

RESEARCH ARTICLE



A new perspective on biogeographic barrier in the flathead grey mullet (Pisces: Mugilidae) from the northwest Pacific

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Received: 16 February 2020 / Accepted: 12 May 2020
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Abstract

Background The flathead grey mullet, *Mugil cephalus* has a global distribution in tropical, subtropical and temperate seas worldwide. Previous studies proposed that globally distributed *M. cephalus* comprises at least 14 different lineages, and divided into two or three groups in the northwest Pacific. Therefore, we hypothesized that there may exist at least two lineages in Korea.

Objective The aim of this study was to determine the number of lineages of flathead grey mullet in Korea, and detail their biogeographic boundaries, and taxonomic implications using various molecular markers.

Methods Two partial mitochondrial DNA (mtDNA) gene sequences (COI and 16S rRNA) and nine microsatellite loci were analyzed in 260 individuals from ten locations.

Results Phylogenetic trees from two partial mtDNA gene sequences revealed two distinct lineages of flathead grey mullet (P distance = 0.016–0.021). Lineage 1 (L1) consisted of mullets from all locations except for the Pacific coasts of Japan, while lineage 2 (L2) consisted of mullets from the Jeju Island, Shanghai, and the Pacific coasts of Japan. The STRUCTURE analyses of msat data also revealed two distinct groups.

Conclusion This study was the first to reveal the coexistence of two lineages of flathead grey mullet in Korea. L2 was confined to the Jeju Island in Korea, implying a biogeographic boundary between the two lineages. The allopatric distribution and genetic heterogeneity between lineages may be affected by an ancient geographic isolation during the glacial period and maintained due to adaptation to oceanic characteristics. Collectively, the two lineages may be comprised of distinct species.

Keywords Biogeographic barrier · Microsatellite · mtDNA · Flathead grey mullet · *Mugil japonicus*

Introduction

The flathead grey mullet, *Mugil cephalus* Linnaeus, 1758, has a global distribution in tropical, subtropical and temperate seas worldwide (Ke et al. 2009; Whitfield et al. 2012). Despite its worldwide distribution, conserved morphology and few diagnostic morphological characteristics have led

to considerable taxonomic confusion of mullets (Heras et al. 2009, 2016; Durand et al. 2012, 2017; Durand and Borsa 2015). Globally distributed *M. cephalus* comprises at least 14 different lineages based on molecular analysis (Durand et al. 2012; Neves et al. 2020). In addition, the flathead grey mullet in the northwest Pacific was divided into two or three groups based on mtDNA and microsatellite (msat) loci (Jamandre et al. 2009; Ke et al. 2009; Liu et al. 2009; Shen et al. 2011; Sun et al. 2012; Viet Tran et al. 2017). Interestingly, three lineages in the northwest Pacific exhibited overlapped distribution range.

Analyzing the spatial distribution of lineages can provide insight into historical and current genetic connectivity patterns (Galarza et al. 2009; D'Aloia et al. 2014; Henriques et al. 2014). Generally, the population structure may be affected by various factors hindering genetic exchange between populations such as historic events, oceanic currents, upwelling, and current habitat fragmentation

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s13258-020-00942-8>) contains supplementary material, which is available to authorized users.

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(Henriques et al. 2014; Mai et al. 2014; Waldrop et al. 2016; Yang et al. 2020). For example, the strong genetic differentiation between populations of *Odontamblyopus lacepedii* in the northwest Pacific may be influenced by isolation of marginal seas and habitat fragmentation during the glacial periods, and these clades were delineated as separate species (Tang et al. 2010). Two distinct genetic lineages of grass puffer could be regarded as different species, indicating that discontinuous distribution and restricted connectivity among populations led to the evolution of distinct genetic lineages (Bae et al. 2020). Therefore, it is important to understand genetic connectivity between populations because intrinsic and extrinsic barriers could be acted as key predicting evolutionary dynamics and evolutionarily significant units for conservations (D'Aloia et al. 2014).

Previous studies suggested that the lineages of *M. cephalus* complex could be cryptic species because the lineages showed similar genetic distances between inter-intra species (Shen et al. 2011; Durand and Borsa 2015; Durand et al. 2017; Neves et al. 2020). Cryptic species and recently diverged species could be classified as a single nominal species because they are at least morphologically indistinguishable. Numerous cryptic lineages and/or newly formed (or forming) species could be found based on molecular markers (Sandoval-Castillo et al. 2004; Ceballos et al. 2012; Martinez-Takeshita et al. 2015; Neves et al. 2020). Durand and Borsa (2015) suggested that the group in the Mediterranean Sea could be designated as true *M. cephalus* because the type locality is European sea. Therefore, it will be necessary to review taxonomic status of the remaining groups of *M. cephalus*. *Mugil japonicus* Temminck and Schlegel (1845) is currently treated as a synonym of *M. cephalus* (Lee and Joo 1994; Whitfield et al. 2012; Kottelat 2013). Chyung (1977) documented that the flathead grey mullet in the Jeju Island coasts have greater body depth than specimens collected in the other Korean localities, resembling *M. japonicus*. Later, Kim (1999) also found greater body depth in flathead grey mullet from the Jeju Island (based on three specimens only), and suggested additional research using more specimens from the Jeju Island is needed to determine whether the flathead grey mullet from the Jeju Island is *M. japonicus* or not. Although Shen et al. (2011) suggested that the flathead grey mullet from Korea may comprise single lineage ("NWP1"). There is possibility for existence of at least two lineages of flathead grey mullet around Korean waters, referring to the previous study results on population genetic structure of marine fish in Korea (Kim et al. 2006; Myoung and Kim 2014; Bae et al. 2020).

Therefore, it is necessary to understand genetic diversity and connectivity among locations of flathead grey mullet around Korea, and re-examine their taxonomic status in the northwest Pacific. This study was performed to determine the population structure of flathead grey mullet around

Korea using mtDNA and microsatellites, and examine the possibility of cryptic species through historical and contemporary gene flow. In addition, this study discusses the potential of biogeographic barriers hindering the genetic exchange among flathead grey mullet in Korea.

