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DNA metabarcoding for diet analysis of an endangered endemic bitterling, *Acheilognathus majusculus* (Teleostei; Cyprinidae)

Biet Thanh Tran[1], Dong-Won Kang[2], Jung Soo Heo[1], Keun-Yong Kim[1], Keun-Sik Kim[2], Ju-Duk Yoon[2,*]

[1]Department of Genetic Analysis, AquaGenTech Co., Ltd, Busan 48228, ROK
[2]Research Center for Endangered Species, National Institute of Ecology, Yeongyang 36531, ROK

ABSTRACT: The bitterling species, *Acheilognathus majusculus*, is a freshwater fish, endemic to the Nakdong and Seomjin rivers in South Korea and listed as an endangered species by the Ministry of Environment in South Korea due to its biological, ecological, and genetic importance. Dietary information is crucial for understanding the role of *A. majusculus* within an ecosystem and planning its restoration strategy. In this study, we employed a non-invasive DNA metabarcoding approach to characterize the diet using 34 *A. majusculus* fecal samples collected from the Nakdong and Seomjin rivers. Analysis of 1 642 037 clean reads generated by applying the next generation sequencing yielded 210 amplicon sequence variants (ASVs). Taxonomic assignment successfully identified 10 phyla, 16 classes, 23 orders, 29 families, 29 genera, and 26 species. Green algae (Chlorophyta) and diatoms (Bacillariophyta) were predominantly detected in all samples, with percentages ranging from 5.56 to 43.59% and 30.00 to 66.67%, respectively. Smaller percentages of other taxa such as parasitic protists of Ichthyosporea (Opisthokonta incertae sedis), ciliates (Ciliophora), mostly eustigmatophytes (Ochrophyta), fungi (Ascomycota), vertebrates (Chordata), flatworms (Platyhelminthes), and green plants (Streptophyta) were also detected. The dietary composition of *A. majusculus* remained consistent irrespective of body size or sex. However, there was pronounced seasonal variation between summer samples and those from spring and fall, as demonstrated by beta-diversity analyses. These findings provide valuable insights into the dietary composition and seasonal variations of *A. majusculus* and highlight the need to protect and restore aquatic habitats and regulate water flow from weirs to ensure the consistent availability of essential food resources for effective habitat management strategies of this endangered species.

KEY WORDS: Metabarcoding · Feces · Diet analysis · Endangered bitterling · Freshwater fish

1. INTRODUCTION

Knowledge of the food source or diet of a species is fundamental and crucial in understanding its ecological function and role within an ecosystem. Over the past decades, extensive studies have focused on dietary analysis to reveal trophic interaction, energy flow, and niche differentiation (Braga et al. 2012, Nielsen et al. 2018, Amundsen & Sánchez-Hernández 2019). These studies focused on identifying and evaluating the availability of potential food items (Hyslop 1980, Manko 2016). Such evaluation reveals the feeding characteristics, patterns, and preferences of a species, which aids in confirming its dietary niche (Gerking 1994, Ward et al. 2006, Sánchez-Hernández et al. 2019). This information can elucidate the

*Corresponding author: zmszmsqkek@hanmail.net

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trophic levels occupied by the species, offering insights into its role in energy and nutrients within food webs (Brodeur & Pearcy 1992, Nielsen et al. 2018), while also determining its habitats, resource dependencies, and metabolic requisites (Larter & Gates 1991, Veloso & Bozinovic 1993, Clare et al. 2011), unveiling competitive interactions and predator–prey relationships (Villsen et al. 2022a,b). These ecological characteristics hold substantial importance for guiding the conservation and management of endangered species.

Various methodologies are available to ecologists investigating food sources (Nielsen et al. 2018). These encompass visual examinations of the digestive tract, stomach, and fecal contents, stable isotope analyses of specific compounds, pigment analysis using high-performance liquid chromatography, and DNA barcoding analyses of prey items. While these approaches offer valuable insights into the diet of a species, each has its own limitations, such as difficulties in identifying food items that leave no hard remains or lack diagnostic taxonomic features, the necessity to sacrifice the studied species for analysis, and low taxonomic resolution (Pompanon et al. 2012). Therefore, the quest for a non-invasive method with higher taxonomic resolution remains essential for dietary studies.

