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Morphological and genetic variation of sea raven, *Hemitripterus villosus* (Hemitripteridae, Pisces) from Korea

by

Seo Ha Jang

Department of Marine Biology

The Graduate School

Pukyong National University

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[한국산 삼세기(*Hemitripterus villosus*)의 형태 및 유전변이]

Advisor: Prof. Jin Koo Kim

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Approved by:

(Chairman) Dr. Hea Ja Baek

(Member) Dr. Jung Ha Kang

(Member) Dr. Jin Koo Kim

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Contents

한국산 삼세기(*Hemitripterus villosus*)의 형태 및 유전변이

장 서 하

부경대학교 대학원 해양생물학과

요 약

삼세기(*Hemitripterus villosus*)는 우리나라 전 연안, 일본 중부 이북, 오호츠크해, 베링해 등지에 분포하는 저서어류로 우리나라와 일본에서는 식용으로 이용하는 어류이다. 본 연구에서는 한국산 삼세기의 개체군 구조를 파악하기 위해 우리나라 6개 지역(인천, 보령, 신안군 흑산도, 부산, 포항, 강원도 고성)에서 채집된 175개체의 삼세기를 대상으로 형태 및 분자 분석을 실시하였다.

삼세기 147개체를 대상으로 6개의 계수형질 및 25개의 계측형질을 이용한 형태분석 결과, 계수형질에서는 척추골 수에서 집단 간 가장 큰 차이를 나타내었다. 계측형질을 사용한 정준판별분석 결과, 서해·남해[인천, 보령, 흑산도]와 동해[포항, 고성]의 두 지역이 잘 구분되었다.

삼세기 175개체를 대상으로 mt DNA Cyt*b* 영역 801bp에 의한 분자분석 결과 총 20개의 단상형(haplotype)이 확인되었다. 최소근접네트워크(Minimum spanning network)는 별 모양으로, 지역에 따라 나뉘는 경향을 보이지 않았다. 하지만, 모든 지역에서 나타난 Haplotype 1의 빈도가 서해-남해-동해로 갈수록 감소하는 경향을

보였다. 지역 집단간 유전적 분화 정도를 나타내는 고정지수 (F_{ST})는 [고성] 지역과 [인천, 보령, 흑산도, 부산] 지역이 유의한 차이를 나타내었다. [포항]은 흥미롭게도 [인천] 지역과만 차이를 보였지만 고성과 같은 하플로타입을 공유하는 점에서 서해·남해[인천, 보령, 흑산도, 부산)와 동해 북부[고성) 사이의 전이지역(transitional zone)일 가능성을 보여주었다. 인천, 보령, 흑산도, 부산, 포항은 낮은 하플로타입 다양성(0.086-0.303)과 낮은 염기 다양성(0.000358-0.000635) 을 가졌으며 고성은 높은 하플로타입 다양성(0.765)과 낮은 염기 다양성(0.001647)을 가져 서로 다른 진화 역사를 가졌다. Tajima's *D* test와 Fu's *Fs* test 결과 대부분의 지역이 통계적으로 유의한 음의 값을 나타내었다. 이는 최근에 집단의 급격한 팽창 현상이 발생하였음을 보여준다.

형태분석과 분자분석 결과, 한국산 삼세기는 서해·남해[인천, 보령, 흑산도, 부산]와 동해[포항, 고성] 간 유전자 흐름이 낮거나 제한적인 것으로 나타났다. 추후 포항과 나머지 지역 간 유전적 구조를 명확히 하기 위해서 최근의 집단 분화 역사를 파악할 수 있는 microsatellite DNA와 같은 마커를 사용한 분석이 필요하다.

I. Introduction

The sea raven, *Hemitripterus villosus* (Pallas, 1814), a member of the family Hemitripteridae, is a cold-water demersal fish. It inhabits the mud, sand, gravel or rocks bottom, ranging in depth to 550 m, off the coast of Korean Peninsula, the Sea of Okhotsk and the Bering Sea (the western part), off the Pacific coast of Japan, the Kuril Islands, and southeastern Kamchatka; off the coasts of North America. It is found only in Alaska Bay near Kodiak Island (Chyung, 1977; Eschmeyer et al., 1983; Masuda et al., 1984; Lindberg and Krasyukova, 1987; Borets, 2000; Novikov et al., 2002; Fedorov et al., 2003; Fadeev, 2005; Kim et al., 2005).

Hemitripterus villosus is characterized by various body color from blackish-brown to reddish-brown, many small tubercles on the body, large head with numerous bony humps and fimbriated flap. In Korea and Japan, this species is caught using different kinds of nets, and its meat is used as food (Choi, 2007; Novikov et al., 2002).

Hemitripterus villosus is winter spawner, inhabits shelf and continental slope and migrate into shallow water (5~10 m) to spawn from November to January (Gomelyuk and Markevich, 1986). Females released adhesive eggs into a rock crevice or polychaete tubes (Gomelyuk and Markevich, 1986; Munehara, 1992). Spawning behavior of *Hexagrammos otakii* (Kim et al., 1993), *Hexagrammos agrammus* (Kim and Myoung, 1983), *Liparis ingens* (Kim et al., 1986a), *Liparis tanakae* (Kim et al., 1986b), and *Aptocyclus ventricosus* (Kim et al., 1987) are similar to this species (Park et al., 2014).

Several studies on the egg development and early life history, distribution and ecology, and reproductive habit of this species have been carried out (Kyushin, 1968; Okiyama and Sando, 1976; Munehara, 1992, 1996; Munehara et al., 1997; Kim et al., 1996; Byun et al., 1997; Tokranov, 2006; Antonenko et al., 2010; Park et al. 2014).

However, studies on genetic diversity and population structure and demographic history research of *H. villosus* have not been conducted yet.

Assessments of population structure provides essential information to underpin resource recovery and aid in delineating and monitoring populations for fishery management (Ricker, 1981; Hilbon and Waters, 1992; Palumbi, 1994; Joseph and Jayasankar, 2001; Han et al., 2008; Reiss et al., 2009; Zhang, 2010).

Multivariate analysis through comparison of countable, morphological structures (e.g., fin rays, gill rakers, scales in rows) have been used for long time for identifying fish stocks (Quddus et al., 1984; Kai and Nakabo, 2002; Tzeng, 2004; Turan et al., 2006; Kim et al., 2008; Murta et al., 2008). Meristic and morphometric characters are applicable for studying environmentally induced short-term changes (Begg et al., 1999).

In addition to this, recently, population genetics for finding genetic variations within species and inferring the evolutionary direction of species have been performed (Liu et al., 2006; Liu et al., 2007; Xiao et al., 2010; Habib et al., 2011). The amount and pattern of polymorphism in DNA sequences is a robust tool for population genetic analysis and has proved effective to identify reproductive isolation among populations and for bringing an evolutionary perspective to conservation, management and speciation (Li, 1997).

Mitochondrial DNA (mtDNA) has been used in population studies because of its compactness, no recombination, almost total maternal inheritance and fast evolutionary rate compared to nuclear DNA (Brown et al., 1982; Wilson et al., 1985; Bartlett and Davidson, 1991; Buonaccorsi et al., 2001). Mt DNA control region is often used to analyze population genetic variations (Stepien, 1999; Bay et al., 2004; Hoolihan et al., 2004; Shui et al., 2009; Habib et al., 2015), cytochrome *b* also used as a maker to analyze population genetic variation (Roberti et al., 1993; Grant and Bowen, 1998; Lecomte et al., 2004; Kim et al., 2003; Hsu et al., 2007; Murray et al., 2008; Myoung et al., 2016).

Marine organisms usually show a low level of genetic differentiation across a wide area (Palumbi, 1994). Dispersion of eggs and larvae by ocean currents, migratory behaviors of

adults contribute to high gene flow, and ultimately many ocean fishes, especially migratory species leading to panmixia (Palumbi, 1994; Walpes, 1998; Grant and Bowen, 1998).

On the other hand, current patterns, oceanic circulation, limited larval dispersion, behavioral characteristics, or selection can act as a barrier to promoting genetic differentiation among regional populations (Grant and Bowen, 1998). Also, historical events such as the glacial period are considered to be important elements in the formation of the genetic structure of species (Hewitt, 2000).

Korean Peninsula is surrounded by the East, West, and South Seas, and each having undergone drastic changes in areas, configuration, and environmental conditions throughout the late Quaternary glacial cycles (Wang, 1999; Kim et al., 2008).

Because *H. villosus* lived all around of Korean Peninsula, we assumed that the populations of this species might have morphological and genetic variation.

The present study aimed to clarify the population structure of *H. villosus* from Korea, based on morphological traits and partial mtDNA cyt*b* gene sequences.

II. Materials and methods

1. Sampling

A total of 175 samples were collected from six localities along the Korean coasts between 2010 and 2016 (Table 1; Fig. 1): Goseong (GS, n=40) and Pohang (PH, n= 30) in the East Sea; Busan (BS, n=25) in the Korea Strait; Heuksando (HS, n=13), Boryeong (BR, n=21), Incheon (IC, n=46) in the Yellow Sea. A piece of muscle tissue was obtained from each individual and preserved in 95% ethanol and stored frozen at -20℃ until DNA extraction; specimens of *H. villosus* were preserved in 70% ethanol after 5% formalin. These specimens were deposited at the Ichthyology Laboratory at Pukyong National University (PKU)

Table 1. List of specimens of the present study.

Species	Voucher no.	No. of specimens	Locality	Date	Standard length (mm)	Remark
Hemitripterus villosus	PKU 3523	1	Pohang	2010.01.27	217.4	
	PKU 4977	1	Guryong-po, Pohang	2010.05.25	165.4	
	PKU 5913	1	Goseong-gun, Gangwon-do	2011.07.07	183.7	
	PKU 6884-6888	5	Incheon	2012.04.16	-	Tissue
	PKU 7700	1	Busan	2012.10.18	167.3	
	PKU 7962-7970	9	Busan	2012.11.13	192.6-252.1	
	PKU 8861-8866	6	Heuksan-do, Sinan-gun	2013.05	190.4-252.9	
	PKU 9401	1	Heuksan-do, Sinan-gun	2013.07.09	217.9	
	PKU 10318-10323	6	Heuksan-do, Sinan-gun	2014.03.08	149.2-252.9	
	PKU 10624	1	Boryeong	2014.04.21	214.1	
	PKU 12787-12791	5	Busan	2015.09.15	166.7-190.6	
	PKU 13012	1	Busan	2015.12.7	223.0	
	PKU 13184-13189	6	Busan	2016.04.09	185.1-247.7	
	PKU 13191-13212	22	Goseong-gun, Gangwon-do	2016.04.18	151.8-252.2	
	PKU 13213-13230	18	Guryong-po, Pohang	2016.04.20	154.1-191.6	
	PKU 13309-13312	4	Boryeong	2016.04.28	113.1-208.6	
	PKU 13313	1	Boryeong	2016.04.28	-	Tissue
	PKU 13315	1	Boryeong	2016.04.26	126.1	
	PKU 13316	1	Boryeong	2016.04.26	-	Tissue
	PKU 13510	1	Incheon	2016.09.26	212.8	
	PKU 13511	1	Incheon	2016.09.26	-	Tissue

Table 1. Continued.