Materials and methods

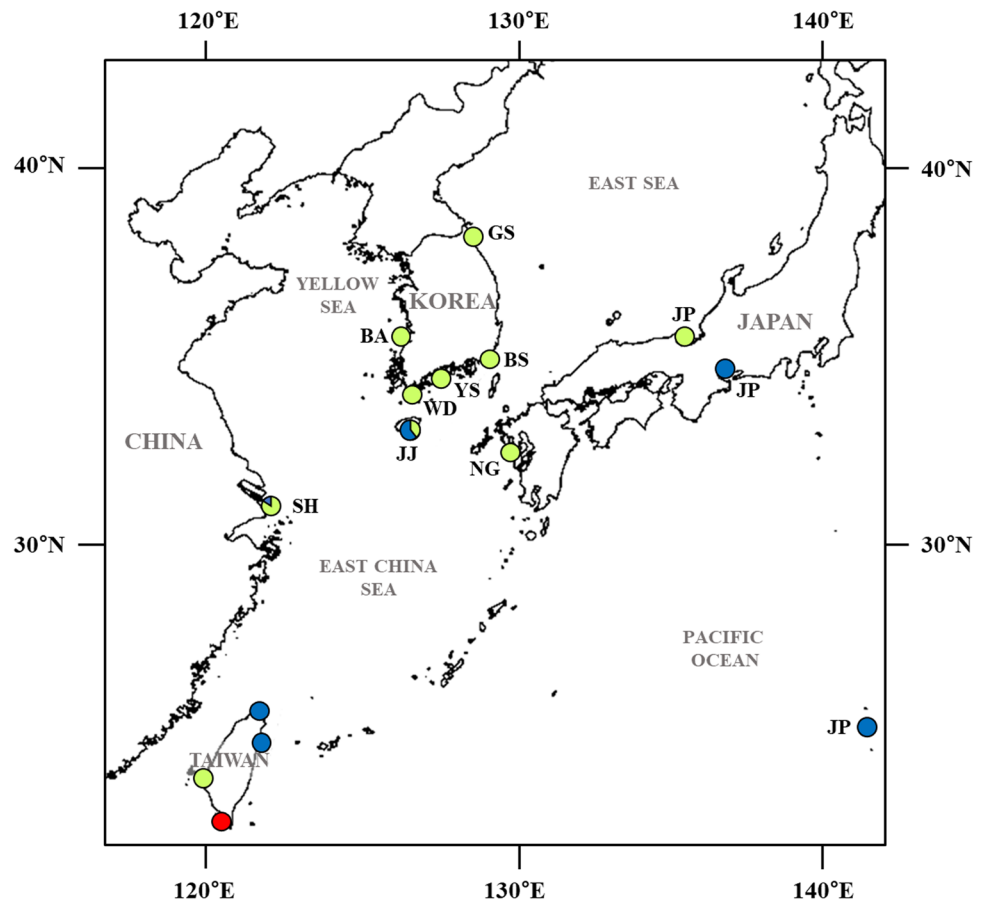
Sample collection

The specimens were collected from six locations along the Korean coasts between 2008 and 2018 (Fig. 1), and the numbers of specimens at each site are shown in Appendix 1. All Korean samples, 7 specimens of Nagasaki (type locality of *M. japonicus*) and 17 specimens of Taiwan were collected by fishing or fish market in 2016 and 2018. Specimens were preserved in 70% ethanol after fixation in 15% formaldehyde for 1 week. The specimens in this study have been deposited in the Ichthyology lab, Pukyong National University (PKU), Korea. The specimens from Shanghai in China (eight specimens) and Taiwan (six specimens; ASIZP0910468, ASIZP0914992, ASIZP7339, and ASIZP092259–2261) were obtained from only fin clips for genetic analyses.

DNA extraction, PCR sequencing and genotyping

Total DNA was extracted from muscle tissue using Chelex 100 resin (Bio-Rad, Hercules, CA). To examine genetic differentiation among locations in each site, the cytochrome oxidase subunit I (COI) and 16S ribosomal RNA (rRNA) sequences of mtDNA were amplified by PCR with universal primers. Primer sets used for amplification were: mtDNA COI primer set with VF2 (5'-TCAACCAACCACAAAGAC ATTGGCAC-3') and FishR1 (5'-TACACTTCTGGGTGG CCAAAGAATCA-3'), and 16S rRNA primer set with 16S BRH (5'-CCGGTCTAGACTCAGATCACGT-3') and 16S ARL (5'-CGCCTGTTTATCAAAAACAT-3') (Ward et al. 2005; Ivanova et al. 2007). The PCR mixture contained 2 µL of genomic DNA, 3 µL of 10× buffer, 2.4 µL of dNTPs, 1 µL of each primer, 0.1 µL of *Taq* polymerase (Biomedic, Korea), and distilled water to bring the final volume to 30 µL. PCR was performed under the following conditions for each gene: for COI sequences, initial denaturation was for 1 min at 95 °C, followed by 35 cycles of 1 min at 95 °C for denaturation, 1 min at 53 °C for annealing, and 1 min at 72 °C for extension, with a final extension at 72 °C for 5 min; for 16S rRNA sequences, the thermal profile began at 95 °C for 11 min, followed by 35 cycles of 94 °C (1 min), 53 °C (1 min), and 72 °C (1 min), with a final extension step of 5 min at 72 °C. The PCR products were purified with a Davinch PCR Purification Kit (Davinch-K, Seoul, Korea), directly sequenced with an ABI BigDye terminator cycle sequencing ready reaction kit v.3.1

Fig. 1 Sampling sites of flat-head grey mullet: *GS* Goseong, *BS* Busan, *YS* Yeosu, *WD* Wando, *BA* Buan, *JJ* Jeju-do, *SH* Shanghai, *JP* Japan, *NG* Nagasaki, *TW* Taiwan. Proportions of the lineages in eight locations. Green: lineage 1, Blue: lineage 2, Red: lineage 3 (color figure online)



(Applied Biosystems, Foster City, CA), and run on an ABI 3730xL sequencer (Applied Biosystems). The mtDNA COI and 16S rRNA sequences were aligned using Clustal W software (Thompson et al. 1994) in BioEdit version 7 (Hall 1999).

In addition, in order to screen genetic variation among 8 different locations, 9 microsatellite (msat) loci were amplified in three multiplex PCR reactions (MCE3, MCE6, MCE10; MCE2, MCE7, MCE11; MCE4, MCE8, and MCE22) designed by Andree et al. (2010) using the fluorescent labeling method. Each multiplex reaction was carried out in a 12.5 μ L of DNA template (20–50 ng), dNTP mix (each 10 mM), 10 $\times$ Buffer (15 mM MgCl₂), 5 U *Taq* polymerase, and 10 pmol each primer. The PCR conditions were as reported by Shen et al. (2011). For genotyping, PCR products were diluted with ddH₂O, mixing with GeneScan 500 LIZ Size Standard and HiDi formamide, and run on an ABI 3500xL Genetic Analyzer (Applied Biosystems).

