Assessment of fish biodiversity in four Korean rivers using environmental DNA metabarcoding

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ABSTRACT

Environmental DNA (eDNA) metabarcoding is a cost-effective novel approach to estimate the biodiversity in an ecosystem. In this study, the MiFish pipeline was employed to test if th system methodology is sufficiently reliable to estimate fish biodiversity in Korean rivers. A total of 125 unique haplotypes and 73 species were identified at the species level from 16 water samples collected from a single survey of four Korean rivers (Hyeongsan, Taehwa, Seomjin, and Nakdong). Among the four rivers, highest species richness was recorded in Seomjin river (52 species), followed by Taehwa river (42 species), and Hyeongsan river (40 species). The Nakdong river (26 species) presented the lowest values of species richness and of endemic species presumably due to its metropolitan location and anthropogenic impacts such as dams or weirs present in the river. We were also able to detect that five exotic species (*Carassius cuvieri, Cyprinus carpio, Cyprinus megalophthalmus, Lepomis macrochirus,* and *Micropterus salmoides*) are widely distributed in all surveyed rivers, a situation that might be problematic in terms of conservation. Our findings indicate that the eDNA metabarcoding technique is one of the most cost-effective scientific tools available for the management and conservation of freshwater fish resources available in Korean. However, low 12S sequences of endemic species in the database and low resolution of MiFish region for differentiating several taxa should be upgraded for their wide use.

Keywords: biodiversity, Korea, next-generation sequencing, MiFish, metabarcoding, eDNA

INTRODUCTION

Fish communities have been considered as reliable bioindicators of ecosystem status due to their vulnerability to environmental or anthropogenic stresses such as pollution, climate change, or other disturbances in habitats (Dudgeon, 2010). Traditional monitoring methods

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for fish biodiversity, which have relied on the direct capture or observations of specimens, are often costly and time-consuming due to a lack of taxonomic expertise and necessity of extensive fieldwork. Environmental DNA (eDNA) metabarcoding (detection of multispecies by using degraded DNA from environmental sample) has been proposed as an alternative strategy to analyze fish biodiversity, also demonstrating a potential to improve the traditional methods in a cost-effective way (Foote et al., 2012; Kelly et al., 2017; Kelly et al., 2014; Shaw et al., 2016; Stoeckle et al., 2017; Yamamoto et al., 2017). This technique has been shown to be sensitive as to allow the identification of rarely identified species (Pilliod et al., 2013), invasive species (Ardura et al., 2015; Cai et al., 2017; Clusa et al., 2017; Dejean et al., 2012; Klymus et al., 2017; Takahara et al., 2013; Williams et al., 2018) or migratory species (Gustavson et al., 2015; Pont et al., 2018; Yamamoto et al., 2016; Yamanaka and Minamoto, 2016).

Since eDNA metabarcoding analysis of fish biodiversity is mainly based on the amplicon of homologous genes by PCR, universal primers with high taxon-specificity and wide taxon-coverage are essential. Three fish-specific universal primer sets are currently reported, two sets for 12S rRNA regions [Eco Primers (Riaz et al., 2011) and MiFish (Miya et al., 2015b)] and one for 16S rRNA region (Shaw et al., 2016). Among them, the MiFish primer set demonstrated its reliability for eDNA metabarcoding analysis of fish biodiversity both in marine (Ushio et al., 2017; Yamamoto et al., 2017) and continental waters (Sato et al., 2018). More recently, the web-based MiFish pipeline in MitoFish was publicly open (http://mitofish.aori.u-tokyo.ac.jp/mifish/), alleviating the time-consuming bioinformatic analysis for the users (Sato et al., 2018).

Although metabarcoding analysis by the MiFish pipeline is one of the most reliable tools at the moment, numbers of MiFish sequences in the database are still one of the last hurdles to overcome for the global use of MiFish pipeline. Since the average length of the MiFish region

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is approximately 170 bp, which is much smaller than the typically used 670 bp of the COI barcodes, a high-quality database is critical for successful species assignment. Species identification by MiFish primer could not discriminate closely related species in several genera including *Sebastes* spp. and *Takifugu* spp. (Yamamoto et al., 2017). In particular, considering the tremendous diversity of freshwater fishes (Seehausen and Wagner, 2014), direct application of MiFish platform may produce a high amount of 'unidentified' records. In addition, a relatively much lower amount of MiFish sequence data (12S region) is currently deposited compared with those of COI region. Therefore, before the direct application of the MiFish pipeline, the MiFish DNA sequence data for the local freshwater species should be tested for the accurate fish biodiversity analysis using eDNA metabarcoding.

In this study, we firstly employed eDNA metabarcoding analysis of water samples collected from four rivers using the MiFish in order to improve the knowledge on freshwater fish biodiversity in Korea. After that, we analyzed the haplotypes obtained by the MiFish pipeline to assess their compatibilities in the identification of endemic species of fishes inhabiting Korean rivers. We also calculated the Shannon-Wiener (H') indices derived from the eDNA metabarcoding results to estimate fish biodiversity in four Korean rivers. Finally, the relationship between the fish assemblage according to the locations in the river was analyzed using a heat-map clustering analysis.

