineages, A and B (see Fig. 2), with a genetic distance of 5.5%–6.6%. We found no shared haplotypes between lineages A and B, suggesting that at least two differentiated source populations had been successfully recruited (Evans et al., 2010). Lineage B probably contains two sublineages (b1 and b2), with a genetic distance of 1.0%–2.0%, suggesting that extended spatial sampling is required, as mentioned by Evans et al. (2010).

The gizzard shad showed high levels of haplotype diversity in lineage A (0.992 ± 0.003) and lineage B (0.943 ± 0.016), but low levels of nucleotide diversity in both lineage A (0.007 ± 0.004) and lineage B (0.023 ± 0.011). According to Grant and Bowen (1998), if a population shows high haplotype diversity and low nucleotide diversity, it might have undergone a genetic bottleneck event, followed by its sudden expansion. The levels of haplotype and nucleotide diversity in the gizzard shad (0.943–0.992 and 0.007–0.023, respectively) are similar to the levels in the silver pomfret (*Pampus argenteus*) in the China Sea (0.88 and 0.006, respectively) (Peng et al., 2009), and *Plectropomus maculatus* (0.941 and 0.014, respectively) and *Lutjanus carponotatus* (0.742 and 0.011, respectively) on the Great Barrier Reef (Evans et al., 2010). These species may have undergone rapid

population expansion after a period of low effective population size (Santos et al., 2006; Liu et al., 2008; Peng et al., 2009; Evans et al., 2010), possibly resulting from climatic changes, such as glacial and interglacial cycles (Petit et al., 1999). Using an mtDNA CR sequence divergence rate of 4%–8% per million years, the divergence between lineages A and B was estimated to have occurred 69,000–138,000 years ago. During the glacial period, the East Sea was isolated from the Pacific Ocean by the reduced sea level (Lambeck et al., 2002; Kitamura and Kimoto, 2006). As well as the isolation of the two seas, the habitats of the Korean gizzard shad might also be separated in the two different seas, potentially resulting in lineage sorting. Similarly, Han et al. (2012) calculated that the divergence of the two lineages of *Ammodytes personatus* in Japan and China occurred about 453,000 years ago, suggesting that water temperature might be the main factor differentiating the two lineages. The Japanese anchovy (*Engraulis japonicus*) and Australian anchovy (*E. australis*) also diverged into different lineages 105,000–420,000 years ago (Liu et al., 2006b) and *Chelon haematocheilus* 155,000–803,000 years ago (Liu et al., 2007). Those studies are consistent with our results, showing that the two lineages of *K. punctatus* differentiated in the late Pleistocene.

2. Demographic history

The Korean gizzard shad shows two differently shaped mismatch distributions, a unimodal distribution in lineage A but a bimodal distribution in lineage B. The unimodal shape may be attributable to a recent population expansion (Michaux et al., 2004), probably following a population bottleneck (Harpending 1994).

However, the bimodal shape may be sensitive to the age of the expansion, with the right peak representing an older expansion and the other peak a recent expansion (Bos et al., 2008; Schneider et al., 2010). From this perspective, lineage B of the Korean gizzard shad might have a more complex evolutionary history than lineage A. A bimodal mismatch distribution can also be seen in the Japanese *S. ariakensis* (Kim et al., 2006a) and the eastern population of the Korean *A. personatus* (Kim et al., 2006b). Dawson et al. (2002) suggested that a bimodal mismatch distribution is attributable to a historically differentiated allopatric population. Kim et al. (2006b) also suggested that the eastern population of *A. personatus* comprises a historically larger and older population than the western and southern populations. It is likely that lineage B of the Korean gizzard shad comprises two allopatrically differentiated populations resulting from their different habitats, such as the northern and southern parts of the East Sea. The species is distributed north to Vladivostok, Russia, in the East Sea (Froese and Pauly, 2014). Therefore, the bimodal mismatch distribution of the Korean gizzard shad may have occurred in response to the cold-current ecosystem formed in the middle East Sea of the subpolar front (Kim, 2009; Hong et al., 2012; Yoon et al., 2013).

The expansion of the lineage A population was estimated to have occurred approximately 89,000 years ago during the Late Pleistocene. During the Late Pleistocene (the past one million years), the climate was punctuated by a series of large glacial–interglacial changes (Imbrie et al., 1992). Population expansions in the late Pleistocene have been reported for other species, including *Mazocraeoides gonialosae* 17,000 years ago (Li et al., 2011), the Japanese

anchovy *E. japonicus*, 79,000–317,000 years ago, the Australian anchovy *E. australis* 45,000–178,000 year ago (Liu et al., 2006b), and the fat greening *Hexagrammos otakii* 91,000–327,000 years ago (Habib et al., 2011). It seems that marine fish populations around the Korean peninsula may have declined or almost become extinct during the glacial period, but increased and recovered rapidly during the interglacial period.

3. Population structure

AMOVA showed strong structure (F_{ST} = 0.856; P < 0.0001) between lineages A and B. Wright (1965) suggests that a pairwise F_{ST} range of 0.00–0.05 indicates little differentiation, a range of 0.05–0.25 indicates moderate differentiation, and a range > 0.25 indicates very great differentiation. In this study, high levels of structuring were observed between the two lineages of the gizzard shad with AMOVA (F_{ST} = 0.856; P < 0.0001). Therefore, the gizzard shad in Korean waters shows very great differentiation, according to Wright (1965).