Data analyses

Genetic diversity, phylogenetic relationship and demographic history in mtDNA sequences

The genetic distances were calculated using the Kimura-2-parameter model (Kimura 1980) in MEGA 7 software (Kumar et al. 2016). The phylogenetic tree was constructed using the neighbor-joining (NJ) method in MEGA 7 and its confidence was assessed via 1000 bootstrap replications. The sequences of lineages, suggested by Ke et al. (2009) and Durand et al. (2012, 2015), were used to confirm the lineages of flathead grey mullet in Korea: COI-JQ060540, JQ060541, JQ060553, JQ060534; 16S rRNA-JQ060778, JQ060789, JQ060801, and JQ060818. The molecular diversity indices such as haplotype diversity and nucleotide diversity, analysis of molecular variance (AMOVA), pairwise fixation index

(F_{st}) values, and Tajima's D and Fu's F_s test of neutrality were calculated in Arlequin 3.5.1.2 software (Excoffier and Lischer 2010). Historic demographic expansion was analyzed using mismatch distribution analysis in Arlequin v.3.5.1.2, and a Minimum Spanning Network (MSN) was constructed using the TCS v.1.21 program (Clement et al. 2000). The genetic barriers associated with each geographical location were investigated using pairwise F_{st} values in BARRIER version 2.2 software (Manni et al. 2004).

Genetic diversity, population structure and gene flow in microsatellites loci

For genetic diversities, the number of alleles (N_a), observed heterozygosity (H_o), expected heterozygosity (H_e) and F_{st} were calculated using Arlequin ver. 3.5.1.2. Polymorphic information contents (PIC) was calculated from Cervus 3.0 software. STRUCTURE v.2.3.4 was used to determine the probability that each of the individuals belonged to a distinct population. Ten replicates were performed with the following parameters: the number of groups (cluster, K) varied from 1 to 15 with a burn-in period of 100,000 and Markov chain Monte Carlo (MCMC) of 1,000,000. To infer the most likely number of genetic clusters in the data set, K values were calculated using STRUCTURE HARVESTER software (Earl and von Holt 2012). BAYESASS 1.3 software was used to estimate the recent migration rates and directions of migration between locations (Wilson and Rannala 2003).

Results

Genetic diversity, phylogenetic relationship and demography history (mtDNA)

Concatenated mtDNA of 1096 base pair (bp) (COI 560 bp + 16S rRNA 536 bp) was amplified to compare ten locations. A total of 40 haplotypes were found; among them, haplotype 1 (H1) was predominant, followed by haplotype 21 (H21) (Fig. 2). H1 was shared by all locations except Japan, but H21 was shared by only the Jeju Island, Japan and Taiwan. Haplotype diversity ranged from 0.9000 (Japan) to 0.2452 (Buan), and among Korean locations, Jeju was the highest haplotype diversity ($h=0.7496$). The nucleotide diversity varied from 0.0104 (Jeju Island) to 0.0002 (Goseong). Among Korean locations, haplotype diversity was highest in the Jeju Island ($h=0.7496$) but lowest in Buan ($h=0.2452$). Nucleotide diversity was highest in the Jeju Island ($\pi=0.0104$) and lowest in Goseong ($\pi=0.0002$) (Appendix 1).

An MSN based on distances among mtDNA haplotypes showed two clearly distinct lineages (Fig. 3). Lineage 1 (L1) consisted of haplotypes from all locations (H1–H20,

H29–H31, H33–H34), and lineage 2 (L2) consisted of haplotypes from the Jeju Island, Shanghai, Japan and Taiwan (H21–H28, H32, H35, H37–H40). L1 formed a star-like haplotype network containing 25 haplotypes. The neighbor joining (NJ) tree showed that the flathead grey mullet around Korea was divided into two major lineages with regard to mtDNA (Fig. 2). The P distance within each lineage was $d=0.000$ – 0.004 , but between two lineages was $d=0.016$ – 0.021 , which was supported by high bootstrap values and posterior probabilities. Consistent with the phylogeographic analysis, the existence of two lineages in flathead grey mullet around Korea was supported by NJ and MSN, and this result indicated that all locations contained L1, but L2 was found only in the Jeju Island of Korea as well as Shanghai and off the Pacific coasts of Japan. Especially, Taiwan include all three lineages (Fig. 1).