MATERIALS AND METHODS

Sample collection and environmental DNA extraction

The eDNA water samples were collected on June 11 and 12, 2018 from 16 stations in the Hyeongsan river, Taehwa river, Seomjin river, and Nakdong river, which are four large rivers in the southern part of the Korean peninsula (Fig.1 and Table 1). In this study, sampling stations of each river were categorized as upstream (station 1 and 2), midstream (station 3),

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and downstream (Station 4). One liter of water sample was collected at each station with disposable plastic bottles. After collecting water, the bottles were immediately stored in an icebox and were taken to the laboratory for filtration. Water temperature and salinity were measured with a conductivity meter (CD-4307SD, LUTRON). The water collected was filtered (250 ml X 4) with a 0.45 µm pore-sized GN-6 membrane (PALL Life sciences, Mexico). The filtration system was cleaned up with 10 % commercial bleach containing sodium hypochlorite to prevent cross-contamination. After filtration, the membranes were put into 2.0 ml tubes and stored at -20˚C before DNA purification.

The genomic DNA was extracted directly from the membrane filters through the DNeasy® Blood and Tissue Kit (Qiagen, Germany) according to the producer's manual. The membrane filters were cut into smaller pieces before homogenization by TissueLyser II motorized homogenizer (QIAGEN, Hilden, Germany). The extracted genomic DNA was quantified by ND-1000 NanoDrop (Thermo Scientific, Waltham, MA, USA), aliquoted, and stored at -20˚C.

Construction of library and MiSeq sequencing

In order to assess the fish biodiversity, amplicon libraries of partial 12S rRNA region by the MiFish universal primer sets were constructed (Miya et al., 2015a). The first PCR was performed to amplify MiFish regions with an overhanging linker sequence for each Nextera XT index (Illumina, USA). The PCR mixture (20 µL) contained1.0 µL of the MiFish (forward & reverse) primers (5pmol each), 2.0 µL template, 2.0 µL dNTPs (2.5mM), 2.0 µL of 10X EX Taq buffer, 0.6 µL DMSO (3 %), 0.2 µL of EXTaq Hot Start polymerase (TaKaRa Bio Inc. Japan) and 11.20 µL of ultra-pure water. The PCR reaction began with denaturation temperature at 95˚C for 3 min, followed by 30 cycles of 94˚C for 20 sec, 65˚C for 15 sec, and 72˚C for 15 sec with a final extension at 72˚C for 5 min. The amplicon with the expected size

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(250 bp~350 bp) was purified with the AccuPrep® Gel Purification Kit (Bioneer, Republic of Korea) after 1.5 % agarose gel electrophoresis. The purified amplicons were subjected to additional PCR to link each amplicon with the corresponding Nextera XT index. The second PCR mixture (20 µL) contained 5 µL template, 1 µL of a couple of index primers (10 pmol), 0.5 µL dNTPs (10 mM), 4 µL 5X Phusion HF Buffer, 8.3 µL ultrapure water, and 0.2 µL Phusion Hot Start Flex DNA polymerase (New England Biolabs, Hitchen, UK). The second PCR started at 94˚C for 5 min followed by 15 cycles of 94˚C for 30 sec, 55 ˚C for 30 sec, and 72˚C for 30 sec, and an additional 5 min at 72 ˚C. No noticeable bands were detected in the desired ranges for 16 field negative controls in the 1.5 % agarose gel electrophoresis. Consequently, the 16 negative controls were discarded from the next analysis. After gel purification, the quality and quantity of the indexed PCR products with the expected sizes were analyzed by qubit dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA, USA) followed by the sequencing using MiSeq platform (2 X 300 bp).

Bioinformatics analysis of NGS data

The MiSeq raw reads were paired by Python 2.7 software (Zhang, 2015), and the paired reads were uploaded to the MiFish pipeline (http://mitofish.aori.u-tokyo.ac.jp/mifish/) for further analysis. In the MiFish pipeline, low-quality tail of reads (QV ≤ 20) was trimmed in FASTQC. After taxonomic assignments from the MiFish pipeline, the sequences assigned to OTUs were compared with the GenBank database. If the sequence identity of the query sequence and top BLASTN hit was ≥ 99 %, then the sequence was ascertained as a particular species. If the sequence identity from 97 % to 99 %, the sequence was ascertained to the genus level, whereas sequences with 97 % to 95 % identity to the GenBank database were assigned as 'unidentified' genera. The habitat distribution of each species was assessed on the FishBase website (https://www.fishbase.org/). Alpha biodiversity was measured using the

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normalized read numbers from each sampling station of the four rivers sampled. The Shannon-Wiener (H') index indicates the heterogeneity of species or the richness of total species in an ecosystem (Gray, 2000; Magurran, 1988). The H' index and the heat map clustering analysis were enumerated with the PRIMER® software v7 (Clarke and Gorley, 2015).

RESULTS

Physico-chemical parameters

Water temperature of the sample sites ranged from 18.6 ˚C to 24.20 ˚C (Table 1). The Hyeongsan river showed the highest difference (5.4 ˚C) in temperature from upstream (HS1) to downstream (HS4), whereas lowest levels of temperature variation were observed in the Seomjin river (0.8 ˚C) and Nakdong river (1.5 ˚C). The lowest salinity (0.15 PSU) was measured at station 1 (upstream) of the Seomjin river, while the highest (20.20 PSU) was recorded at station 4 (downstream) of the Hyeongsan river. Salinity level increased from upstream to downstream in all rivers sampled, except for the Nakdong river, where an artificial dam has been constructed to block water from the ocean (Table 1).