Species	Voucher no.	Number of specimens	Locality	Date	Standard length (mm)	Remark
Hemitripterus villosus	PKU 13512-13531	20	Incheon	2016.09.26	168.6-223.9	
	PKU 13536	1	Incheon	2016.09.26	174.3	
	PKU 13648	1	Goseong-gun, Gangwon-do	2012.10.24	-	Tissue
	PKU 13649	1	Goseong-gun, Gangwon-do	2013.05.07	-	Tissue
	PKU 13650	1	Goseong-gun, Gangwon-do	2013.12.24	-	Tissue
	PKU 13651-13656	1	Goseong-gun, Gangwon-do	2014.02.26	-	Tissue
	PKU 13657	1	Goseong-gun, Gangwon-do	2014.03.04	-	Tissue
	PKU 13658	1	Goseong-gun, Gangwon-do	2014.03.11	-	Tissue
	PKU 50351-50363 (odd no.)	7	Pohang	2014.01.17	151.4-237.4	
	PKU 50366-50368 (even no.)	2	Pohang	2014.01.17	-	Tissue
	PKU 50372-50378 (even no.)	4	Boryeong	2014.01.13	-	Tissue
	PKU 50379-50383 (odd no.)	3	Boryeong	2014.01.13	172.7-184.0	
	PKU 50386-50390 (even no.)	3	Boryeong	2014.01.13	-	Tissue
	PKU 51181-51183 (odd no.)	2	Boryeong	2014.04.18	112.8-123.3	
	PKU 51187	1	Boryeong	2014.04.17	205.4	
	PKU 53509-53513 (odd no.)	3	Busan	2015.01.20	172.0-235.1	
	PKU 56649-56653 (odd no.)	3	Goseong-gun, Gangwon-do	2015.10.21	167.4-180.9	
	PKU 56815-56819 (odd no.)	3	Goseong-gun, Gangwon-do	2015.10.24	167.3-185.7	
	PKU 58789	1	Pohang	2016.09-09	197.5	
	PKU 58713-58747 (odd no.)	18	Incheon	2016.09.03	175.2-232.6	

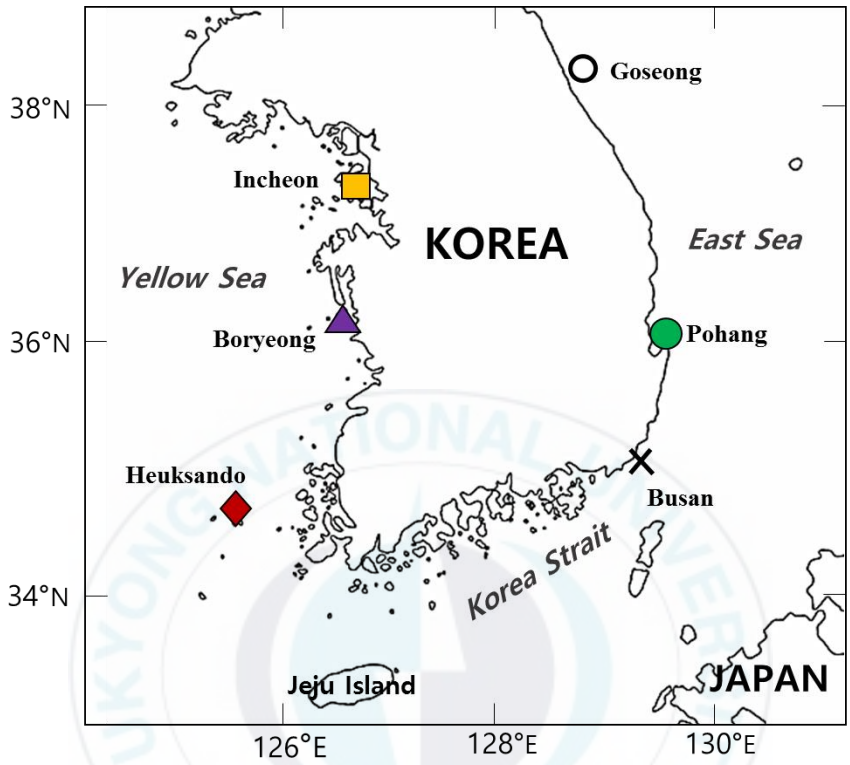

Fig. 1. Map showing the sampling area of *Hemitripterus villosus* from Korea.

2. Morphological analysis

(1) Measurement method

The morphological analysis was based on 147 specimens of *H. villosus*: Goseong (n=29), Pohang (n=28) in the East sea; Busan (n=25) in the Korea Strait; Heuksando (n=13); Boryeong (n=12); Incheon (n=40) in the West Sea. A total of 25 morphometric and 6 meristic characters were analyzed according to the method of Nakabo (2013): morphometric- total length, standard length, preanus length, body depth, body width, head length, head width, snout length, orbit diameter, postorbital length, interorbital width, upper jaw length, lower jaw length, predorsal fin length, presecond dorsal fin length, preanal fin length, prepectoral fin length, prepelvic fin length, first dorsal fin height, first dorsal fin base length, second dorsal fin base length, anal fin base length, pectoral fin length, pelvic fin length, caudal peduncle length, caudal peduncle depth, caudal peduncle width; meristic-first dorsal fin spines, second dorsal fin rays, anal fin rays, pectoral fin rays, pelvic fin spines and rays, vertebrae. Six meristic characters were counted by radiograph (SOFTEX HA-100; Hitex Co., Tokyo, Japan). 25 morphometric were taken to the nearest millimeter using followed by vernier calipers.

(2) Statistical analysis

Six meristic characters were analyzed by the Kruskal-Wallis nonparametric test (Zar, 1999). 25 morphometric characteristics were analyzed by linear regression, analysis of variance (ANOVA) and Tukey's test. Each morphometric data log-transformed and corrected in order to remove the size effect and possible allometric relationships between variables given the different length ranges of the samples (Ricker, 1973; Ihssen et al., 1981;

Hurlbut and Clay, 1998; Murta et al., 2008). A principal component analysis (PCA) and canonical discriminant analysis (CDA) were conducted to identify shape-related differences among six localities populations of *H. villosus*. All statistical analysis was performed using SPSS version 21.

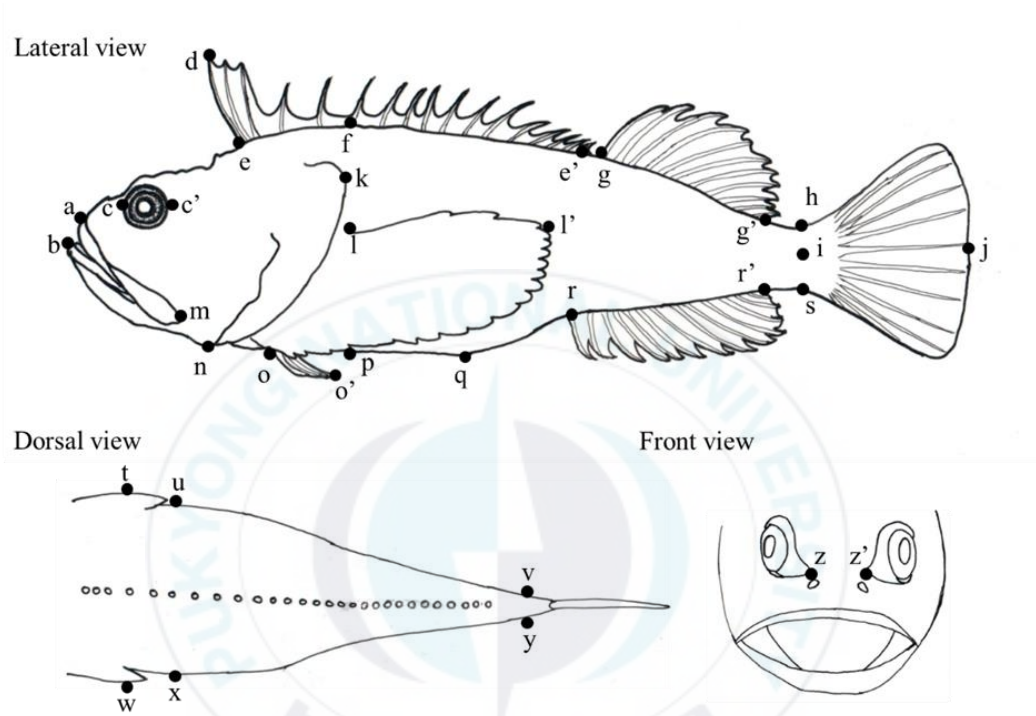

Fig. 2. The measurements of *Hemitripterus villosus*. b-j: Total length (TL); a-i: Standard length (SL); a-q: greanus length; f-p: Body depth; u-x: Body width; a-k: Head length; t-w: Head width; a-c: Snout length; c-c': orbit diameter; c'-k: Postorbital length; z-z': Interorbital width; a-m: Upper jaw length; b-n: Lower jaw length; a-e: Predorsal fin length; a-g: Presecond dorsal fin length; a-r: Preanal fin length; a-l: Prepectoral fin length; b-o: Prepelvic fin length; e-f: First dorsal fin height; e-e': First dorsal fin base length; g-g': Second dorsal fin base length; r-r': Anal fin base length; l-l': Pectoral fin length; o-o': Pelvic fin length; h-s: Caudal peduncle depth; v-y: Caudal peduncle width; r'-s: Caudal peduncle length.

3. Molecular analysis

(1) Genomic DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from the muscle tissues of fish using Genomic DNA Extraction Kit (Bioneer, Daejion, Korea). An 801-bp fragment of the mtDNA cyt*b* was amplified with universal primers (Palumbi et al. 1996): L-GLUDG (5'- TGA CTT GAA RAA CCA YCG TTG-3') and H-CB3 (5'- GGC AAA TAG GAA RTA TCA TTC-3'). Y (pyrimidine, C+T) and R (purine, G+A) were the degenerate (mixed) base. The degenerate base means more than one base possibility at a particular position, this is a very useful tool when the DNA sequence for a PCR target is not available.

Polymerase chain reaction (PCR) amplification was carried out in a reaction mix of 30 μl (10X PCR buffer 3 μl, 2.5mM dNTP 2.4 μl, L-GLUDG primer 1 μl, H-CB3 primer 1 μl, Takara Taq polymerase 0.1 μl, total DNA 3 μl, and 19.5 μl of sterile distilled H_2O). The thermal cycle followed: Initial denaturation at 95℃ for 5 min, 35 cycles of denaturation at 95℃ for 1 min, primer annealing at 52℃ for 1 min, extension at 72℃ for 1 min, final extension at 72℃ for 5 min. The PCR products were purified using DNA purification kit (LaboPass™ Gel and PCR Clean-up Kit, Cosmo Genetech, Korea).