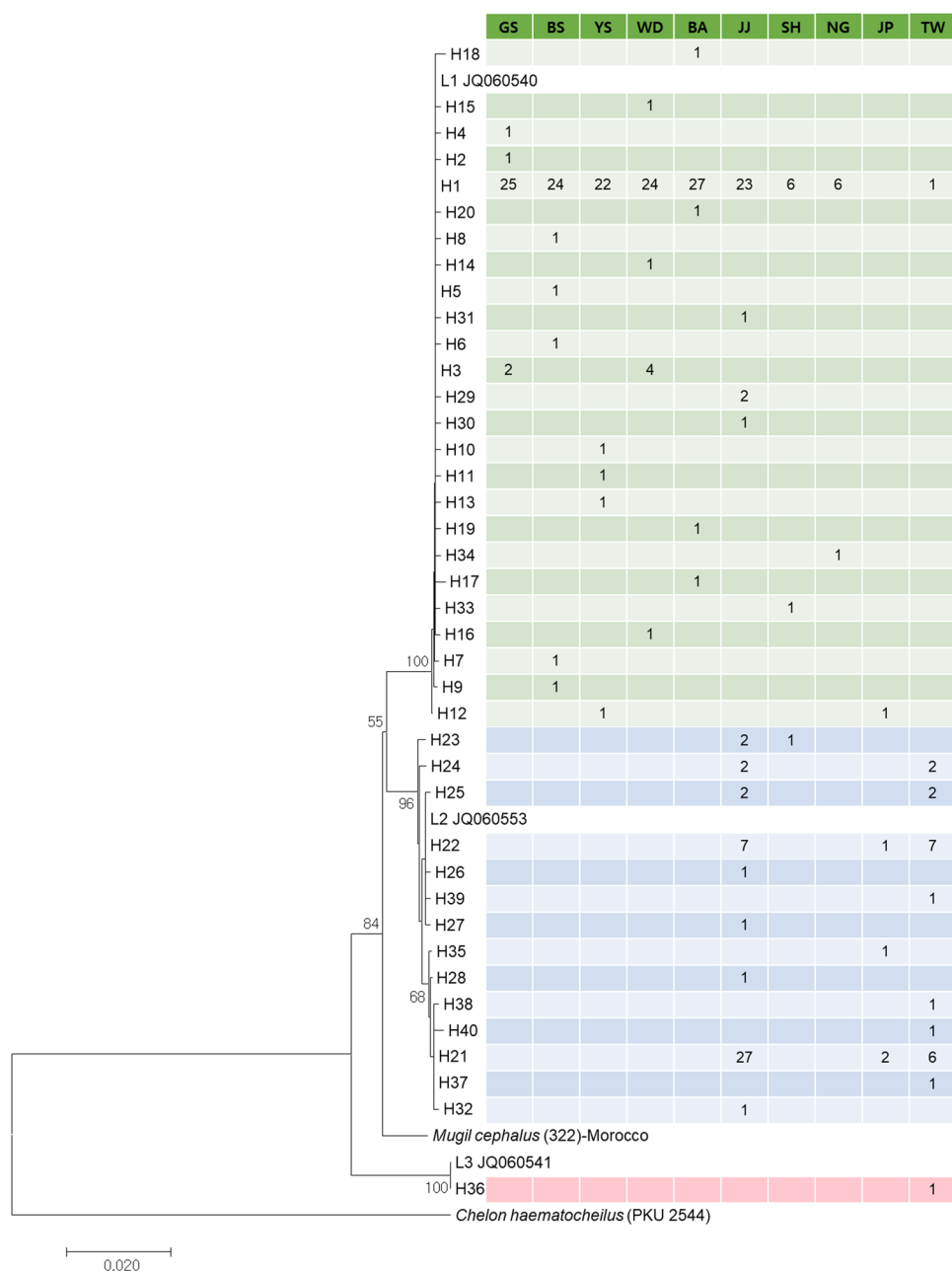
Comparison of pairwise F_{st} between the Jeju Island and other locations in Korea showed a range between 0.48608 and 0.50031, which indicated significant differentiation ($P<0.01$). In addition, pairwise F_{st} comparison of Japan, Taiwan and Jeju Island showed significant differentiations from most locations ($P<0.01$) (Table 1). Based on F_{st} values, there may exist three barriers between particular populations; the first barrier may be located between Pacific side of Japan and three other locations (Goseong and Busan in Korea; Nagasaki in Japan), and the second may exist between Shanghai and Taiwan. The last barrier may be located between the Jeju Island and four other locations (Yeosu and Wando in Korea; Shanghai in China; Nagasaki in Japan). These barriers were supported by 90%, 68% and 50% of genes, respectively (Appendix 2).

The tau value (τ) provides a rough estimate of the time at which rapid population expansion began. Although L1 and L2 had the similar τ value (3.0000 in L1 and 3.0234 in L2), the expansion value (θ_1) in L2 (3.3521) was higher than L1 (0.4729) (Table 2). The mismatch distribution was unimodal in lineage 1, but bimodal in lineage 2 (Appendix 3). Neutrality analysis was performed to obtain more precise estimates. L1 had significant values ($P<0.01$) in both Tajima's D test. L2 showed negative values in Tajima's D test, but they didn't show statistical significance ($P<0.01$) (Table 2).

Genetic diversity, population structure and gene flow (Microsatellites)

The analysis was performed using nine microsatellites (msat) loci from four locations in Korea [Busan (BS), Yeosu (YS), Wando (WD) and the Jeju Island (JJ in adult, JJ-j in juvenile)], and three locations in China, Taiwan and Japan [Shanghai (SH), Taiwan (TW), Japan (JP) and Nagasaki (NG)]. The mean number of alleles per location_lineage ranged from 1.4 (JP_L1) to 13.0 (WD_L1). The mean observed heterozygosity (H_o) ranged from 0.444 (JP_L1) to

Fig. 2 Phylogenetic tree for mtDNA COI + 16S rRNA haplotypes of flathead grey mullet. The numbers above the branches are NJ bootstrap values and posterior probabilities for the Bayesian analysis. *Chelon haematocheilus* was used as an outgroup. Green: lineage 1, Blue: lineage 2, Red: lineage 3 (color figure online)



0.794 (SH_L1), and the mean expected heterozygosity (H_e) varied from 0.444 (JP_L1) to 0.778 (SH_L2). With regard to the mean expected heterozygosity (H_e) in Korean locations, Busan had the highest value (0.737 in BS_L1) and the Jeju Island had the lowest value (0.671 in JJ_L2). The mean PIC showed that most locations had high genetic diversity > 0.5 , with the exception of five locations (NG_L1, JP_L1, SH_L2, TW_L3 and TW_L1), which may have resulted from the small number of samples (Appendix 4). Compared to allele frequencies except for two locations (JP_L1 and SH_L2) having homozygote, two lineages have different allele compositions in two locus (Appendix 5). For example, in MCE-22, L1 have higher frequencies of 117 allele than L2,

whereas L2 have higher frequencies of 121 allele. In addition, in MCE-4, there are no-presence or lower frequencies of 187 allele in L2.