Analysis of fish haplotypes obtained by the MiFish pipeline

The reliability of MiFish pipeline (http://mitofish.aori.u-tokyo.ac.jp/mifish/workflows/new) for biodiversity assessment of species of fishes inhabiting the sampled rivers was analyzed (Table 2). From 2,315,605 raw reads, 2,280,850 merged reads were obtained by the MiFish pipeline showing 98.50 % yields from the raw reads. A total of 238 representative haplotypes were assigned at the default cutoff sequence identity. Among the 238 haplotypes, 125 unique haplotypes were found, which were identified using the phylogenetic tree analysis in the MEGA7 software (Kumar et al., 2016) with a Maximum likelihood algorithm (Fig. 2-5). A

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total of 2,241,130 reads (98.26 %) were assigned to 73 confirmed species, 46 genera and 13 families of the Teleostei at 99 % as cutoff identity. The remaining 39,720 reads (49 haplotypes), which showed less than 99 % identity, were further assigned into 11 genera and 8 unidentified genera (Table 3). A total of 34,755 reads (1.50 %) were discarded from further analyses. The highest species number was identified in the family Cyprinidae (35), followed by Gobiidae (11), Cobitidae (8), and the remaining (19) are from other families of the Teleostei. Among them, the highest species numbers (4 species) were identified in the genus *Acheilognathus*, followed by *Carassius, Misgurnus, Tridentiger*, and *Squalidus* with 3 species in each of those genus (Table S1).

Cyprinidae

A total of 65 haplotypes was identified in the family Cyprinidae. Among the 65 haplotypes, 51 were assigned to 35 species of fishes with 99 % or a higher percentage of sequence identity to the GenBank database (Fig. 2). Two haplotypes in the genus *Hemibarbus* from the Seomjin river (SJ1) and the Nakdong river (ND2) showed 100 % and 99 % identity to the haplotype of *Hemibarbus labeo* (GenBank Number: DQ347953) and *Hemibarbus maculatus* (LC146032) sampled in Korea and Japan, respectively. Among four endemic species in the genus *Hemibarbus*, *H. labeo* and *H. longirostris* are the most widely distributed species in Korea (Lee et al., 2012). Two haplotypes identified from Seomjin river (SJ1 and SJ2) and one from Taehwa river (TH1) showed 97 % and 95 % identity to *H. longirostris* (LC049889), respectively, which suggests that those three haplotypes may be either *H. longirostris* or *H. mylodon* (Fig.2).

Five haplotypes were identified in the genus *Squalidus*. Four species of the genus are reported from Korean waters: *Squalidus gracilis*, *S. japonicus*, *S. multimaculatus*, and *S. chankaensis* (Kim and Park, 2002). Two haplotypes from the Taehwa (TH3) and Hyeongsan

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rivers (HS1) showed 100 % identity to *S. japonicas coreanus* (GenBank Number: KR075134) and *S. multimaculatus* (GenBank Number: KT948081). Another haplotype from the Hyeongsan river (HS3) showed 100% identity to a sequence of *S. japonicas* (GenBank Number: LC277782) sampled in Japan. Two haplotypes from Seomjin river showed 99 % identity to a sequence of *S. chankaensis tsuchigae* (GenBank Number: KT948082) sampled in Korea.

Fishes of the subfamily Acheilognathinae, commonly known as bitterlings, deposit eggs in the gill cavities of freshwater mussels (Kitamura, 2007; Kitamura et al., 2012). About 60 species of bitterlings are considered as valid in the genera *Acheilognathus*, *Tanakia*, and *Rhodeus* (Arai, 1988). *Acheilognathus intermedia, A. macropterus, A. majusculus, A. rhombeus, Rhodeus suigensis, R. uyekii, Tanakia somjinensis,* and *T. signifier* were herein identified with a sequence identity higher than 99 % when compared to the GenBank database. Three haplotypes from the Seomjin river showed 99 % sequence identity to haplotypes of *A. intermedia* (EF483933), *T. somjinensis* (FJ515921), and *T. signifier* (EF483930) sampled in Korea. Among them, *T. somjinensis* and *T. signifier* are endemic to Korea (Kim and Park, 2002). One haplotype from Taehwa river (TH3) showed 100 % identity to *Rhynchocypris semotilus* (KT748874) sampled in Korea. This species is currently categorized as Critically Endangered in the Red Data Book of endangered fishes in Korea (Ko et al., 2011).

Two sub-species of *Sarcocheilichthys* are known in Korea, *S. nigripinnis morii* and *S. variegates wakiyae* (Kim and Park, 2002). Two haplotypes from Seomjin river (SJ2) and Hyeongsan river (HS2) showed 100 % and 97 % sequence identity to *S. variegates wakiyae* (GenBank Number: KU301744) sampled in Korea. One haplotype from Hyeongsan river (HS2) showed 100 % and 99.43 % sequence identity to *S. soldatovi* (LC146036) and the Korean haplotype of *S. nigripinnis morii* (AP017653) sampled in Japan and Korea

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respectively. However, *S. soldatovi* is not currently reported for Korean waters. Therefore, further studies are needed to confirm the occurrence of this species in the Hyeongsan river for conservation purposes.