The genetic distance between the two lineages of the Korean gizzard shad was 5.5%–6.6%. Nei (1975) suggested that a genetic distance of 0%–5% indicates differences within the same population, and a range of 2%–20% indicates differences at the subspecies level. In the present study, the Korean gizzard shad is considered to form subspecies, but this could not be confirmed because the two lineages coexist in the East Sea and Korea Strait. To further understand recent events, such as the secondary contact between the two lineages, more samples must be collected from the far-northern East Sea and additional studies conducted

using more sensitive DNA markers, such as microsatellite DNA.

The NJ tree showed that the Korean gizzard shad clusters into two reciprocal monophyletic groups, lineages A and B. Lineage A is distributed along all the coasts of Korea, with occurrence rates of 100% in the Yellow Sea and western Korea Strait, but declines steadily from the middle Korea Strait (Yeosu, 81%) to the East Sea (Jumunjin, 4.2%) (Fig. 3). Previous studies have suggested that such phenomena may be attributable to the influence of seawater temperatures or currents (Santos et al., 2003; Kim et al., 2006b; Kim et al., 2010; Han et al., 2012; Hong et al., 2012; Yoon et al., 2013). Santos et al. (2003) suggested that seawater temperatures and currents are the main factors distinguishing the two *Macrodon* groups. Kim et al. (2006b, 2010) demonstrated very restricted gene flow between the eastern population and the western + southern population of *A. personatus*, suggesting that this may be attributable to their unique adaptation to each habitat during the glacial period. Han et al. (2012) also suggested that the distributions of the two lineages of *A. personatus* are strongly influenced by seawater temperature. The distributions of lineages A and B of the Korean gizzard shad are probably maintained by the complexity of various currents, e.g., the Kuroshio Current (KC), North Korean Cold Water (NKCW), and Western Korea Coastal Water (WKCoW) (Zhang et al., 2000; Johnson and Teague 2002; Chen, 2009). The KC is a generally high-temperature (winter, T = 20–24 °C; summer, T = 28–29 °C) and high-salinity current (winter, S = 33.5–34.75; summer, S = 33.5–34.5) (Chen, 2009), whereas the WKCoW is a generally low-temperature (winter, T = 2–5 °C; summer, T = 20–26 °C) and low-salinity current (winter, S = 31.5–32.5; summer, S < 30) (Zhang et al., 2000; Chen, 2009). Like WKCoW, the annual sea

temperature of the NKCW is 1–7 °C and its salinity < 34.1 psu (Yun et al., 2004). Based on a comprehensive comparison of genetic and environmental data, we speculate that a part of lineage A might have moved to the east with the WKCoW, and thereafter a subpart might have moved to Jumunjin (northernmost area of the East Sea here), with the KC. However, the occurrence of lineage A declines abruptly at Hupo and Jumunjin, which are strongly affected by the NKCW. Interestingly, lineage B does not occur west from Yeosu, implying that lineage B is more sensitive to seawater temperature or salinity than lineage A.

4. Morphological analysis

Principal components analysis (PCA) showed that eight localities populations overlapped from each other. As a result of canonical discriminant analysis (CDA), the postorbital length being largest in CAN1, and it is divided into two groups (Fig. 7). Busan population has both group and show same trend with genetic analysis. This result means Busan population has two lineages which have different morphological characteristics by different spawning ground. Shen et al. (2011) suggested from msDNA analysis that three lineage about *Mugil cephalus* having different spawning ground. From these results, spawning ground investigation about msDNA of Busan population need in the future.

The body width being largest in CAN2 that Yeosu was separated in other populations (Fig. 7). And most of the Yeosu population is distinguished from other populations by wider body (Fig. 9). Body width influenced from spawning season.

It is known that gizzard shad spawning season from April to August (Kim et al., 2007a; Lee et al, 2010). Except the populations from Hupo and Goseong, we used this survey were captured at spawning season (Table 1). Therefore, wider body width of Yeosu population not related to spawning season. It is known that morphometric and meristic characters are influenced by environmental conditions (Lindsey, 1988; Swain et al., 1999; Turan, 2000). The further study is required to analyze environmental conditions of sampling site of gizzard shad.

Result of genetic data, the Korean gizzard shad was divided into two distinct lineages. Based on these result, percentage of correctly classified was obtained 90.2% by canonical discriminant analysis (CDA). A study conducted by Murta (2000) showed that horse mackerel (*Trachurus trachurus*), for the morphometric data, a clear distinction between Cadiz (97%) and all other groups. Our result of canonical discriminant analysis (CDA) was smaller than Murta's result, but it was similar to a study of Silva (2003), the sardine (*Sardina pilchardus*) correctly classified was 87% (the northern Atlantic-Mediterranean group) and 86% (the southern Iberia-Morocco group) between the two groups (Table 12). And Silva (2003) suggested that discrimination of the two morphotypes were confirmed statistically by the high percentage of correct classification (>85%) of new fish. Therefore, the gizzard shad in Korean waters shows divided two lineages, based on the result and suggestion of Silva (2003).

The genetic and morphological analysis of Portuguese sole (*Synaptura lusitanica*) indicated the differences between the west coast (Setùbal) and the south-eastern coast by Cabral et al. (2003). According to these authors, Portuguese sole conservative approach to the fisheries management of this species should consider

to be two independent stocks. In this study, Korean gizzard shad is separated into lineage A and lineage B, in a dimension of genetic and morphological results. So, we suggest a need for divided management strategies for each lineage.

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