The overall AMOVA showed significant genetic structure ($F_{st} = 0.065$, $P < 0.001$). Comparison of pairwise F_{st} values indicated that L2 in the Jeju Island (JJ_L2 and JJ-j_L2) was significantly differentiated from L1 in all other locations (Table 3). This result is similar to mtDNA results. From STRUCTURE analysis, the highest LnP (D) value was achieved for $K = 2$, and there was clear delineation of population with high assignment probabilities except two locations (SH_L2 and TW_L3) due to limited number of specimens. The assignment probability of individuals in the

Fig. 3 Minimum spanning trees showing genetic relationship among COI + 16S rRNA gene haplotypes. The sizes of the circles are proportional to haplotype frequency. Thick marks on the lines joining haplotypes represent the number of nucleotide substitutions. *GS* Goseong, *BS* Busan, *YS* Yeosu, *WD* Wando, *BA* Buan, *JJ* Jeju Island, *SH* Shanghai, *JP* Japan, *NG* Nagasaki, *TW* Taiwan

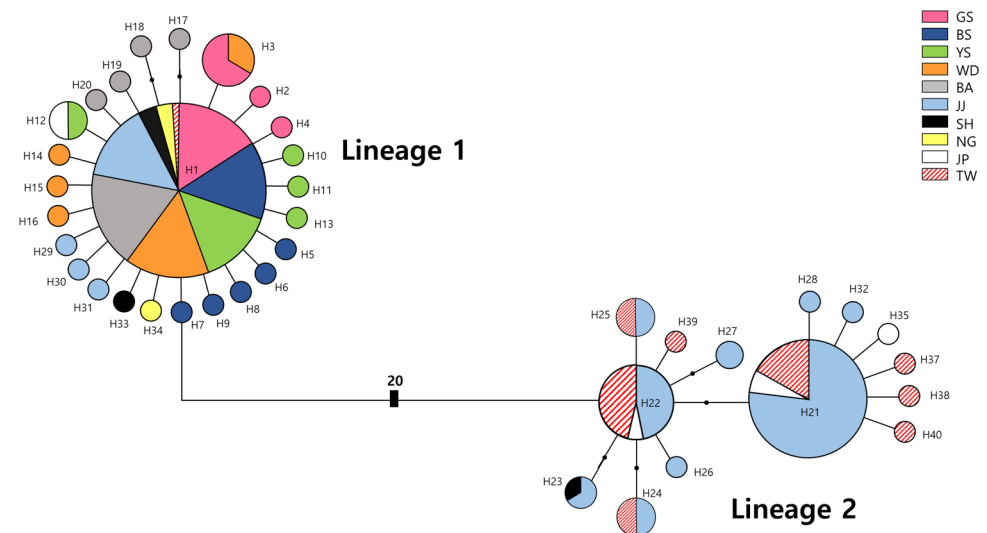


Table 1 Pairwise estimates of F_{st} (below diagonal) and P values (above diagonal) among locations of flathead grey mullet from mtDNA COI + 16S rRNA sequences

Location	GS	BS	YS	WD	BA	JJ	SH	NG	JP	TW
Goseong (GS)		0.4658	0.3066	0.6377	0.2549	0.0000*	0.0820	0.5088	0.0000*	0.0000*
Busan (BS)	0.0003		0.7070	0.0977	0.6865	0.0000*	0.1690	0.7617	0.0000*	0.0000*
Yeosu (YS)	0.0087	− 0.0002		0.0664	0.7148	0.0000*	0.1465	0.7275	0.0000*	0.0000*
Wando (WD)	− 0.0075	0.0212	0.0326		0.1309	0.0000*	0.0938	0.4629	0.0000*	0.0000*
Buan (BA)	0.0070	− 0.0001	− 0.0007	0.0308		0.0000*	0.1465	0.6865	0.0000*	0.0000*
Jeju Island (JJ)	0.4961	0.4928	0.4861	0.5003	0.5003		0.0010*	0.0010*	0.4356	0.0020*
Shanghai (SH)	0.1574	0.1393	0.1333	0.1552	0.1524	0.3059		0.8662	0.0010*	0.0000*
Nagasaki (NG)	0.0119	− 0.0100	− 0.0039	0.0121	− 0.0158	0.4155	− 0.0165		0.0020*	0.0000*
Japan (JP)	0.9089	0.9044	0.8995	0.9048	0.9068	− 0.0308	0.5776	0.7756		0.4180
Taiwan (TW)	0.8582	0.8557	0.8498	0.8590	0.8598	0.1587	0.6854	0.7755	− 0.0302	

Significant P values are indicated by * $P < 0.01$

two inferred clusters showed > 90%, respectively (Fig. 4; Table 4).

Gene flow, as determined from msat data, suggested that the flathead grey mullet from almost all locations may be non-migrants (mean non-migration rate = 0.833). Especially, Wando showed the highest non-migration rate (98.54%), and Busan had the lowest rate (67.71%). Contemporary migration rates ranged from 0.001 to 0.2843, and were typically asymmetrical. The highest levels of migration were from Wando to Busan (28.43%) and from adult in Jeju Island to juvenile in Jeju Island (28.41%) (Appendix 6). Although the Jeju Island and Wando were geographically proximate, the migration between them occurred only asymmetrical direction. This implies that the flathead grey mullet freely migrate among locations in southern coast of Korea, whereas only some individuals migrate between the Jeju Island and southern coast of Korea across the Korean/Tsushima Strait,

indicating the directional gene flow between them occurred (Fig. 5).

Discussion

Shen et al. (2011) suggested that only one lineage (“NPW1”) represents the flathead grey mullet in the East China Sea. However, this study revealed that the flathead grey mullet in Korea appeared to belong to both L1 (“NPW1” and “*Mugil* sp. C”) and L2 (“NWP2” and “*Mugil* sp. I”). Especially, L2 was distributed around the Jeju Island in Korea, and the genetic heterogeneity between the two lineages could be reflected by some combination of historical and contemporary factors. These observations raise questions about the factors influencing the relationships between the two lineages.

Table 2 Summary of molecular diversity for flathead grey mullet from mtDNA COI + 16S rRNA sequences

Lineage	N	n	h	π	k	Tajima's D		Fu's F_s		Mismatch distribution		
						D	P	F_s	P	τ	θ_0	θ_1
Lineage 1 (L1)	188	25	0.2935 ± 0.0443	0.0003 ± 0.0003	0.3383 ± 0.3377	-2.5853	0.0000	N.A.	0.0000	3.0000	0.0000	0.4279
Lineage 2 (L2)	70	14	0.7039 ± 0.0493	0.0016 ± 0.0011	1.7938 ± 1.0485	-1.5551	0.0320	N.A.	0.0120	3.0234	0.0000	3.3521

Number of individuals (n), number of haplotype (N), nucleotide diversity (π), haplotype diversity (h), nucleotide diversity (π), mean number of pairwise differences (k) for each location of samples. Tajima's D and Fu's F_s , corresponding P value, and mismatch distribution parameter estimates for each location were also indicated