Gobiidae

We identified 16 haplotypes of the family Gobiidae, which represent 7 genera and 11 species (Fig. 3). Five haplotypes were identified in the genus *Tridentiger*, which represents the five known species of the genus recorded in Korea (Kim et al., 2005). One haplotype from the Taehwa river (TH4) showed a 100 % identity with *Tridentiger obscures* (GenBank Number: KT601092) sampled in Korea. One haplotype from the Hyeongsan river (HS4) showed 100 % identity to *T. trigonocephalus* (GenBank Number: LC385175) sampled in Japan and another haplotype from Seomjin river (SJ3) showed 100 % identity with *T. trigonocephalus* (GenBank Number: KM030481) sampled in Korea. According to the phylogenetic tree recovered, the *T. trigonocephalus* haplotype from the Seomjin river is different from that of the Hyeongsan river (Fig. 3). All three haplotypes of the genus *Rhinogobius* showed 100 % identity to the database. First and second haplotype showed 100 % identity to *Rhinogobius brunneus* sampled in Korea (KM030471) and Japan (LC049760), respectively. Third haplotype showed 100 % identity with *Rhinogobius giurinus* sampled in Korea (KM030475). Two haplotypes of *Gymnogobius* sp. from the Taehwa river and Hyeongsan river showed a 98 % sequence identity to *Gymnogobius taranetzi* (GenBank Number: LC385155). Nine species of the genus *Gymnogobius* are currently reported in Korea (Kim et al., 2005) and their MiFish sequences should be supplemented to the GenBank database.

Cobitidae

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Sixteen species in five genera of the family Cobitidae are currently reported from Korean rivers (Kim, 2009). A total of 18 haplotypes, which represent five genera of the family, were identified (Fig. 4). Two haplotypes in the genus *Cobitis* identified in the Seomjin river were most closely related to *C. tetralineata* (LC146139) sampled in Japan, with 100 % and 99 % identity. Two haplotypes from the Taehwa river showed 98 % and 97 % identity to *C. hankugensis* (LC146140). Two species of *Misgurnus* are reported from the Korean waters, *M. mizolepis* and *M. anguillicaudatus* (Kim, 2009). Interestingly, two phylogenetically distinct clades in *M. anguillicaudatus* were identified in the phylogenetic analysis (Fig. 4). One of them was grouped with the haplotype of *M. bipartitus* (KF562047) sampled in China, while the other one was clustered with *M. mizolepis* (AP017654) sampled in Korea. *Misgurnus bipartitus* is currently reported as endemic to China and sequence data of Korean freshwater fishes in GenBank data should be reexamined.

Two haplotypes from the Hyeongsan river (HS1; KJ699181) and the Taehwa river (TH4; KM186182) showed 100 % identity with haplotypes of *Paramisgurnus dabryanus* sampled in China (Fig. 4). This species is regarded as endemic to China, but *P. dabryanus* is often imported to Korea together with *Misgurnus anguillicaudatus* due to their phenotypic similarity. Shimizu and Takagi (2010) concluded that there are different populations of *P. dabryanus* (Shimizu and Takagi, 2010) and the two haplotypes of the species identified herein suggests that *P. dabryanus* has been imported from various locations in China. One haplotype from the Taehwa river (TH1) showed 100 % sequence identity to *Niwaella multifaciata* (EU670806) sampled in Korea, while another from the Hyeongsan river (HS1) showed lower (96 %) identity to *Niwaella* sp. Therefore, further studies should be conducted to confirm the presence of species of that genus in the Hyeongsan river.

Other families of the Teleostei

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Excluído: *M. …ipartitus* ... [18]
Excluído: …M186182) showed 100 % identity with the distantly located ... [19]
Excluído: Chinese
Excluído: *…aramisgurnus dabryanus* sampled in China (Fig. 4). This species is regarded as endemic to China, but and …. *dabryanus* is often imported to Korea together with *Misgurnus anguillicaudatus* ... [20]
Excluído: morphological …henotypic similarity. Shimizu and Takagi (2010) concluded Previous study showed ... [21]
Excluído:
Excluído: those
Excluído:
Excluído: indicated
Excluído: …hat *P. …abryanus* has been imported from various locations in China. One haplotype from the Taehwa river (TH1) showed 100 % sequence identity to the ... [22]
Excluído: Korean haplotype of
Excluído:
Excluído: So
Excluído:
Excluído: study
Excluído:
Excluído: haplotype of the

Besides the three main families of the Teleostei identified in this study, 27 additional haplotypes, representing 19 species belonging to14 genera and 11 families, were also identified in the following families: Bagridae (5 haplotypes), Mugilidae (4), Anguillidae (1), Centrarchidae (3), Channidae (1), Clupeidae (2), Odontobutidae (3), Pleuronectidae (1), Siluridae (3), Sinipercidae (3), and Amblycipitidae (1). All the haplotypes of the family Bagridae were clearly identified, and include *Pseudobargrus ussuriensis*, *P. koreanus*, *Tachysurrus nitidus*, and *T. fulvidraco* (Fig. 5). Two species of *Silurus* are currently known in Korean rivers, *S. microdorsalis*, and *S. asotus* (Park and Kim, 1994). One haplotype from the Taehwa river (TH1) showed 99 % sequence identity with *Silurus microdorsalis* (GenBank Number: KT350610) sampled in Korea, whereas another haplotype from the Seomjin river (SJ1) showed lower identity (96 %) with *Silurus microdorsalis* (KT350610).