The PCR products were sequenced with the ABI Prism BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) on an ABI 3730XL DNA Analyzer (Applied Biosystems Inc.).

(2) Sequence alignment and data analysis

The mtDNA cyt*b* sequences were edited and aligned using ClustalW (Thompson et al., 1994) in Bioedit 7 (Hall, 1999). Molecular diversity indices such as the number of haplotypes, polymorphic sites, transitions, transversions, and indels were obtained using the program Arlequin 3.5.1.2 (Excoffier et al., 2010). Haplotype diversity (*h*), nucleotide

diversity (π; Nei, 1987), and the mean number of pairwise differences (k; Tajima 1983), with their corresponding variances were estimated. Genetic relationships among mtDNA cyt*b* haplotypes of the species were reconstructed using the neighbor-joining (NJ) method with Tamura-Nei model (Tamura and Nei, 1993), and confidence was assessed with 1000 bootstrap replications. Minimum Spanning Network (MSN) were constructed using TCS v.1.21. program (Clement et al., 2000).

To quantify the genetic differentiations between local populations, F_{ST} values were calculated and their significance tested by Arlequin 3.5 (Excoffier et al., 2010). Hierarchical analysis of molecular variance (AMOVA) was performed in Arlequin 3.5. The sampling localities were grouped by 'two-categories'. First, the Yellow Sea and Korea Strait group (Incheon, Boryeong, Heuksando, Busan) and the East Sea group (Pohang, Goseong) were grouped respectively. Second, we added Pohang to the Yellow Sea and Korea Strait group (Incheon, Boryeong, Heuksando, Busan) and compared it to the Goseong group. To determine whether there was a relationship between genetic distance and geographic distance, we used Mantel test on the IBD website (Jensen and Bohonak, 2005). The geographic distance between each pair of sampling localities was measured following the coastline (https://www.google.com/maps).

The historical demographic patterns of *H. villosus* were examined by two approaches. Evidence of population expansion was estimated using Tajima's *D* test (1989) and Fu's *F*s test (1997). The significant negative value of *D* and *F*s statistics can be interpreted as signatures of population expansion or selective hitchhiking (Rand, 1996). A positive value indicates either balancing selection or secondary contact among previously isolated populations. Possible historical demographic expansion of *H. villosus* populations was estimated from the mismatch distribution, which is based on three parameters: θ_0, θ_1 (θ before and after the population growth), and τ (time since expansion expressed in the unit of mutational time) (Rosers and Harpending, 1992). The model of sudden population expansion was applied. The concordance of the observed with the expected distribution

under the sudden expansion model was evaluated using Harpending's raggedness index (Harpending, 1994) and the sum of squared deviations (SSD; Schneider and Excoffier, 1999). The values for τ were transformed to estimate the real time since expansion with the equitation $\tau=2ut$ (Rosers and Harpending, 1992)., where u is the mutation rate for the whole sequence under study and t is the time since expansion. An appropriate nucleotide substitution rate has not been calibrated for this species. The evolutionary rate calibrated for cyt*b* in other teleosts (2% per million years) was used to estimate population expansion time among different *H. villosus* populations (Avise, 1994; Bowen et al., 2006; Han et al., 2008).

III. Results

1. Morphological analysis

Among the specimens used in this study, most of them showed dark-brown, but some showed reddish-brown and yellowish-brown. However, we could not find the difference of meristic and morphometric characteristic relating to the body color.

Meristic and morphometric characters of the six localities populations of *H. villosus* are shown in Table 2. The frequency distribution of meristic characters of each population is shown in Table 3. The Kruskal-Wallis test showed significant differences in the number of first dorsal fin spines, anal fin rays, and vertebrae (P<0.05) (Table 4). However, the frequency distributions of first dorsal fin spines and anal fin rays were almost overlapping, these meristic characters were considered insufficient to population discrimination of *H. villosus*. On the other hand, the mean number of vertebrae revealed the largest significant difference between Goseong and the rest localities (Incheon, Boryeong, Heuksando, Busan, Pohang), and the frequency distributions showed also clear differences. The difference of mean number was especially pronounced between specimens of Goseong (mean, 39.7) and those of Pohang (mean, 38.4).

Most morphometric characters (Tukey's test) were found to be significantly different except first dorsal fin base length and presecond dorsal fin length (P<0.05) (Table 5). Results of PCA showed that 54.16% of the total variance was explained by the first principal components axis (PC1, evenvalue=13.540). The second and third components show that 9.82% (PC2, evenvalue=2.455) and 7.63% (PC3, evenvalue=1.910) of the total variance, respectively (Table 6). The loadings for head length (0.980), pelvic fin length (0.625), and body width (0.687) were largest absolute value on PC1, PC2, and PC3, respectively (Table 6). Plots of scores on PC1 and PC2 showed that all localities were

overlapped. Plots of scores on PC2 and PC3 showed similar results, six localities were not clearly distinguished; however, Incheon slightly separated from Pohang and Goseong (Fig. 3).

The CDA generated three discriminant functions; the first canonical variables (CAN1) contributed 50.7% of total variance (eigenvalue=4.511), the second canonical variables (CAN2) contributed 21.6% of total variance (eigenvalue=1.918), and the third canonical variables (CAN3) contributed 15.7% of total variance (eigenvalue =1.396) (Table 7). The loadings for prepectoral fin length (-2.355), preanus length (1.439) were largest absolute value on CAN1, CAN2, respectively (Table 7). Only 11 specimens were misclassification from among the 147 specimens (7.4%). The highest percentage of misclassification was observed at Boryeong (16.7%), the lowest at Heuksando and Busan (0%). Unlike the PCA results, the CDA results showed clear separation, with two groups segregated by the center value (‘0’) of CAN1 (Fig. 4). One is populations of Incheon, Boryeong, Heuksando, and Busan (with positive loadings on CAN1), and the other includes populations of Pohang and Goseong (with negative loadings on CAN1), the first group having a larger head length and smaller prepectoral fin length than the latter (Table 2). In Busan, most individuals overlapped with populations of Incheon, Boryeong, and Heuksando, and 3 specimens overlapped with Pohang. There is more overlap among populations on CNA2, however, Pohang distinct from Goseong except for two specimens of Goseong based on loadings on CAN2 (Fig. 4). Pohang having a larger preanus length than the rest of the populations.

Table 2. Comparison of counts and measurements of *Hemitripterus villosus* among six localities.

	Incheon	Boryeong	Heuksando	Busan	Pohang	Goseong
Number of specimens	40	12	13	25	28	29
Total length (TL)	213.5-290.6 (253.6)	189.3-319.5 (252.9)	189.3-319.5 (252.8)	212.2-320.3 (264.4)	191.5-300.3 (231.2)	165.8-322.4 (248.1)
Standard length (SL)	168.6-232.6 (193.6)	149.2-257.0 (202.0)	149.2-252.9 (201.6)	166.7-252.1 (205.6)	151.4-237.4 (183.3)	151.8-252.2 (196.4)
Count						
1st dorsal fin spines	XVI -XVIII	XVII-XVIII	XVII-XVIII	XVI-XVII	XVI-XVIII	XVII-XVIII
2nd dorsal fin rays	10-12	11-12	11-13	11-13	11-12	11-13
Anal fin rays	13-15	13-14	13-15	12-15	12-14	12-15
Pectoral fin rays	18-19	18-19	18-20	18-20	18-20	18-20
Pelvic fin spines and rays	I, 3	I, 3	I, 3	I, 3	I, 3	I, 3
Vertebrae	37-40	38-39	38-39	38-40	37-39	39-41
In %of SL						
Preanus length	49.8-58.1 (54.4)	49.3-72.2 (57.5)	44.6-60.5 (51.8)	47.8-60.5 (52.9)	49.5-65.8 (59.1)	40.4-59.1 (52.4)
Body depth	27.7-37.6 (33.2)	26.3-38.4 (32.2)	24.5-35.1 (28.5)	27.0-37.4 (32.6)	27.0-35.7(31.9)	23.1-34.8 (29.9)
Body width	23.1-30.4 (26.0)	21.0-35.1 (24.5)	22.9-29.0 (25.6)	22.3-34.9 (27.8)	23.4-29.1 (26.0)	18.4-31.3 (26.5)
Head length	35.7-42.3 (38.4)	36.0-43.1 (38.5)	34.6-41.0 (38.5)	35.8-41.3 (38.4)	36.7-40.7 (38.3)	31.6-40.6 (38.0)
Head width	26.1-32.2 (29.0)	25.5-31.8 (28.2)	25.3-31.5 (28.3)	27.4-35.9 (31.9)	26.5-34.2 (29.2)	24.4-33.9 (30.3)
Snout length	8.4-10.0 (9.2)	8.9-11.1 (9.6)	8.7-10.4 (9.5)	8.8-10.5 (9.6)	9.0-11.8 (9.9)	8.5-11.4 (10.2)
Orbit diameter	6.7-7.7 (7.1)	6.8-9.7 (7.9)	6.2-8.3 (7.1)	6.3-7.5 (7.0)	6.4-7.5 (6.8)	5.6-7.4 (6.6)
Postorbital length	21.0-24.1 (22.1)	20.6-24.7 (22.6)	20.2-26.5 (23.1)	21.5-24.8 (22.9)	21.4-25.2 (23.2)	18.8-25.0 (22.7)
Interorbital width	10.0-11.5 (10.5)	10.6-16.1 (12.0)	10.2-12.3 (11.1)	10.0-11.6 (10.8)	10.2-12.9 (11.3)	9.2-12.7 (11.0)

Values of mean in parentheses.

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Table 2. Continued.