Historical separation of the two lineages

Two distinct lineages of flathead grey mullet around Korean waters were identified that did not share mtDNA haplotypes, and exhibited a genetic distance of 1.6–2.1%. This indicated the significant genetic differentiation between two lineages ($F_{st} = 0.9670$, $P < 0.0001$), and inferred long-term genetic isolation. Many previous studies suggested that historical events, such as sea level changes during the Plio-Pleistocene, had impacts on the population genetic structure and genetic connectivity among marine species in the northwest Pacific (Liu et al. 2006, 2007; Shen et al. 2011; Cheng et al. 2019). During the glacial periods, the sea levels in the marginal sea of the northwest Pacific was declined at 120–140 m, and this could act as historical barrier for marine organisms (Liu et al. 2006, 2007; Gao et al. 2014; Cheng et al. 2019; Bae et al. 2020). Our study revealed three barriers around the northwest Pacific, and especially, barrier 1 (between JP in the Pacific and GS and BS in the East Sea) and 2 (between Shanghai in Yellow Sea and Taiwan in the East China Sea) were supported by high F_{st} values, and these barriers might be correlated with geographic fluctuations during the glacial periods. During the Pleistocene, the East Sea was a semi-enclosed marginal sea, and the East China Sea was reduced to a narrow Okinawa trough. In addition, the Yellow Sea and the southern coast of Korea were exposed during that time with the latter being connected to the Jeju Island by land (Liu et al. 2006; Song et al. 2010; Lee et al. 2012). The intertidal limpet (*Cellana toreuma*) from Southeast Asia and China formed well-supported phylogenetic clades with high genetic divergences that might have been affected by changes in the topography of that region during the glacial period (Wang et al. 2016). Similarly, two distinct lineages of *Coilia nasus* showed strong differences in the geographical distribution, and they might be diverged during Pleistocene low sea levels (Gao et al. 2014). These genetic and geographical/environmental differentiations could be inferred as the results of single population that remained through selection acting during glacial periods or two ancestral and divergent populations that became connected through a zone of secondary contact (Bekkevold et al. 2005; Gaggiotti et al. 2009).

Various refugia emerged during the glacial periods, and the geographical isolation might be contributed to genetic isolation and within-species divergence (Cheng et al. 2019). According to Shen et al. (2011), the NWP1 and NWP2 originated during the Pleistocene, with the East Sea and the South China Sea being the refugium for L1 and L2, respectively. A recent study, however, suggested that the L2 refugium occurred in the Okinawa trough and South China Sea, while that of L1 was in the Okinawa trough, South China Sea, and the East Sea (Viet Tran et al. 2017). Maggs et al. (2008) suggested four models to explain the population structures,

Table 3 Pairwise F_{st} (below diagonal) and associated P values (above diagonal) in each location_lineage of flathead grey mullet from microsatellites

Locality	BS_L1	YS_L1	WD_L1	JJ_L1	JJ_L2	JJ-L2	NG_L1	JP_L1	JP_L2	SH_L1	SH_L2	TW_L3	TW_L2	T2_L1
BS_L1		0.7842	0.5732	0.9707	0.0000*	0.0000*	0.0039*	0.9990	0.0000*	0.9033	0.9990	0.9990	0.0000*	0.9990
YS_L1	-0.0024		0.6250	0.8945	0.0000*	0.0000*	0.0039*	0.9990	0.0000*	0.4590	0.9990	0.9990	0.0000*	0.9990
WD_L1	0.0000	-0.0010		0.7656	0.0000*	0.0000*	0.0029*	0.9990	0.0000*	0.6152	0.9990	0.9990	0.0000*	0.9990
JJ_L1	-0.0110	-0.0086	-0.0048		0.0000*	0.0000*	0.0176	0.9990	0.0010*	0.1719	0.9990	0.9990	0.0010*	0.9990
JJ_L2	0.1147	0.1358	0.1104	0.1291		0.0127	0.0176	0.9990	0.0772	0.0000*	0.9990	0.9990	0.0127	0.9990
JJ-L2	0.0987	0.1169	0.0855	0.1055	0.0203		0.0020*	0.9990	0.8262	0.0000*	0.9990	0.9990	0.3857	0.9990
NG_L1	0.1022	0.1208	0.1132	0.1234	0.2435	0.1984		0.9990	0.0547	0.0293	0.9990	0.9990	0.0625	0.9990
JP_L1	0.0666	0.1134	0.1149	0.0969	0.2513	0.1730	0.2000		0.1875	0.1504	0.9990	0.9990	0.2041	0.9990
JP_L2	0.1025	0.1372	0.1060	0.1241	0.0311	-0.0131	0.1969	0.2054		0.0010*	0.9990	0.9990	0.6621	0.9990
SH_L1	-0.0093	-0.0003	-0.0021	0.0088	0.1369	0.1176	0.0817	0.1333	0.1376		0.9990	0.9990	0.0059*	0.9990
SH_L2	0.0096	0.0382	0.0452	0.0323	0.1042	0.0514	0.0962	0.1200	0.0826	0.0193		0.9990	0.8086	0.9990
TW_L3	0.2107	0.2402	0.2177	0.2590	0.2414	0.1949	0.3143	0.5294	0.2385	0.2351	0.2667		0.1953	0.9990
TW_L2	0.1065	0.1288	0.1094	0.1190	0.0424	0.0035	0.2008	0.2035	-0.0074	0.1366	-0.0046	0.2230		0.9990
TW_L1	-0.0189	-0.0034	-0.0148	0.0517	0.1237	0.0894	0.0797	0.2308	0.1389	-0.0067	0.0370	0.2857	0.1168	

Significant P values are indicated by * $P < 0.01$

haplotype networks, and allele/haplotype frequencies following glaciation. The phylogeographic structure of the *M. cephalus* complex was consistent with Model IV where two or more refugiums-derived populations became mixed due to secondary contact. The key genetic signature for this model was the increase in diversity in the secondary contact zone as the two refugiums merged.

The significantly negative values in neutrality test and star-like MSN for L1 suggested a recent demographic expansion. For L2, a bimodal distribution in the mismatch analyses indicated that L2 may have been dispersed and isolated upon the appearance of land bridges. This corresponded to the results of Heras et al. (2016) which suggested that two lineages have been affected by different evolutionary process. Two lineages may have been reconnected following the glacial period. The barrier surrounding the Jeju Island supported that the lineages resided in different refugiums during the glacial period. Following glaciation and reconnection of the marginal seas, the flathead grey mullet extended their range from the refugiums and experienced secondary contacts. Thus, two admixture zones may have existed in the northwest Pacific. Admixture zone I was in the South China Sea, including Vietnam, southern China, and Taiwan (all lineages), while admixture zone II ranged from Shanghai to the Pacific coasts of Japan to the Jeju Island in Korea (L1 and L2). Similarly, *C. nasus* in the East China Sea and Yellow Sea showed limited gene flow among locations, suggesting that the Yellow Sea/Yangtze River Estuary were a post-glacial contact zone between China (along the China coastline) and Japan groups (along the Korean Peninsula coastline) (Han et al. 2015). Therefore, L1 and L2 were geographically divided during the glacial periods, and sympatrically existed in various regions including the Jeju Island around Korean waters after glacial periods.