One haplotype of the Amblycipitidae from the Seomjin river showed 97 % and 96 % identity to *Liobagrus styani* (KX096605) and *L. mediadiposalis* (KR075136), sampled in China and Korea, respectively. This result indicates that haplotypes of the family should be supplemented for their accurate identification. Three species of *Odontobutis* are currently known in Korea, *O. interrupta*, *O. platycephala*, and *O. obscura* (Kim et al., 2005). Two of them (*O. interrupta* and *O. platycephala*) were identified in this study. Two haplotypes of the genus *Coreoperca* showed 100 % and 97 % sequence identity to *Coreoperca herzi* (KR075132) sampled in Korea. Since two species of *Coreoperca* are reported as endemic to the Korean peninsula (Kim et al., 2005), the second haplotype is most likely *C. kawamebari*, but further studies should be conducted for confirmation of this identification. Two invasive species of the family Centrarchidae, the Bluegill (*Lepomis macrochirus*) and the Largemouth bass (*Micropterus salmoides*) were also identified in this study. Those two species are endemic to North America but were introduced in the Korean peninsula for aquaculture purposes without considering the impacts on the ecosystem.

Excluído: ,
Excluído: in
Excluído: which
Excluído: *Pseudobargrus*
Excluído:
Excluído: *Tachysurus*
Excluído:
Excluído: the
Excluído: waters
Excluído: *Silurus*
Excluído:
Excluído: *Silurus*
Excluído:
Excluído: the
Excluído: Korean haplotype of
Comentado [FDD9]: From where?
Excluído: Further studies should be made to identify this haplotype.
Excluído: the
Excluído: Chinese haplotype
Excluído: species of
Excluído: the Korean haplotype of *Liobagrus*
Excluído: in
Excluído: *Odontobutis*
Excluído: *Odontobutis*
Excluído: *Odontobutis*
Comentado [FDD10]: What is the level (percentage) of identity?
Excluído: in
Excluído:
Excluído: the
Excluído: Korean haplotype of
Excluído: *Coreoperca*
Excluído: y
Excluído: haplotype

Fish biodiversity in the four rivers

Fish assemblage in the four rivers included in this study were analyzed. Among the 73 confirmed species of fishes detected in this study, 13 were identified in all four rivers: *Rhinogobiu sbrunneus, Mugil cephalus, Misgurnus mizolepis, Konosirus punctatus, Hemibarbus labeo, Zacco platypus, Rhynchocypris lagowskii, Pseudorasbora parva, Anguilla japonica, Silurus asotus, Micropterus salmoides, Tridentiger obscurus, Opsariichthys uncirostris* (Fig. 6). Regardless of sample stations, species of the Cyprinidae appear to be dominant, with average proportions of 47.02 ± 6.73 %, followed by the Gobiidae (15.24 ± 3.07 %) and Cobitidae (9.95 ± 4.09 %) (Fig.7). However, proportions of species of those families were different between upstream and downstream. The proportion of species of the Cyprinidae was higher (45.27 ± 9.1 %) at the upstream of rivers (stations 1 and 2) compared with downstream (33.78 ± 18 % at station 4). Contrastingly, the proportion of species of the Gobiidae was lower (14.53 ± 8.28 %) at the upstream of rivers than downstream (station 4, 19.90 ± 14 %).

The highest number of species was recorded in Seomjin river (52 species), followed by Taehwa river (42 species), Hyeongsan river (40 species), and Nakdong river (26 species). A total of 17 species were exclusively recorded in the Seomjin river: *Cobitis tetralineata, Squalidus gracilis, Tanakia somjinensis, Acanthogobius hasta, Siniperca scherzeri, Pseudobagru skoreanus, Acheilognathus majusculus, Sarcocheilichthys variegatus, Coreoleuciscus splendidus, Tanakia signifier, Acheilognathus rhombeus, Microphysogobio yaluensis, Rhodeus suigensis, Kareius bicoloratus, Rhodeus uyekii, Phoxinus oxycephalus,* and *Acheilognathus intermedia*. Five species were, in turn, recorded in the Taehwa River: *Pseudogobius masago, Mugilogobius abei, Acanthogobius lactipes, Rhynchocypris semotilus,* and *Silurus microdorsalis*, followed by four species identified in the Nakdong River:

Excluído: commonly
Excluído: , which included
Comentado [FDD11]: This should be in alphabetical order or in some other clearly defined sequence
Excluído: fish in
Excluído: and its
Excluído: were
Excluído: ,
Excluído: its
Comentado [FDD12]: Not clear what proportion you're talking about. Clarify in the text, here, if my suggestion is not correct
Excluído: By contrast
Excluído: , which include
Comentado [FDD13]: Again, some order is necessary – alphabetical, for instance
Excluído: By contrast, f
Excluído: from
Comentado [FDD14]: Same here
Excluído: from

Tachysurus nitidus, Rhinogobius giurinus, Pseudobagrus ussuriensis, and *Plagiognathops microlepis*. Only three species (*Nipponocypris koreanus*, *Squalidus multimaculatus,* and *Sarcocheilichthys soldatovi*) were exclusively recorded in Hyeongsan river (Fig. 6).

The highest Shannon Index (SI) was identified in the Seomjin river (3.480) followed by the Taehwa (3.067), Hyeongsan (2.954), and Nakdong rivers (2.864). Among the 16 surveyed stations, station 1 of Seomjin river (SJ1) showed the highest species richness (2.197), whereas the lowest (1.008) was recorded in station 4 of the Nakdong river (ND4). From upstream to downstream, average species richness decreased from 1.951 to 1.415 (Table 4).