	Incheon	Boryeong	Heuksando	Busan	Pohang	Goseong
In %of SL						
Upper jaw length	19.6-22.7 (21.2)	20.8-24.2 (21.9)	19.8-22.7 (21.6)	18.7-24.1 (22.4)	21.1-23.8 (22.3)	19.4-24.0 (22.5)
Lower jaw length	21.8-25.4 (23.7)	23.5-27.8 (24.8)	22.8-25.5 (24.2)	23.3-26.8 (25.2)	23.2-26.9 (24.6)	21.0-26.5 (25.0)
Predorsal fin length	21.1-25.0 (23.3)	22.6-27.8 (24.9)	22.9-26.4 (24.4)	22.6-25.6 (24.2)	23.0-27.8 (24.7)	20.0-26.1 (23.9)
Presecond dorsal fin length	67.3-73.8 (69.9)	66.5-81.8 (70.4)	66.9-72.4 (69.8)	66.8-72.9 (70.0)	67.7-76.4 (70.6)	56.4-71.4 (68.7)
Preanal fin length	64.5-73.5 (69.3)	63.4-81.4 (69.1)	61.7-70.4 (67.5)	64.4-73.2 (69.2)	66.2-74.0 (70.5)	54.0-73.7 (67.7)
Prepectoral fin length	35.3-39.6 (37.4)	36.1-39.7 (38.0)	36.0-39.9 (38.5)	34.1-41.3 (38.1)	36.5-41.9 (39.3)	32.0-41.1 (39.1)
Prepelvic fin length	27.9-34.9 (31.8)	28.9-38.6 (33.6)	29.8-36.2 (32.8)	28.2-37.1 (31.0)	28.8-38.9 (33.3)	24.4-36.9 (32.2)
First dorsal fin height	12.0-19.7 (17.2)	15.2-20.7 (18.1)	16.2-22.7 (18.3)	15.4-22.1 (18.6)	15.3-24.5 (19.0)	13.5-22.6 (18.0)
First dorsal fin base length	43.0-49.4 (46.2)	42.0-52.7 (46.6)	41.9-49.1 (45.3)	42.1-50.9 (47.3)	42.2-49.1 (46.4)	38.2-49.8 (46.2)
Second dorsal fin base length	21.0-27.7 (24.2)	20.9-28.2 (24.7)	21.1-24.7 (22.7)	22.7-26.8 (24.5)	19.8-24.5 (22.7)	15.5-25.5 (20.1)
Anal fin base length	25.9-32.9 (29.2)	25.9-31.8 (28.9)	26.1-29.9 (27.7)	24.6-31.9 (29.4)	24.8-29.8 (26.9)	25.0-32.1 (28.5)
Pectoral fin length	23.9-29.4 (26.6)	23.8-30.4 (26.8)	25.1-29.6 (27.4)	25.3-29.0 (27.0)	22.8-27.9 (25.3)	23.5-29.4 (26.1)
Pelvic fin length	15.5-20.4 (17.5)	15.3-19.8 (17.4)	14.1-18.4 (16.3)	16.7-20.5 (18.6)	13.1-18.4 (16.1)	12.8-18.3 (15.9)
Caudal peduncle length	6.4-9.4 (8.0)	7.5-12.1 (9.2)	7.5-10.2 (8.7)	7.2-9.9 (8.3)	5.6-10.1 (8.0)	6.2-10.0 (8.2)
Caudal peduncle depth	8.1-9.5 (9.0)	8.0-9.4 (8.5)	7.4-8.7 (8.3)	7.4-10.0 (9.0)	8.5-9.6 (8.9)	6.3-9.4 (8.2)
Caudal peduncle width	3.9-6.4 (5.3)	3.9-5.9 (4.8)	3.9-5.9 (4.8)	4.0-7.0 (5.3)	4.3-5.9 (5.3)	3.9-6.5 (5.3)

Values of mean in parentheses.

Table 3. Frequency distributions of counts of *Hemitripterus villosus* among six localities.

Locality	N	1st dorsal fin spines			2nd dorsal fin rays				Anal fin rays				Pectoral fin rays			Vertebrae				
		XVI	XVII	XVIII	10	11	12	13	12	13	14	15	18	19	20	37	38	39	40	41
Incheon	40	3	30	7	1	18	21			7	30	3	3	37		2	13	23	2	
Boryeong	12		8	4		3	9			4	8		2	10			3	9		
Heuksando	13		10	3		9	3	1		1	10	2	2	10	1		4	9		
Busan	25	4	21			13	11	1	2	3	18	2	8	13	4		13	11	1	
Pohang	28	11	15	2	1	16	10	1	2	16	10		11	16	1	3	10	15		
Goseong	29	1	20	8		9	17	3	1	5	16	7	4	21	4			10	16	2

Table 4. Kruskal-Wallis test for counts of *Hemitripterus villosus* among six localities.

Locality	1st dorsal fin spines	2nd dorsal fin rays	Anal fin rays	Pectoral fin rays	Vertebrae
Incheon	17.1^{a}	11.5^{a}	13.9^{a}	18.9^{a}	38.6^{a}
Boryeong	17.3^{ac}	11.8^{a}	13.7^{ac}	18.8^{a}	38.8^{a}
Heuksando	17.2^{ace}	11.4^{a}	14.1^{acd}	18.9^{a}	38.7^{a}
Busan	16.8^{bdeg}	11.5^{a}	13.8^{acdf}	18.8^{a}	38.5^{a}
Pohang	16.7^{bdfgi}	11.4^{a}	13.3^{bcegh}	18.6^{a}	38.4^{a}
Goseong	17.2^{acehj}	11.8^{a}	14.0^{acdfi}	19.0^{a}	39.7^{b}

Values and different superscript letters indicate means and significant difference ($P<0.05$) among six localities, respectively.

Table 5. The result of one-way ANOVA with Tukey's test for morphometric characters among six localities.

	Incheon	Boryeong	Heuksando	Busan	Pohang	Goseong
Mean standard length (mm)	199.7	156.5	201.5	205.6	183.3	196.4
In % of SL						
Preanus length	54.4^{a}	57.6^{ac}	51.7^{ade}	52.9^{adeg}	59.1^{bcfhi}	52.8^{adegj}
Body depth	33.2^{a}	32.2^{ac}	28.4^{bde}	32.6^{acfg}	31.9^{acfgi}	29.9^{bcehj}
Body width	26.0^{a}	24.5^{ab}	25.5^{abd}	27.8^{acde}	26.0^{abdef}	26.5^{abdef}
Head length	38.4^{a}	38.5^{ac}	38.5^{ace}	38.4^{aceg}	38.3^{bcehi}	38.0^{bdfhi}
Head width	29.0^{a}	28.2^{ac}	28.3^{ace}	31.9^{bdfg}	29.2^{acehi}	30.3^{adfhi}
Snout length	9.2^{a}	9.6^{ac}	9.5^{ace}	9.6^{aceg}	9.9^{bcegi}	10.2^{bdfhi}
Orbit diameter	7.1^{a}	7.9^{bc}	7.0^{ade}	7.0^{adeg}	6.8^{adegi}	6.6^{bdfhi}
Postorbital length	22.1^{a}	22.6^{ac}	25.0^{bde}	22.9^{acfg}	23.2^{acegh}	22.6^{acfgh}
Interorbital width	10.5^{a}	12.0^{bc}	11.1^{ace}	10.8^{adef}	11.3^{bcefg}	11.0^{adefg}
Upper jaw length	21.2^{a}	21.9^{ac}	21.6^{acd}	22.4^{bcdf}	22.3^{bcdfg}	22.5^{bcefg}
Lower jaw length	23.7^{a}	24.8^{bc}	24.3^{acd}	25.2^{bcef}	23.6^{bcdfg}	25.0^{bcdfg}
Predorsal fin length	23.3^{a}	24.9^{bc}	24.4^{bcd}	24.2^{bcde}	24.7^{bcdef}	23.9^{acdef}
Presecond dorsal fin length	69.9^{a}	70.1^{a}	69.7^{a}	70.0^{a}	70.6^{a}	69.0^{a}
Preanal fin length	69.3^{a}	68.8^{ab}	67.4^{abc}	69.2^{abce}	70.5^{abdef}	68.2^{abceg}
Prepectoral fin length	37.4^{a}	38.0^{bc}	38.5^{bcd}	38.1^{acde}	39.3^{bcdfg}	39.1^{bcdeg}
Prepelvic fin length	31.8^{a}	33.6^{ac}	32.8^{ace}	31.0^{adef}	33.5^{bcegh}	32.2^{acefh}
First dorsal fin height	17.2^{a}	18.1^{ac}	18.4^{acd}	18.6^{bcde}	19.0^{bcdef}	18.0^{acdef}
First dorsal fin base length	46.2^{a}	46.6^{a}	45.2^{a}	47.3^{a}	46.4^{a}	46.2^{a}
Second dorsal fin base length	24.2^{a}	25.1^{ac}	22.6^{ade}	24.5^{acfg}	22.7^{bdehi}	20.1^{bdfhj}
Anal fin base length	29.2^{a}	28.9^{ac}	27.6^{bce}	29.4^{acfg}	26.9^{bdehi}	28.5^{acegj}
Pectoral fin length	26.6^{a}	26.8^{ac}	27.3^{ace}	27.0^{aceg}	25.3^{bdfhi}	26.1^{acegi}
Pelvic fin length	17.5^{a}	17.4^{ac}	16.3^{bce}	18.6^{bdfg}	16.1^{bdehi}	15.9^{bdehi}
Caudal peduncle length	8.0^{a}	9.2^{bc}	8.7^{ace}	8.3^{adef}	8.0^{adefg}	8.2^{adefg}
Caudal peduncle depth	9.0^{a}	8.5^{bc}	8.3^{bce}	9.0^{adfg}	8.9^{adfhi}	8.2^{bcegj}
Caudal peduncle width	5.3^{a}	4.8^{ac}	4.6^{bcd}	5.3^{acef}	5.3^{acefg}	5.3^{acefg}

Values and different superscript letters indicate the means and significant difference ($P<0.05$) among six localities, respectively.

Table 6. Eigenvectors for the first three principal components (PC) based on 25 morphometric characters of *Hemitripterus villosus.*

Measurements	PC1	PC2	PC3
Head length	.980	-.068	-.031
Prepectoral fin length	.954	-.154	-.061
Presecond dorsal fin length	.954	.005	-.155
Preanal fin length	.938	-.039	-.156
Lower jaw length	.928	-.154	.120
First dorsal fin base length	.920	.087	-.058
Predorsal fin length	.915	-.213	.017
Postorbital length	.896	-.207	.048
Upper jaw length	.875	-.200	.227
Prepelvic fin length	.842	-.248	-.018
Pectoral fin length	.800	.143	-.078
Anal fin length	.749	.355	-.135
Preanus length	.740	-.010	.016
Second dorsal fin base length	.714	.491	-.150
First dorsal fin height	.705	-.195	.045
Interorbital width	.699	-.447	.168
Body depth	.682	.432	.122
Orbit diameter	.664	.275	-.317
Head width	.625	.270	.489
Pelvic fin length	.499	.625	-.035
Snout length	.128	-.612	.595
Body width	.285	.286	.687
Caudal peduncle width	-.408	.342	.577
Caudal peduncle length	.042	-.279	.076
Caudal peduncle depth	-.020	.450	.431
Eigenvalues	13.540	2.455	1.910
Proportions	0.542	0.098	0.076
Cumulative values	0.542	0.640	0.716

Table. 7. Standardized canonical coefficients (CAN) based on 25 morphometric characters of *Hemitripterus villosus.*

Measurements	CAN1	CAN2	CAN3
Body depth	.478	.127	.344
Body width	.184	-.306	-.179
Least caudal peduncle depth	.265	.857	.113
Least caudal peduncle width	-.389	-.005	.048
Caudal peduncle length	.153	-.281	-.120
Head length	1.201	.059	.288
Head width	-.287	.135	.317
Interorbital width	-.071	-.278	-.073
Snout length	-.235	-.200	-.073
Upper jaw length	-.256	.180	.397
Lower jaw length	-.242	-.410	1.194
Orbit diameter	1.019	-.025	-.334
Postorbital length	-.055	-.120	.006
Predorsal fin length	-.735	.823	-.592
First dorsal fin base length	-.302	-.676	.620
Pre-second dorsal fin length	.270	1.000	-1.364
Second dorsal fin base length	.303	-.198	-.322
Preanus length	-.903	1.439	-.571
Preanal fin length	.621	-.663	1.792
Anal fin length	.049	-.244	.123
Prepectoral fin length	-2.355	.318	-.863
Pectoral fin length	.374	-.668	-.294
Pelvic fin length	.336	-.038	.402
Pre-pelvic fin length	.921	-.753	-.607
First dorsal fin height	-.101	.212	.216
Eigenvalues	4.511	1.918	1.396
Proportions	0.507	0.216	0.157
Cumulative values	0.507	0.723	0.880

Table 8. Number (and percentage) of individuals reclassified correctly into their original group by canonical discriminant function. Rows are the original group and columns are the reclassified group.