Inference of biogeographic barriers between the two lineages

Two lineages had significant differences at microsatellite loci ($F_{st} = 0.7232$, $P < 0.0001$) and different frequencies in some alleles, suggesting that little gene flow occurred between them. Such limited gene flow between lineages was supported by the low migration rates ($< 10\%$) and the high assignment rate of each population in STRUCTURE analysis ($> 90\%$). Gene flow plays a complex evolutionary role since it can either promote or constrain adaptation, and is related to various factors, such as physical or ecological barriers, unique life stages, natural selection, and the geographic distances between populations (Garant et al. 2007; Avise 2012). Three lineages of flathead grey mullet in the northwest Pacific sympatrically existed in Taiwan, Japan, and China, and the gene flow among them may be restricted by the oceanic currents and/or specific reproductive behaviors

Fig. 4 STRUCTURE analyses of flathead grey mullet in seven locations **a** estimation of the number of genetic clusters (K) using delta K as suggested by Evanno et al. (2005). **b** STRUCTURE bar plots for K = 2. Each bar plot indicated population assignment of individuals from each location, and two colored clusters were represented as lineage 1 (L1, red) and lineage 2 (L2, green), respectively. The abbreviated geographic location is given at the horizontal axis: lineage 1 of Busan (BS_L1), Wando (WD_L1), Yeosu (YS_L1), Jeju (JJ_L1), Nagasaki (NG_L1), Japan (JP_L1), Shanghai (SH_L1), and Taiwan (TW_L1); Lineage 2 of Jeju (JJ_L2), juvenile in Jeju (JJ-j_L2), Japan (JP_L2), Shanghai (SH_L2), and Taiwan (TW_L2); Lineage 3 of Taiwan (TW_L3) (color figure online)

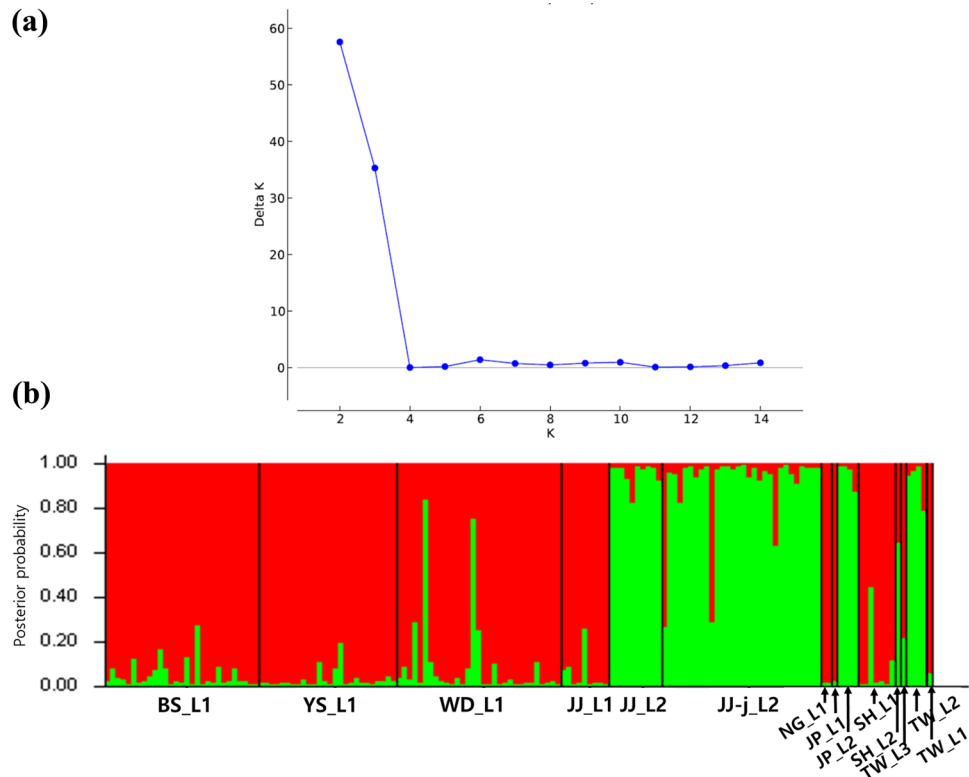


Table 4 The number of individuals in each location_lineage of flat-head grey mullet, and proportion of individuals in two clusters from microsatellites inferred by STRUCTURE analysis

Location_lineage	Inferred clusters		Number of individuals
	Cluster 1	Cluster 2	
BS_L1	0.947	0.053	29
YS_L1	0.969	0.031	26
WD_L1	0.901	0.099	31
JJ_L1	0.944	0.056	9
JJ_L2	0.044	0.956	10
JJ-j_L2	0.092	0.908	30
NG_L1	0.984	0.016	2
JP_L1	0.972	0.028	1
JP_L2	0.044	0.956	4
SH_L1	0.908	0.092	7
SH_L2	0.355	0.645	1
TW_L3	0.779	0.221	1
TW_L2	0.075	0.925	4
TW_L1	0.937	0.063	1

(Shen et al. 2011, 2015; Whitfield et al. 2012). Previous studies showed that the distribution of L1 (NWP1, *Mugil* sp. C) ranged from Russia to Vietnam, while L2 (NWP2, *Mugil* sp. I) was distributed from the Pacific coast of Japan to the Philippines. Our finding suggested that two lineages co-existed in the northwest Pacific (Korea, Japan, China,

Taiwan and Vietnam). Generally, species with overlapping, or sympatric ranges tend to have a more complex evolutionary history (Shen et al. 2015). There are various oceanic currents around the Jeju Island, such as the Jeju Warm Current, Yellow Sea Bottom Cold Water, and Korean Southern Sea Cold Water (Son et al. 2003; Han et al. 2013). Many studies suggested that strong S-shaped thermohaline fronts north-west of the Jeju Island usually block water transport from the East China Sea to either the Yellow Sea or the Tsushima/Korean Strait (Rebstock and Kang 2003; Hwang et al. 2014). Mullet larvae are passively transported by oceanic currents, and the local oceanographic conditions regarding spawning and marine boundaries can be acted as barriers to the dispersal of larvae and gene flow (Ke et al. 2009; Shen et al. 2015; Liu et al. 2019a). Therefore, the migration patterns of two lineages may be disrupted by strong biogeographic barriers, such as these oceanic fronts (Barrier A in Fig. 5).