Clustering analysis

In order to assess the correlation between the fish assemblage and sample stations, a heat-map analysis with the 30 most abundant species was conducted with the Primer software (Clarke and Gorley, 2015). The result indicates the species distribution in different sampling stations (Fig. 8). In upstream (Station 1 and 2), dominant species are *Zacco platypus*, *Odontobutis interrupta, Odontobutis platycephala, Nipponocypris temminckii, Rhynchocypris lagowskii, Misgurnus mizolepis, Coreoperca herzi, Acheilognathus intermedia,* and *Tanakia signifier*. In station 3, the dominant species are *Pseudorasbora parva, Gymnogobius breunigii, Rhinogobius giurinus, Rhinogobius brunneus,* and *Mugil cephalus*, whereas in the downstream (Station 4), *Tridentiger obscurus, Tridentiger trigonocephalus, Konosirus punctatus, Mugil cephalus, Anguilla japonica, Planiliza haematocheila* were identified as the dominant species, all of which are either euryhaline or anadromous (https://www.fishbase.org).

DISCUSSION

Comentado [FDD15]: Same here
Excluído: were identified, respectively
Excluído: including
Excluído: , and *Nipponocypris koreanus*
Excluído: detected
Excluído: river
Excluído: river
Excluído: observed
Excluído: the
Excluído: the
Excluído: know
Excluído: we conducted
Excluído: using
Excluído: clearly demonstrated
Comentado [FDD16]: Is there a reason for this particular sequence of species? If not, place them in alphabetical order, but if there's, this should be clarified in the text.
Comentado [FDD17]: Same here
Excluído: its
Comentado [FDD18]: Same here
Excluído: ,
Excluído: This result indicated that salinity is one of the essential factors to determine the fish assemblage at the downstream of the rivers.

Result indicate that eDNA metabarcoding using the MiFish pipeline is a useful tool for the fish biodiversity assessment in Korean freshwater ecosystems, since a total of 125 unique haplotypes including at least 73 species were successfully identified by a single-day survey of 16 sampling stations in te four rivers (Fig. 2-5). According to the "Survey and Evaluation of Aquatic Ecosystem Health (SEAEH)", a total of 130 freshwater species of fishes were identified from 953 sampling sites that covered most Korean rivers and lakes (Yoon et al., 2012). The total number of species confirmed by eDNA metabarcoding was equivalent to approximately 56% of those obtained by the year-long conventional survey. The efficiency of eDNA barcoding might actually be considered even higher, considering also the number of haplotypes successfully identified at the genus and/or family level. This result indicates that eDNA metabarcoding with the MiFish pipeline can significantly contribute to the assessment of the freshwater fish biodiversity of Korea, especially considering its relatively lower cost of implementation when compared with more conventional morphology-based surveys. Although the methodology in each research group may be slightly different, similar conclusions have been achieved in other studies (Bista et al., 2017; Deiner et al., 2016). eDNA metabarcoding analysis is also adequate for surveying aquatic species in protected areas, since it minimizes disturbance of vulnerable communities as well (Fernandez et al., 2018).

In spite of its relevance as a methodology for assessment of biodiversity, there are still several shortcomings for a more widespread use of the eDNA metabarcoding by MiFish pipeline. First, MiFish sequence data for endemic species of Korea should be supplemented to the GenBank database. According to the Archive of Korean species (https://species.nibr.go.kr), 67 species of freshwater fishes are endemic to Korea, and many of their MiFish sequences are still not available in the GenBank database. Beside the lack of sequence data, freshwater fishes typically have intra-species genetic distances generally

Excluído: In present study, we were able to know

Excluído:

Excluído: would be

Excluído:

Excluído: analysis which recovered

Excluído:

Excluído: simply …y a single- ... [23]

Excluído: of …n th… four rivers (Fig. 2-5). According to the "Survey and Evaluation of Aquatic Ecosystem Health (SEAEH)", a total of 130 freshwater species of fishes were identified from 953 sampling sites in …hat covered the …ost of…Korean rivers and lakes (Yoon et al., 2012). The total numbers…of confirmed fish …pecies confirmed by eDNA metabarcoding were …as equivalent to approximately 56.15 % of those obtained by the year-long conventional survey. The efficiency of eDNA barcoding might actually be considered even higher, considering also, and its proportions would be higher considering ... [24]

Comentado [FDD19]: I don't think identifications at the family level are significant…

Excluído: 'unidentified' species.

Excluído: strongly

Excluído: suggested

Excluído:

Excluído: that a freshwater fish biodiversity survey in Korea would be possible using eDNA metabarcoding platform with the MiFish pipeline for its incomparable cost and labors compared with a conventional morphological based surveys in Korea.

Excluído: suggests…ndicates that …DNA metabarcoding with the MiFish pipeline would…an significantly contribute to the assessment of the freshwater fish biodiversity of Korea, especially considering its relatively lower cost of implementation when compared with more conventional morphology-based surveys. Although the methodology in each research group may be slightly different, similar conclusions have been drawn from the ... [25]

Excluído: This

Excluído: inside …n in …rotected areas, since it to ... [26]

Excluído: Notably, most of rivers in Korea are the main source for the drinking water in metropolitan cities, and eDNA metabarcoding would be more importantly used for those rivers.