Original group	Reclassified group (percentage)						
	Re- Incheon	**Re- Boryeong**	**Re- Heuksando**	**Re- Busan**	**Re- Pohang**	**Re- Goseong**	**Total**
Incheon	34 (85.0%)	0 (0%)	2 (5%)	4 (10%)	0 (0%)	0 (0%)	40
Boryeong	1 (8.3%)	10 (83.3%)	1 (8.3%)	0 (0%)	0 (0%)	0 (0%)	12
Heuksando	0 (%)	0 (%)	13 (100.0%)	0 (0%)	(0%)	0 (0%)	13
Busan	0 (0%)	0 (0%)	0 (0%)	25 (100.0%)	(0%)	0 (0%)	25
Pohang	0 (0%)	0 (0%)	1 (3.6%)	1 (3.6%)	26 (92.9%)	0 (0%)	28
Goseong	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (3.4%)	28 (96.6%)	29

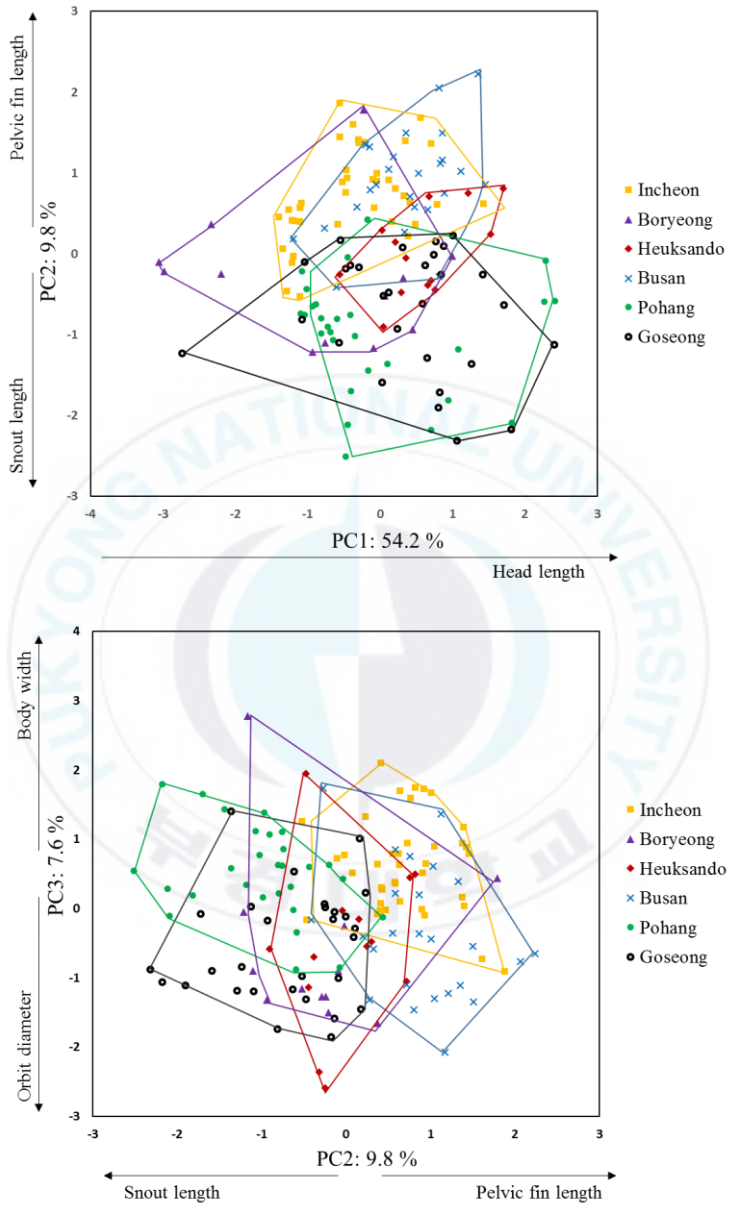

Fig. 3. Plots of principal component (PC) scores (a) PC1 versus PC2 and (b) PC2 versus PC3 based on 25 morphometric characters of *Hemitripterus villosus*.

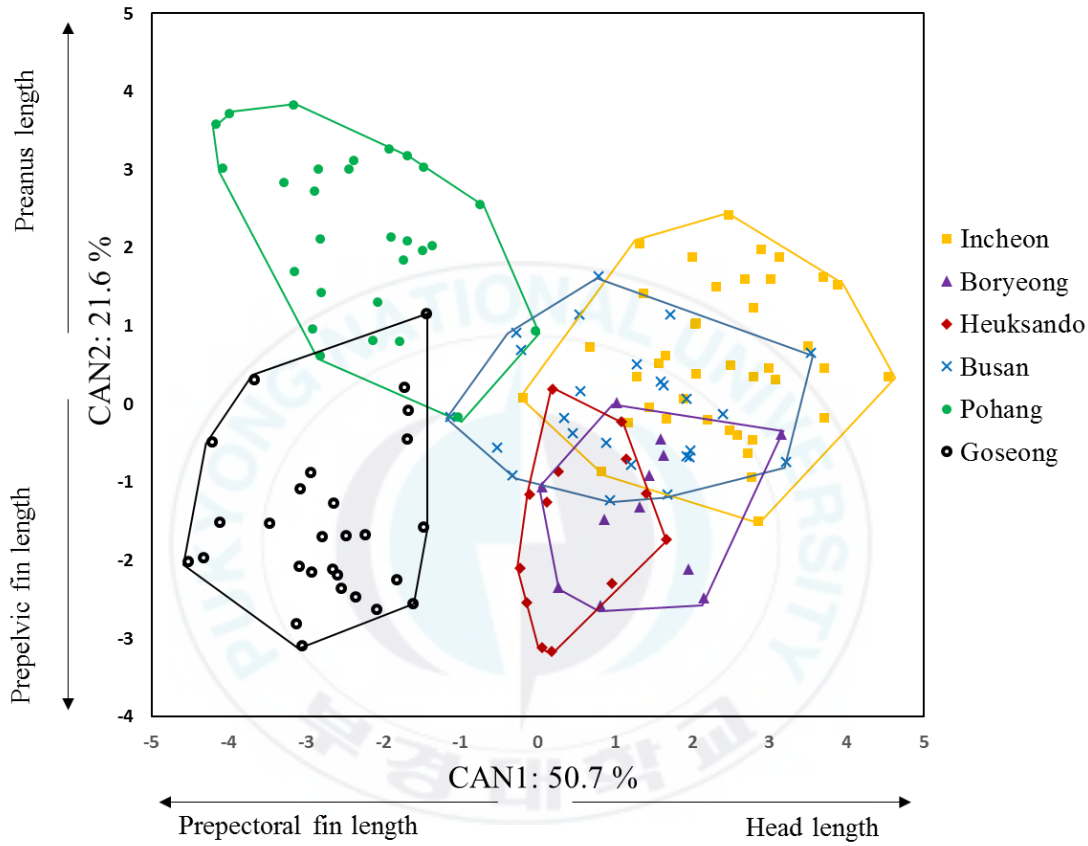

Fig. 4. Plots of discriminant scores on the first and second canonical (CAN) axes based on 25 morphometric characters of *Hemitripterus villosus*.

2. Molecular analysis

(1) Genetic variation

The analysis of 801 bp fragments of the mitochondrial cyt*b* gene in 175 *H. villosus* specimens from six local populations identified 20 haplotypes (Table 9). Within the 801 bp aligned region, a total of 23 variable sites were detected, with 18 transitions, 5 transversions. Insertions and deletions were not detected. Although most individuals have G at the locus of 327 and A at the locus of 411, three individuals of Pohang and five individuals of Goseong have an at the locus of 372 and G at the locus of 411 (Table 9). Also, most individuals have an at the locus of 489, one individual of Pohang and nine individuals of Goseong have G at the same locus (Table 9; Table 10). Low haplotype diversity (0.086±0.0563−0.303±0.1041) and low nucleotide diversity (0.000109 ±0.000224− 0.000635±0.000615) were found in Incheon, Boryeong, Heuksando, Busan, Pohang. Goseong showed high haplotype diversity (0.765±0.0526) and low nucleotide diversity (0.001647±0.001164) (Table 10).

Table 9. Variable sites of the 20 mitochondrial DNA cytochrome *b* haplotypes of *Hemitripterus villosus.*

	Position of differences																	
Haplotype	**1 0**	**1 3**	**8 7**	**9 0**	**1 7**	**3 4**	**3 5**	**3 7**	**3 7**	**4 0**	**4 1**	**4 8**	**6 0**	**6 4**	**6 5**	**7 3**	**7 4**	**7 9**
HVcytb1	A	C	G	A	C	A	T	G	A	C	A	A	A	A	A	C	C	G
HVcytb2	·	A	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
HVcytb3	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	·	·
HVcytb4	·	G	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·
HVcytb5	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
HVcytb6	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·
HVcytb7	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·
HVcytb8	·	·	·	·	·	G	·	·	·	·	·	·	·	·	·	·	·	·
HVcytb9	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·
HVcytb10	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	A
HVcytb11	·	·	·	·	·	·	·	A	·	·	G	·	·	·	·	·	·	·
HVcytb12	G	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
HVcytb13	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·
HVcytb14	·	·	·	G	·	·	·	A	·	·	G	·	·	·	·	·	·	·
HVcytb15	·	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·
HVcytb16	·	·	·	·	·	·	·	A	·	·	·	·	·	·	·	·	·	·
HVcytb17	·	·	·	·	T	·	·	·	·	·	G	·	·	·	·	·	·	·
HVcytb18	·	·	·	·	·	·	·	·	·	·	·	·	·	·	G	·	·	·
HVcytb19	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	A	·
HVcytb20	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	T	·	·

Table 10. Genetic diversity indices based on the mitochondrial DNA cytochrome *b*, and a number of individual of dominant haplotypes of *Hemitripterus villosus*.