The differences in ecological environment can be one of the main factors that drove genetic divergence and spatially limited gene flow (Hollenbeck et al. 2019; Li et al. 2019). For example, the salinity can be main factor to promote genetic variation in *Sillago japonica* populations, suggesting that the differences in genetic diversity is correlated with salinity changes (Yang et al. 2020). The diversification of the *M. cephalus* complex could be related the adaption in local environments, indicating that this species is actually ecologically specialized lineages that have colonized different regions and have been subject to different selective

Fig. 5 A map showed two barriers around the northwest Pacific: barrier A is located around Jeju Island and barrier B is located around south-west of Kyushu. The map was obtained from the online (https://upload.wikimedia.org/wikipedia/commons/d/dc/East_China_Sea_location_map_OSM.svg)



pressure (Whitfield et al. 2012; Neves et al. 2020). The distribution patterns of three lineages of the flathead grey mullet can be related to different environmental preferences and adaptation. L1 (NWP1) is dominant in marine environments, whereas L2 (NWP2) preferred to the estuaries in early life stages (Shen et al. 2011, 2015). Additionally, L1 was distributed along the Chinese Coastal Current, whereas the distribution of L2 was related to the Kuroshio Currents with high salinity and temperature (Shen et al. 2011), indicating that the asymmetric migration among populations could be barriers to gene flow. The Kuroshio Current, one of the strongest currents, can play an important role in transporting eggs, larvae or juveniles of several fish species (Liu et al. 2018, 2019b; Cheng et al. 2019). However, Liu (2013) indicated that the Taiwan Warm Current, which is branched from the Kuroshio Currents, can transport many warm-water marine species from their tropical centers to the north. That current also connects the coasts of China to Korea (Chen and Sheu 2006; Chen and Wang 2006; Song et al. 2010). The Taiwan Warm Current dominates in the summer, and spreads into the Yangtze River mouth, instead of the Kuroshio Current, which intensifies gradually during the winter (Guo et al. 2006; Zhou et al. 2015). According to Li et al. (2014), the Taiwan Warm Current helped mackerel larvae

migrate from the continental shelf in the East China Sea to the Tsushima/Korean Strait. Thus, the distribution of L2 on the Jeju Island can be related to Taiwan Warm Current instead of the Kuroshio or Tsushima Warm Currents. The adaptation of different habitats can lead to the evolution that one lineage rapidly diversified into multiple lineages which specialized into many different niches (Marques et al. 2018, 2019). The different selection pressure can affect the gradual accumulation of genetic variation between *Pampus* species, and they evolved into different valid species (Li et al. 2019). Our study found the possibility that two lineages have been adapted to different environments, and the oceanic currents have also played an important role in species dispersion and divergence of flathead grey mullet.

Taxonomic implications

Our study revealed significant differentiation in mtDNA and msat loci, indicating very restricted gene flow between two lineages. Three genetically distinct lineages are reproductively isolated, and this reproductive isolation prevented further genetic exchange between lineages, potentially leading to speciation (Ke et al. 2009; Shen et al. 2011; Durand and Borsa 2015). *Mugil liza*, which is a much debated taxon, has

been recognized as a single species based on the results of various studies of ecological, genetic, and morphological characters, but a recent study based on msat analysis indicated that *M. liza* along the Atlantic coast of South America is composed of two different clusters (Mai et al. 2014; Heras et al. 2016). Durand and Borsa (2015) reported the similarity in ratios of inter- to intra lineage nucleotide distance, and all lineages within *M. cephalus* complex may be regarded as putative, distinct species. Compared to actual *M. cephalus* in Morocco reported by Durand and Borsa (2015), L1 and L2 are distinct from actual *M. cephalus* ($d=0.017\text{--}0.021$). The genetic distances were similar between the two lineages, suggesting that the two lineages in the northwest Pacific should be distinct species that differ from *M. cephalus*.

Although many morphological characters have been proposed by many authors to reveal the taxonomic status of mugilid fishes, few characters can clearly establish the relationships among these fishes (Nirchio et al. 2005; Heras et al. 2009, 2016). This confusing taxonomy in the family Mugilidae is due to the complicated synonyms, and although the family Mugilidae includes up to 233 nominal species, only 80 have been recognized as valid species (Nirchio et al. 2005; Siccha-Ramirez et al. 2014; Froese and Pauly 2019). Among the synonyms of *M. cephalus*, the scientific name of *M. japonicus* (type locality: Nagasaki, Japan) has been used by many ichthyologists (Lee and Joo 1994; Chyung 1977; Whitfield et al. 2012; Kottelat 2013). In this study, L1 including Nagasaki individuals might be *M. japonicus*. In order to support the taxonomic status of two lineages, it is necessary for additional studies involving detailed morphological comparisons by comparing the lectotype and paralectotypes of *M. japonicus*.

In conclusion, this study revealed that two distinct lineages of flathead grey mullet exist, and especially, two lineages coexisted in Jeju Islands due to historical and biogeographical barriers. We suggest the new possibility that L1 is *M. japonicus*, which should be revived from a junior synonym of *M. cephalus*, and L2 is an undescribed species, tentatively named *Mugil* sp. (corresponding to *Mugil* sp. I in Durand and Borsa 2015).

Acknowledgements This research was supported by the Marine Biotechnology Program of the Korea Institute of Marine Science and Technology Promotion (KIMST) funded by the Ministry of Oceans and Fisheries (MOF) (no. 20170431). We are grateful to T. Nakabo (Kyoto University), Y. Kai (Kyoto University) for providing the flathead grey mullet in Japan and valuable comments for the manuscript. We also thank the Biodiversity Research Museum, Academia Sinica, Taiwan and Forestry Bureau, Council of Agriculture, Taiwan for providing samples.

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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