Excluído: Although

Excluído: eDNA metabarcoding analysis using the MiFish pipeline seems to be can be regarded as a useful tool to monitor assess the biodiversity of freshwater fish. However…pite of its relevance as a methodology for assessment of biodiversity,, ... [27]

Comentado [FDD20]: Sure it's "several"?

Excluído: of…or a more widespread use of the eDNA metabarcoding by MiFish pipeline. ... [28]

Excluído: , several drawbacks still need to be overcome

Excluído: ….First, MiFish sequence data for the …ndemic species in …f Korea should be supplemented to the GenBank ... [29]

higher than those of marine species (Seehausen and Wagner, 2014). Therefore, it is necessary to establish the haplotype database for the endemic fish species. Secondly, MiFish primer amplifies the 12S rRNA gene (163-185 bp) region of mitochondrial DNA, which is much smaller in size as well as lower in sequence variance compared with the COI region, which is typically used in species identification (IVANOVA et al., 2007). In fact, the MiFish region was unable to differentiate several closely related marine fish taxa, such as those in the genus *Sebastes* and *Takifugu* (Sato et al., 2018; Yamamoto et al., 2017). We also found that the average genetic distance of several genera in the family Cyprinidae was low in the MiFish region. For example, the average genetic distance of species of *Carassius* was too low (0.01), therefore identification at the species level was not possible (Fig. 2).

Further studies using eDNA metabarcoding might also be relevant at should be conducted to obtain more than biodiversity uch as the quantitative analysis of fish species. It is difficult to estimate the spatial abundance of eDNA in lotic environments. In fact, many factors should be considered for the quantitative analysis of eDNAs in rivers, including water dynamics (Deiner and Altermatt, 2014; Jerde et al., 2016; Wilcox et al., 2016) or different decaying times due to different physical, chemical, or biological factors (Shapiro, 2008). Although several studies about the decaying times of eDNAs in the laboratory and natural conditions (Alvarez et al., 1996; Matsui et al., 2001; Zhu, 2006), it is generally known that the short fragments of DNA are degraded slower than larger ones increasing the probability of detection in the natural environments (Deagle et al., 2006). Therefore, it is still too early to adopt eDNA metabarcoding for the quantitative analysis of fish species in natural condition. For the quantitative study, standardized collection methods and pretreatment procedures for the NGS sequencing analysis should be established as well. One of the strongest points in biodiversity survey by eDNA metabarcoding is the quantity of information it can generate compared with more conventional surveys, since large data sets are useful for statistical

Excluído: species have been fragmented and isolated for long time, and the intra-species genetic distance is generally higher than those for the marine species

Excluído: strongly required

Excluído:

Comentado [FDD21]: This has been mentioned before, what's the connection of this sentence with the previous one?

Excluído: than

Excluído: the typically used

Excluído: for the

Excluído: species

Excluído: as

Excluído:

Excluído: spp.

Excluído:

Excluído:

Excluído: spp.

Excluído: in the genus

Excluído: to discriminate against one another identify each species in the MiFish region

Excluído: The supplemented strategy should be designed for those taxa to obtain accurate results.

Excluído: Although we here analyzed fish biodiversity, f

Excluído:

Excluído: y

Comentado [FDD22]: This sentence is still confusing, not clear what you guys mean here

Excluído: made to adopt

Excluído: using eDNA metabarcoding s

Excluído: the

Excluído: with

Comentado [FDD23]: There's something strange in the first portion of the sentence. It's not clear what is the connection to the second part, after the comma. Please rewrite it.

Excluído: from

Excluído: However

Excluído: ,

Excluído:

Comentado [FDD24]: Not clear what "quantitative analysis" ... [30]

Excluído: far from establishing the reliable methods for the [31]

Excluído: the

Comentado [FDD25]: Please, always bear in mind the ... [32]

Excluído: the

Excluído: large amount number of data sets

Excluído: , which would be

Excluído: the

analyses. However, large amounts of data have been produced using different water collection methods, eDNA preparation, sequencing and bioinformatics analyses platforms by different research groups in different countries. Therefore, the interconversion of data is currently not possible. The establishment of an international standard in the overall methodology of eDNA metabarcoding would help researchers to produce more comparable data.

According to results obtained herein, the highest species richness was found in the Seomjin river (3.48) compared with those of the other three rivers: Taehwa river (3.06), Hyeongsan river (2.95), and Nakdong river (2.86). The lower values of species richness detected in Nakdong, Hyeongsan and Taehwa rivers is presumably related to the higher anthropogenic alteration of the natural conditions in those rivers. Like most other Korean rivers, those three rivers run through highly populated metropolitan cities, in which rivers are exposed to various human impacts which directly or indirectly promote changes in diversity and distribution of freshwater fishes (Finkenbine et al., 2000). In particular, lowest values of species richness (2.86) and number of endemic species (only one, *Odontobutis interrupta*) were identified in the Nakdong river, where the highest numbers of constructions and population exist among the sampled rivers. Lee et al. (2015) reported only two endemic species (*Coreoperca herzi* and *Odontobutis platycephala*) in the Nakdong river by a conventional catch survey. Eight endemic species, in turn (*Coreoleuciscus splendidus, Iksookimia longicorpa, Microphysogobio koreensis, Microphysogobio yaluensis, Odontobutis interrupta, Odontobutis platycephala, Pseudobagrus koreanus,* and *Squalidus gracilis*) were identified in this study in the Seomjin river, a number that is similar to those obtained in previous results (Jang et al., 2003; Lee et al., 2015). The several constructions along the urbanized watershed, including dams and weirs, have caused the simplification and reduction of habitats, decreasing the biodiversity in the river (Nilsson et al., 2005; Riley et al., 2005). Different from those three rivers, there is no metropolitan city along the Seomjin river, which is therefore less exposed to anthropogenic impacts. A long-term