Locality	n	N*h*	Ti/Tv	*h*	*π*	*k*	Number of individual HVcytb1 (%)	Number of individual HVcytb13 (%)
Incheon	46	3	1/1	0.086±0.0563	0.000109±0.000224	0.087143±0.161398	44 (95.7)	-
Boryeong	21	3	1/2	0.186±0.1102	0.000358±0.000442	0.286382 ±0.316965	19 (90.5)	-
Heuksando	13	2	1/0	0.154±0.1261	0.000192±0.000321	0.154166±0.228924	12 (92.3)	-
Busan	25	4	2/1	0.230±0.1095	0.000300±0.000396	0.240515±0.284963	21 (84.0)	-
Pohang	30	4	4/0	0.303±0.1041	0.000635±0.000615	0.508819±0.442985	25 (83.3)	1 (3.3)
Goseong	40	11	8/1	0.765± 0.0526	0.001647±0.001164	1.319478±0.839574	17 (42.5)	9 (22.5)

n, the number of samples; N*h*, the number of haplotypes; Ti/Tv, the number of transitions per transversions; *h*, haplotype diversity; *k*, mean pairwise differences; π, nucleotide diversity

(2) Population structure

The dominant haplotype HVcytb1was found in all the populations, which was shared by 79.43% (139/175) of individuals (Table 11). The minimum spanning network (MSN) showed a star-like with a dominant haplotype (HVcytb1) (Fig. 5A). All other haplotypes differed from HVcytb1 by only one to three mutations. Of this, 17 haplotypes were singletons and the other 2 haplotypes were shared among two different sampling sites; of these, HVcytb11 and HVcytb13 shared between the populations of Pohang and Goseong. The proportion of haplotype HVcytb1 in all the populations ranged from 42.5-95.7 % and declined along with the coast of Korea peninsula, from the Yellow Sea to the East Sea (Fig 5B). Besides the frequency of HVcytb1, there was also a geographic trend for the frequency of haplotype HVcytb13. Haplotype HVcytb13, second dominant haplotype, detected in only the populations of Pohang and Goseong. The proportion of HVcytb13 increase from Pohang to Goseong (3.3-22.5 %) (Fig 5B). The NJ tree constructed with 20 cyt*b* region haplotypes showed five haplotypes of Pohang and Goseong separated from the rest of haplotypes (Fig. 6).

Pairwise F_{ST} values between six populations ranged from -0.00851 to 0.15523, and most of the Pairwise F_{ST} values were low and not significant among the localities. However, pairwise F_{ST} values were significant between Goseong and other localities (0.10-0.16, $P<0.05$) except Pohang (0.03, $P>0.05$). Other significant differentiation occurred between Pohang and Incheon (0.06, $P<0.05$) (Table 12).

The AMOVA for two groups (category I) showed that a significant component of genetic differentiation (11.4%, $P=0.0003$) between (Incheon, Boryeong, Heuksando, Busan) group and (Pohang, Goseong) group (Table 13). An additional AMOVA for two groups (category II) also showed a significant proportion of genetic variation (18.4%, $P=0.0001$) between the (Incheon, Boryeong, Heuksando, Busan, Pohang) group and (Goseong) group, indicated more clear separation of two groups than the former. The proportion of genetic

diversity among population within groups showed negative value (-0.501), which indicated an absence of population structure or that more alleles are shared between rather than within populations (Roesti et al., 2012).

The Mantel test indicated a significant regression (R^2=0.442, P<0.05) between genetic distance [F_{ST}/(1-F_{ST})] and the geographic distance among six localities, indicating IBD, with geographic distance explaining 44% of the variation in genetic differentiation for *H. villosus* (r = 0.665; Fig. 7).

(3) Demographic history

Mismatch distributions for *H. villosus* were unimodal and well fitted the expected distribution under the sudden expansion model (Fig. 9). This interpretation was also supported by Harpending's raggedness index and the sum of squared deviations (SSD) values. (Table 14). The estimated current effective population size was higher than before the expansion except for Heuksando. Neutrality test statistics, Tajima's *D* and Fu's *F*s, for the cytochrome *b* haplotypes showed significantly negative values (*D*= -1.473~-1.733, *P*<0.05; *F*s= -1.199~-3.085, *P*<0.05) for the Incheon, Boryeong, Busan. For the Goseong, the negative value of Tajima's *D* (-1.111) was not significant (*P*>0.05), but the Fu's *F*s value was significantly negative (*F*s= -5.746, *P*<0.05) indicating that this regional group has also undergone recent population expansion. For the Heuksando and Pohang, both Tajima's *D* (-1.149~-1.28) and Fu's *F*s (-0.537~-1.184) was a negative value, but not significant (*P*>0.05) (Table 14). Therefore, the hypothesis of neutrality was rejected except for the Heuksando and Pohang. The observed values for the expansion age parameter (τ) were 2.000 for Incheon, Boryeong, Busan, and Pohang, 1.900 for Heuksando, 3.000 for Goseong. Based on the method of Rogers and Harpending (1992), population expansion for this species may occur about 43,632~93,632 years ago. Goseong characterized by larger τ and differences between θ_0 and θ_1, suggesting more stable demographic history during a recent period (Table 14).

Table 11. distribution of the mitochondrial DNA cytochrome *b* haplotype of *Hemitripterus villosus*

Haplotype	Sampling site						n	%
	IC	BR	HS	BS	PH	GS		
HVcytb1	44	19	12	22	25	17	138	79.43
HVcytb2	1	-	-	-	-	-	1	0.57
HVcytb3	1	-	-	-	-	-	1	0.57
HVcytb4	-	1	-	-	-	-	1	0.57
HVcytb5	-	1	-	-	-	-	1	0.57
HVcytb6	-	-	1	-	-	-	1	0.57
HVcytb7	-	-	-	1	-	-	1	0.57
HVcytb8	-	-	-	-	-	1	1	0.57
HVcytb9	-	-	-	1	-	-	1	0.57
HVcytb10	-	-	-	1	-	-	1	0.57
HVcytb11	-	-	-	-	3	5	8	4.57
HVcytb12	-	-	-	-	1	-	1	0.57
HVcytb13	-	-	-	-	1	9	10	5.71
HVcytb14	-	-	-	-	-	1	1	0.57
HVcytb15	-	-	-	-	-	2	2	1.14
HVcytb16	-	-	-	-	-	1	1	0.57
HVcytb17	-	-	-	-	-	1	1	0.57
HVcytb18	-	-	-	-	-	1	1	0.57
HVcytb19	-	-	-	-	-	1	1	0.57
HVcytb20	-	-	-	-	-	1	1	0.57
Total	46	21	13	25	30	40	175	100

Table 12. Pairwise F_{ST} values (below the diagonal) and associated *P*-values (above the diagonal) for the mtDNA cytochrome *b* among six localities of *Hemitripterus villosus*.

Locality	Incheon	Boryeong	Heuksando	Busan	Pohang	Goseong
Incheon		0.16503	0.39263	0.11979	0.02713	0.00000
Boryeong	0.01061		0.77517	0.45936	0.13900	0.00406
Heuksando	0.01825	-0.00851		0.61736	0.44332	0.03475
Busan	0.01005	0.00076	-0.00774		0.15800	0.00149
Pohang	0.0607*	0.02854	0.01428	0.03237		0.08227
Goseong	0.15523**	0.09978*	0.07778*	0.10889*	0.0342	

*p<0.05; **p<0.001.

Table 13. Analysis of molecular variance (AMOVA) for the mtDNA cytochrome *b* sequences among six localities of *Hemitripterus villosus*.

Source of variation	Variance	Percentage of variation	*F* statistics	*P* value
Between two groups				
I (Incheon, Boryeong, Heuksando, Busan) vs. (Pohang, Goseong)				
Among groups (F_{CT})	0.03189	11.34352	0.11344	0.00020**
Among populations within groups (F_{SC})	0.00356	1.26720	0.01429	0.05119
Within populations (F_{ST})	0.24571	87.38923	0.12611	0.00000**
II (Incheon, Boryeong, Heuksando, Busan, Pohang) vs. (Goseong)				
Among groups (F_{CT})	0.05502	18.38784	0.18388	0.00089**
Among populations within groups (F_{SC})	-0.0015	-0.50087	-0.00614	0.00505*
Within populations (F_{ST})	0.24571	82.11303	0.17887	0.00000**

*p<0.05; **p<0.001.

Table 14. Results of the mismatch analysis and neutrality tests of *Hemitripterus villosus*.

Locality	τ	θ_0	θ_1	Raggedness index	SSD (*P*)	Tajima's *D* (*P*)	Fu's *Fs* (*P*)
Incheon	2.000	0	0.097	0.69428 (0.77410)	0.00006 (0.275)	-1.473 (0.028)*	-2.918 (0.003)*
Boryeong	2.000	0	0.203	0.53127 (0.65570)	0.00723 (0.280)	-1.727 (0.018)*	-1.199 (0.046)*
Heuksando	1.900	0.450	0.450	0.50296 (0.42080)	0.02811 (0.183)	-1.149 (0.163)	-0.537 (0.119)
Busan	2.000	0	0.450	0.34670 (0.63330)	0.00661 (0.448)	-1.886 (0.015)*	-3.085 (0.001)*
Pohang	2.000	0	0.432	0.36793 (0.51500)	0.02155 (0.261)	-1.282 (0.080)	-1.184 (0.191)
Goseong	3.000	0	99999.000	0.06404 (0.40010)	0.00184 (0.625)	-1.111 (0.142)	-5.747 (0.002)*

τ, expansion parameter; θ_0, mutation parameter before expansion; θ_1, mutation parameter after expansion; SSD, sum of squared deviations, observing a less good fit between the model and the observed distribution by chance.
*$p<0.05$

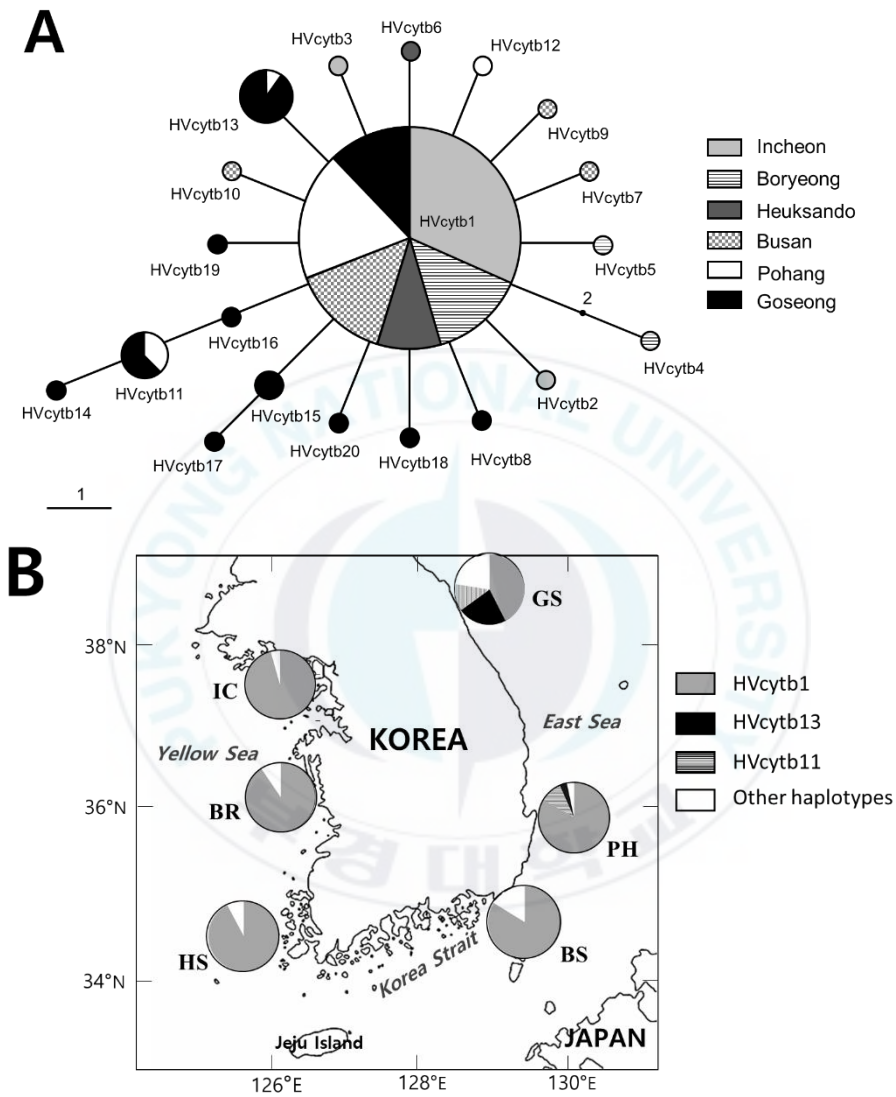

Fig. 5. A single minimum spanning network (A) and percentages for HVCytb1, HVCytb 11, HVCytb13 and other haplotypes (B) for populations of *Hemitripterus villosus*. Circle size reflects haplotype abundances (A). The length of the line between the haplotypes indicates the number of nucleotide substitutions. Bars reflect a 1-nucleotide difference (A).