Excluído: i… compared with the conventional surveys ... [33]
Excluído: by the
Excluído:
Excluído: in
Excluído: respective …esearch groups in different countries. Therefore, the interconversion of data is currently not possible. and it is required to ... [34]
Excluído: . As one of them, MiFish pipeline would be a feasible bioinformatic platform for eDNA metabarcoding analyses of fish biodiversity with a little modifications and supplementation for the regional application.
Excluído: …ould help researchers to produce the ... [35]
Excluído: We here identified
Excluído:
Excluído: Low
Excluído:
Excluído: found …n Nakdong, Hyeongsan and Taehwa rivers is presumably related maybe for ... [36]
Excluído: due
Excluído: effects …n these ... [37]
Excluído:
Excluído: the
Excluído: …ost other Korean rivers, those three rivers run through highly populated metropolitan cities, in which rivers are exposed to various human impacts which directly or indirectly promote changes in diversity and distribution of freshwater fishes (Finkenbine et al., 2000). In particular, the ... [38]
Excluído: numbers …only one, *Odontobutis interrupta*) were identified in the Nakdong river, where along which ... [39]
Excluído: …he highest numbers of constructions and population exist among the sampled rivers. Lee et al., ... [40]
Excluído: from
Excluído:
Excluído: the traditional
Excluído: … conventional catch survey ... [41]
Excluído: method
Excluído: On the other hand, e…ight endemic species, in turn (including : ... [42]
Excluído: including
Excluído: :
Formatado: Fonte: Não Itálico
Excluído:
Excluído: which was
Excluído:
Excluído: the
Excluído:
Excluído: various
Excluído: with …he Seomjin river, which is,…therefore,…less exposed to anthropogenic impacts. As ... [43]

survey should be conducted to establish the clear correlation between anthropogenic factors and fish assemblages in the Korean rivers.

The eDNA metabarcoding analysis also indicates that some exotic species of fishes are widely distributed in Korean rivers. We were able to identify at least five exotic species (*Carassius cuvieri, Cyprinus carpio, Cyprinus megalophthalmus, Lepomis macrochirus*, and *Micropterus salmoides*; Table S3). Those exotic species may impact the native fishes in terms of shelter and spawning sites. They can also disturb the food chain, preying on the native fishes. In addition, thpse species have a high reproductive capacity, which makes them highly potential invasive species (Keller & Lake, 2007; Koster *et al*, 2002; Nico & Fuller 2010). Our results also surprisingly revealed that the largemouth bass, *M. salmoides*, and the bluegill, *L. macrochirus* are likely present in all sampled four rivers. Those two species, which are native to North America, were artificially introduced in the 1970s in Korea without any further considerations of the effects on the freshwater ecosystems of the country. They are now widely distributed throughout the Korean peninsula, competing with the native species. A long-term survey on those rivers should be conducted to more properly assess the potential impacts of those introduced species (Jang et al., 2002; Yoon et al., 2012). Freshwater ecosystems are much more vulnerable to invasive species, causing biodiversity loss and global change (Clavero and García-Berthou, 2005), and eDNA metabarcoding analyses would be useful to monitor the distribution patterns of invasive species in Korean rivers.

Excluído: →
Excluído: revealed
Excluído: inland
Excluído: waters
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Excluído: As
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Excluído: eastern
Excluído:
Excluído: those two species
Excluído:
Excluído: ,
Excluído: as freshwater fish stock
Excluído: in Korea
Excluído:
Excluído: The species has spread
Excluído: and t
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Excluído: know
Excluído: ir
Excluído: s on the ecosystems
Excluído: Since
Excluído:
Excluído: f
Comentado [FDD26]: What do you mean by global change?
Excluído:
Excluído: the
Excluído: i
Excluído: the
Excluído: Collectively, we firstly analyzed the fish ... [44]

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Excluído: thankful

Excluído: and paying gratitude to

Excluído: enrich

Additional information and declarations:

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Competing Interests

The authors declare that they have no competing interests.

Author Contributions

- Md. JobaidulAlamcollected the samples, performed the experiments, analyzed the data, prepared figures and/or tables, wrote the manuscript
- Nack-KeunKim collected the samples, analyzed the data
- SaptoAndriyonoperformed the experiments, analyzed the data, prepared figures and/or tables
- Hee-KyuChoianalyzed the data, prepared figures and/or tables
- Ji-Hyun Lee analyzed the data, prepared figures and/or tables
- Hyun-Woo Kimconceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, authored or reviewed drafts of the manuscript, approved the final draft, wrote the manuscript.

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Not clear what “quantitative analysis” mean here. It’s too general, please be more specific. I assume you’re talking about ecological assessments at higher levels, something like that. Please be more specific.

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Please, always bear in mind the distinction between analysis and analyses

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