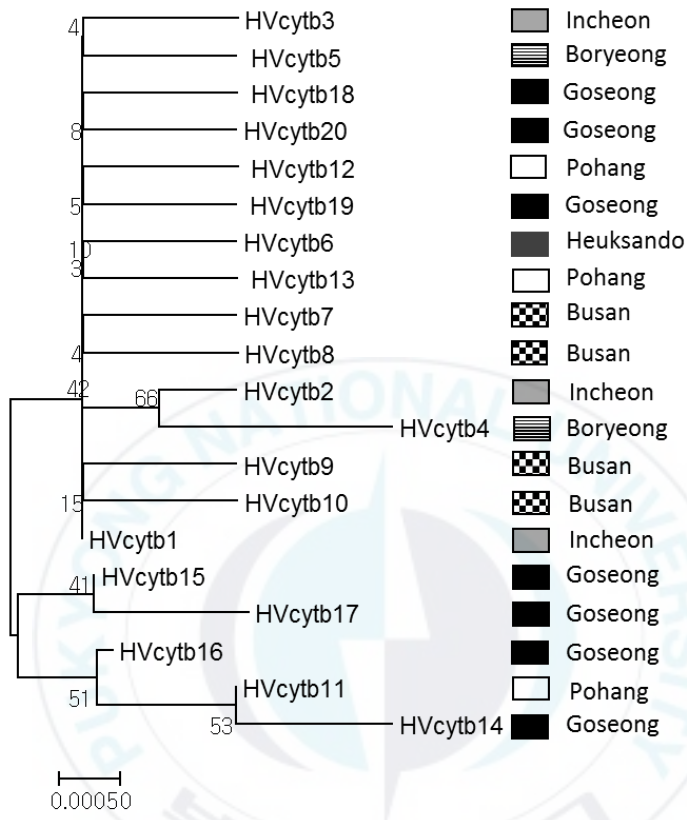

Fig. 6. Neighbor-joining tree based on genetic distance estimated from 20 mitochondrial DNA cytochrome *b* haplotypes of *Hemitripterus villosus*. Numbers at branches indicate bootstrap probabilities in 1000 bootstrap replications. Bar indicates 0.0005 of Tamura-Nei model (1993) genetic distance.

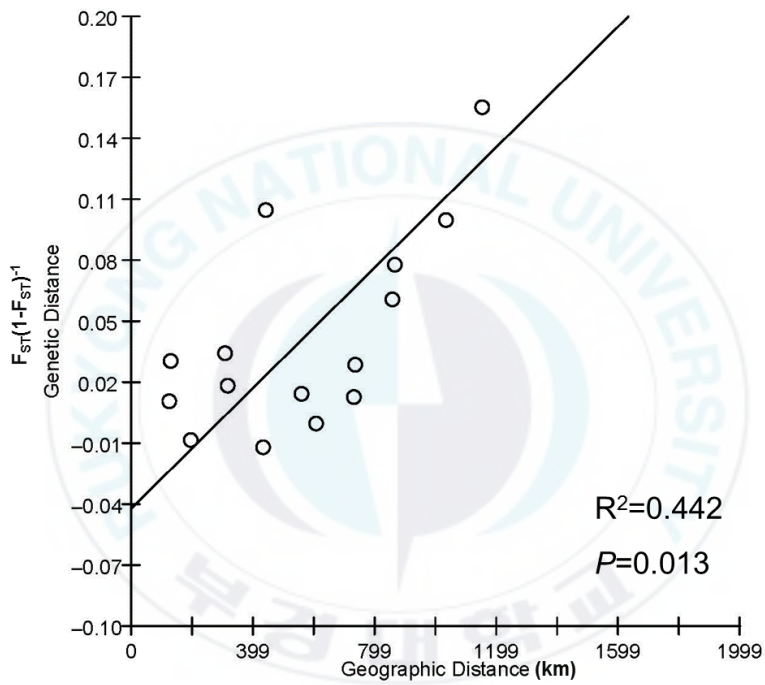

Fig. 7. Correlation between the geographic distance (km) and genetic distance [$F_{ST}/(1-F_{ST})$] among different populations of the *Hemitripterus villosus* among six localities.

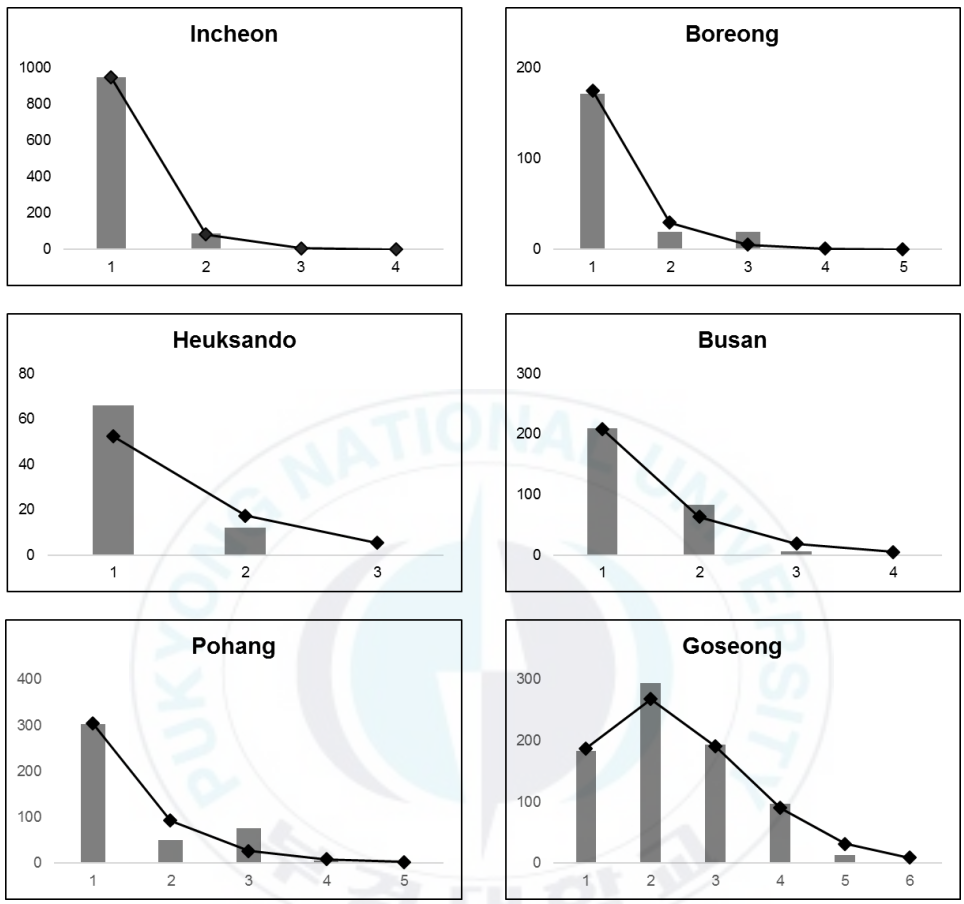

Fig. 8. Mismatch distributions from the mitochondrial DNA cytochrome *b* sequences of *Hemitripterus villosus* from six localities. Bar: observed distributions; Line: expected distributions from the sudden expansion mode

IV. Discussion

1. Morphological analysis

Hemitripterus villosus is demersal fish species, whose body color is somewhat different depending on the habitat environment (Kim et al., 2005). *Hemitripterus villosus* is an ambush predator well disguised among stones and vegetation due to a variegated body pattern and numerous outgrowths on the head (Tokranov, 1992; Novikov et al., 2002). Similar habit also showed in devil stinger, *Inimicus japonicus*. The body color of this species varied depending on the bottom environment; individuals of inhabiting coastal water showed dark brown or yellowish-brown, on the other hand, individuals of inhabiting deep rock bottom showed reddish-brown or yellow (Kim et al., 2005). Therefore, body coloration variation in *H. villosus* from Korea is presumed to individual variation associated with the habitat environment.

The results of PCA showed that extensive overlap between the morphologies of the six localities, however, plots of scores on PC2 and PC3 showed slight separation between (Incheon) and (Pohang, Goseong) (Fig. 2). (Fig. 3). Such a slight separation may be due to the minute difference in pelvic fin length among localities (Table 2). However, the CDA showed that two groups were clearly separated by the center value (‘0’) on CAN1 (East Sea vs. the Yellow Sea + Korea Strait) (Fig. 4). The main characteristics separating the two groups are the head length and pre pectoral fin length.

In this way, if shape differences in different localities of the same species can be used to discriminate morphotypes, they may also be useful in examining the stock structure within a morphotype (Joseph and Jayasankar, 2001). Similarly, with *H. villosus* in the present study, *Ammodytes personatus* from Korea is divided into two morphotypes with different geographic distributions (the East Sea vs. the Yellow Sea + Korea Strait; Kim et al., 2008).

Myoung and Kim (2016) investigated morphological variation of *Konosirus punctatus* of Korea, and detected this species divided into two morphotypes with the intermediate forms occurred located in the eastern Korea Strait (Busan), proposed that further studies are required to clarify whether secondary contact has occurred between the two lineages in Busan (Myoung and Kim, 2016). *Trachurus trachurus* from the Mediterranean Sea and the Atlantic Sea was divided into six morphotypes with different geographic distributions (North Sea, Western Atlantic, Southern Atlantic, Western Mediterranean, Central Mediterranean, Eastern Mediterranean) (Murta et al., 2008). *Scomber scomber* from Mediterranean Seas was divided into two morphotypes with different geographic distributions [(northern Mediterranean Sea) vs. (Aegean Sea, Marmara Sea, Black Sea)] (Erguden et al., 2009). Based on these result, the percentage of correctly classified was obtained 92.5 % by canonical discriminant analysis (CDA). Murta (2000) showed that *Trachurus trachurus*, a clear distinction between Cadiz (97 %) and all other groups based on the morphometric data. Our result of CDA was smaller than that of Murta (*T. trachurus*), but it was similar to that of Silva (2003), who mentioned that *Sardina pilchardus* correctly classified was 87 % (the northern Atlantic-Mediterranean) and 86% (the southern Iberia-Morocco) between two groups (Table 8). Silva (2003) suggested that discrimination of the two morphotypes was confirmed statistically by the high percentage of correct classification (>85 %) of new fish. Thus, *H. villosus* from Korea waters shows two distinct morphotypes, according to the criteria of Silva (2003).

It is known that both the environmental factors (e.g. water temperature, salinity, radiation, dissolved oxygen, water depth, and ocean currents) and the genetic traits influence morphological characters (Hubbs, 1922; Vladykov, 1934; Lindsey, 1988; Swain and Foote, 1999). Sometimes, variation in morphological traits may not be directly caused by genetic factors (Ihssen et al., 1981; Turan, 1999). Meristic characters, unlike the morphometric characters, become more fixed and remain preserve regardless of subsequent changes in the environment or in body size and shape (Lindsey, 1988).

A Kruskal-Wallis test showed a significant difference in the numbers of first dorsal fin spines, anal fin rays, and vertebrae by locality. In particular, individuals from Goseong showed more vertebrae than those of the rest of locality (Table 4). The vertebral counts were more highly correlated with temperature at spawning time, higher vertebral counts tend to be associated with lower water temperature (Hubbs, 1922; Lindsey, 1988). The number of vertebrae is likely to be higher in fish from more polar or cooler waters than in their relatives from tropical or warm waters (Jordan, 1892). Jordan (1892) himself called this trend the 'Law of Vertebrae', and he eventually explored the relationship between the diversity of fishes in a variety of families (Serranidae, Pleuronectidae, Scorpaenidae, Labridae and others) (Jordan, 1922). In particular, differences in vertebral number between Goseong and the rest populations of *H. villosus* may be due to differing water temperatures, the West and South Seas being significantly warmer than the north East Sea (Kim et al., 2008).

In the present study, the morphological analysis showed *H. villosus* divided into two groups (East Sea populations vs. Yellow Sea+Korea Strait populations). However, our results of the molecular analysis showed Pohang did not significantly different from the Yellow Sea+Korea Strait populations except Incheon. Grant and Utter (1984) stated that morphological data is more useful in detecting short-term, environmentally induced variation in fish. Our results support Grant and Utter's assertion.

Furthermore, *H. villosus* performs seasonal and spawning migrations within the shelf and the upper zone of the continental slope (Tokranov and Orlov, 2006). It is known that off the coasts of Primorye, *H. villosus* is always found; it leads a mainly stationary, rather inactive mode of life without considerable migrations, being distributed in the rather wide bathymetric range: 0-555 m (Novikov et al., 2002; Sokolovskii et al., 2007). Therefore, the difference of marine environment by localities and ecological traits results in a morphological difference between regional populations.

2. Molecular analysis

We analyzed 801bp of the mt DNA cyt*b* sequence in 175 individuals of the *H. villosus* collected from six localities of Korea. The NJ tree was constructed using 20 cyt*b* region haplotypes and showed five haplotypes of Pohang and Goseong were separated from the rest of haplotypes (Fig. 6). Grant and Bowen (1998) classified the genetic pattern of marine fishes into four categories based on their haplotype and nucleotide diversities of mtDNA. The genetic patterns of most *H. villosus* populations fell into Grant and Bowen's category one, that is low levels of haplotype diversity (*h*, 0.09-0.30) and extremely low levels of nucleotide diversity (π, 0.000109-0.000635), which was attributed to recent population bottleneck or founder event by single or a few mtDNA lineages. This patterns of haplotype and nucleotype diversity also observed *Merluccius paradoxus* (0.51-0.54 and 0.00014-0.00015, respectively) in the Benguela region (von der Heyden et al., 2010), *Hyporhamphus sajori* (0.14-0.43 and 0.00028-0.00183, respectively) in Korea and Japan (Yu et al., 2016). Goseong population had a different pattern, high levels of haplotype diversity (*h*, 0.76) and very low levels of nucleotide diversity (π, 0.001647), which may indicate undergone a genetic bottleneck event, followed by its sudden expansion (Table 10). This patterns of haplotype and nucleotype diversity also observed *Hexagrammos otakii* (0.96-0.97 and 0.004-0.005, respectively) in the Northwestern Pacific (Habib et al., 2011), *Scomber japonicus* (0.51-0.97 and 0.00056-0.01042, respectively) in the coast of China (Zhu et al., 2014), *Saurida elongate* (0.81-0.94 and 0.004-0.007, respectively) in the coast of China (Tu et al., 2016), and *Crystallichthys matsushimae* (0.464-0.960 and 0.050-0.383, respectively) in the coast of Japan and the Sea of Okhotsk (Tohkairin et al., 2016). Such low genetic diversity is uncommon in marine fishes and are thought to be related to dramatic historical events (von der Heyden et al., 2010).

The haplotype network showed star-like, local population shared dominant haplotype HVcytb1. But, dominant haplotype (HVcytb1) frequency changes among populations

suggest limited genetic exchange between populations of *H. villosus* (Fig. 5). These relationships were also confirmed by pairwise F_{ST}, which showed a significant difference between Goseong and the Yellow Sea + Korea Strait (0.08-0.16, $p<0.05$), except Pohang (0.03, $P>0.05$). Pohang showed a significant difference from only Incheon (0.06, $P<0.05$), differing from the morphological analysis (Table 12). According to the criteria of Wright (1978), only Goseong population shows little but significant differentiation from the Yellow Sea+Korea Strait populations. Isolation by distance (IBD) analysis showed genetic distance was somewhat related to the geographic distance (Fig. 7). However, no IBD pattern was detected within the Yellow Sea+Korea Strait group or the East Sea group, then, the overall pattern appeared to be a result of between-group genetic differentiation. To clarify the relationship between the Pohang population and the rest populations, AMOVA was conducted. The results of AMOVA for two groups showed that a significant component of genetic differentiation both category I and II, which more clear genetic variation revealed (18.4%, P=0.001) when the Goseong population and the rest populations grouped respectively (category II; Table 13). Thus, Pohang is presumed to the transitional zone between the Goseong population and the Yellow Sea+the Korea Strait populations.

The occurrence of genetic differentiation in a species indicates limited dispersal over both contemporary and historical time-scales (Palumbi, 1994). Population genetic structure is influenced by species-specific ecological requirement sand life histories and, especially the genetic differentiation in marine fishes is highly influenced by their dispersal capacity (Waples, 1987; Palumbi, 1992; Song et al., 2010). Also, ocean currents might facilitate the dispersal of larvae between local populations. In the case of *H. villosus* larvae, the habitat change seemed to be taken place from pelagic to benthic soon after transforming into this last stage (after hatching 50-60d) (Okiyama and Sando, 1976). In the study of Kim et al. (2015), *Paralichthys olivaceus* showed that genetic separation between eastern (Yangyang) and pooled western and southern wild populations in Korea, that was probably influenced

by restricted gene flow between regional populations due to the barrier effects of sea currents. Similar genetic population structure pattern also observed the Asian Shose Crab, *Hemigrapsus sanguineus* (Hong et al., 2012) and the sand lance, *Ammodytes personatus* (Kim et al., 2015). The East Sea is similar to the western boundary current, in that polar front forms at the boundary between low-temperature, low salinity waters of the northern East Sea and the high temperature, high-salinity waters of the southern East Sea (Rhein et al., 1995; Pickart et al., 1997). It is known that Jukbyeon (37°N) as oceanographic boundary that divides into middle and southern East Sea, and the results of survey of zooplankton and phytoplankton also reported community separation phenomenon due to front region and ocean currents (Park et al., 1991; Kang et al., 2002; Park, 2014). In the study of species composition and community structure of demersal fish in the middle and southern East Sea of Korea, the results showed a significant difference between the middle and southern East Sea, suggested that temperature and depth of physical environment were the two most important factors structuring demersal fish community between two regions (Park, 2014; Park et al., 2015). The Goseong population is located at the subpolar front, where Northern Korea cold current and Tsushima warm current of the East Sea meet. Thus, genetic differentiation between Goseong population and the Yellow Sea+Korea Strait populations may be explained by the effects of the cold and warm currents in the East Sea.

Individuals of Goseong population have relatively diverse haplotypes those not shared with other regions. These results may be resulted partly from a relatively stable population size prior to subsequent expansion in the East Sea. Such pattern implies that the rapid haplotype differentiations caused by population expansion, or the invasion of a small ancestral population from a neighboring area (Shields and Gust, 1995; Grant and Bowen, 1998). The invasion of a small ancestral population from a neighboring area and demographic expansion afterward under new environmental conditions has often been suggested to have an important role in population formation and/or speciation (Avise, 2000). Therefore, the further study required to clarify the genetic population structure,

analyzing specimens of northern Japan and Russia area. Although Goseong population showed relatively high genetic diversity, overall local populations showed very low genetic diversity, the differentiation period of this species short, it seems that the differentiation period of this species short, there is not enough time for mutation accumulation in mitochondrial DNA. The genetic patterns of most of Tajima's *D* and Fu's *F*s tests were all negative, indicating population size expansion and/or purifying (negative) selection. (Table 14). Also, mismatch analysis showed unimodal distributions (Fig. 8), the unimodal shape may be attributable to a recent population expansion, probably following a population bottleneck (Harpending, 1994). The expansion of *H. villosus* populations could have occurred 43,633~93,632 years ago during the late Pleistocene, according to the estimates of the τ value from the nucleotide mismatch distribution (Table 14). During late Quaternary glacial cycles, the environmental signal induced by sea level was amplified in the marginal seas of the Western Pacific, giving rise to drastic changes in areas and configurations of these seas (Wang 1999). In this time, the sea level in the Yellow Sea area is more than 130 m lower than the present, showing the shape of the land (Lambeck et al., 2002; Kitamura and Kimoto, 2006), the Korea Strait also shape of the land, as a result, the East Sea showed almost separated from the East China Sea (Wang, 1999). Such historical factor may influence current genetic diversity and geographical distribution.

In conclusion, our results revealed low but significant levels of mtDNA variation between the East Sea population and the Yellow Sea+Korea Strait populations. The goseong population showed clear differentiation between the Yellow Sea+Korea Strait populations based on both morphological and genetic analysis. Pohang is presumed to the transitional zone between Goseong population and the Yellow Sea+the Korea Strait populations. We propose that further studies are required using more sensitive DNA markers, such as microsatellite DNA, to understanding current genetic flow among populations.

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