In the recent years, DNA metabarcoding of fecal samples has emerged as a powerful technique for qualitative dietary analyses. By applying next generation sequencing (NGS) technology to fecal samples, this technique has several advantages over traditional methods, as it can identify prey items down to species levels even when they are soft, highly degraded, or present at low concentrations (Lazic et al. 2021). Moreover, owing to its non-invasive nature, this approach eliminates the need for invasive or lethal sampling, particularly important for threatened or endangered species (Snider et al. 2022). Many studies have used this technique to assess the dietary profiles of threatened or endangered birds (Ando et al. 2013, Groom et al. 2017, Huang et al. 2021), sea lions (Berry et al. 2017), pocket mice (Iwanowicz et al. 2016), and fishes (Villsen et al. 2022a,b). However, it has rarely been applied to endangered fishes due to the challenge of obtaining fecal samples from their extremely small population sizes.

Acheilognathus majusculus is a freshwater bitterling that was listed as an endangered species Class II by the Ministry of Environment in South Korea in 2017. Endemic to the southern regions of the Korean Peninsula, it is predominantly found in the Seomjin River and in the Yeong River (a tributary of the Nakdong River) (Kim & Yang 1998). Its distribution is restricted to mid-upper areas of the rivers. This species prefers habitats with numerous large stones along the riverbed in fast-flowing streams, which provide shelter and protection from predators, and typically grows to maximum length of around 10 cm (http://fishillust.com/Acheilognathus_majusculus). It has a unique spawning relationship with freshwater mussels, placing its eggs onto their gills (Dávidová et al. 2008). Recently, *A. majusculus* has attracted much attention because of its biological, ecological, and genetic importance, but its vulnerability to reductions in host shellfish, environmental pollution, and industrialization pose notable threats (Van Damme et al. 2007, Lim & Lee 2017). Adults are known to consume aquatic algae, as indicated by stomach content analysis (Kim & Yang 1998), but there is no information on the detailed food sources. There is a pressing need for research into the food sources of *A. majusculus* to understand its foraging ecology.

This study applied a non-invasive DNA metabarcoding analysis of fecal samples to identify the dietary composition of the endangered *A. majusculus*. Furthermore, we assessed variation in the composition of its diet with respect to seasonality and locality. Understanding the dietary requirements of this endangered species lays the groundwork for determining and implementing effective conservation strategies and management activities to ensure the long-term persistence of *A. majusculus*.

2. MATERIALS AND METHODS

2.1. Ethics statement

As *Acheilognathus majusculus* is listed as an endangered species Class II by Ministry of Environment in South Korea, we obtained approval for its capture from regional branches of the Ministry of Environment in Daegu (Approval No. 2019-10), Saemangeum (Approval No. 2019-6), and Nakdonggang (Approval No. 2019-19). All animal experiments were carried out under the approval of the National Institute of Ecology Institutional Animal Care and Use Committee (Approval No. NIEIACUC-2021-026).

2.2. Study area and field collections

The Nakdong River, one of the major rivers in South Korea, has a length of 525 km and serves as an important water supply source for about 10 million residents. The Seomjin River, located in the central por-

tion of southern Korea, is the fourth largest watershed in the country. This river possesses a main river channel length of 212 km, supplying water for drinking, agricultural, and industrial purposes (Yang et al. 2021).

In this study, 34 adult *A. majusculus* were collected from August 2020 to November 2021 in the Nakdong and Seomjin rivers using cast nets (Table 1, Fig. 1). On-site, the collected fish were carefully identified following descriptions by Kim & Yang (1998) and Kim et al. (2014), measured (total and standard length; mm), weighed (g), and sexed (Table S1 in the Supplement at www.int-res.com/articles/suppl/n055p141_supp.pdf). Specimens were individually placed in a container supplied with 1 l of sterile distilled water at ambient temperature. Upon defecation of fecal pellets in the containers, fish were directly released back to the same site where they were captured. The distilled water containing fecal pellets was immediately vacuum filtered through a 47 mm diameter cellulose membrane filter with a pore size of 0.45 µm (Whatman) on-site. Subsequently, each filtrate was transferred into a 1.5 ml microtube and kept in cold and dark conditions during transportation to the laboratory, where they were stored at −80°C until further processing. Cold and dark storage conditions prevent DNA degradation and microbial growth, ensuring sample integrity for analysis.

2.3. Fecal DNA extraction and PCR amplification

Total DNAs of 34 fecal samples from adult *A. majusculus* were extracted using QIAamp® PowerFecal® DNA kits (Qiagen) according to the manufacturer's instructions. Negative controls (blank extractions) were processed to assess contamination during the extraction. After checking the quality using electrophoresis in a 1.5% agarose gel stained with ChamelGreen™ I (SFC Probes), the extracted fecal DNA was stored at −20°C for later analysis.

To avoid the result bias of prey DNA caused by predator DNA co-amplification, it is necessary to

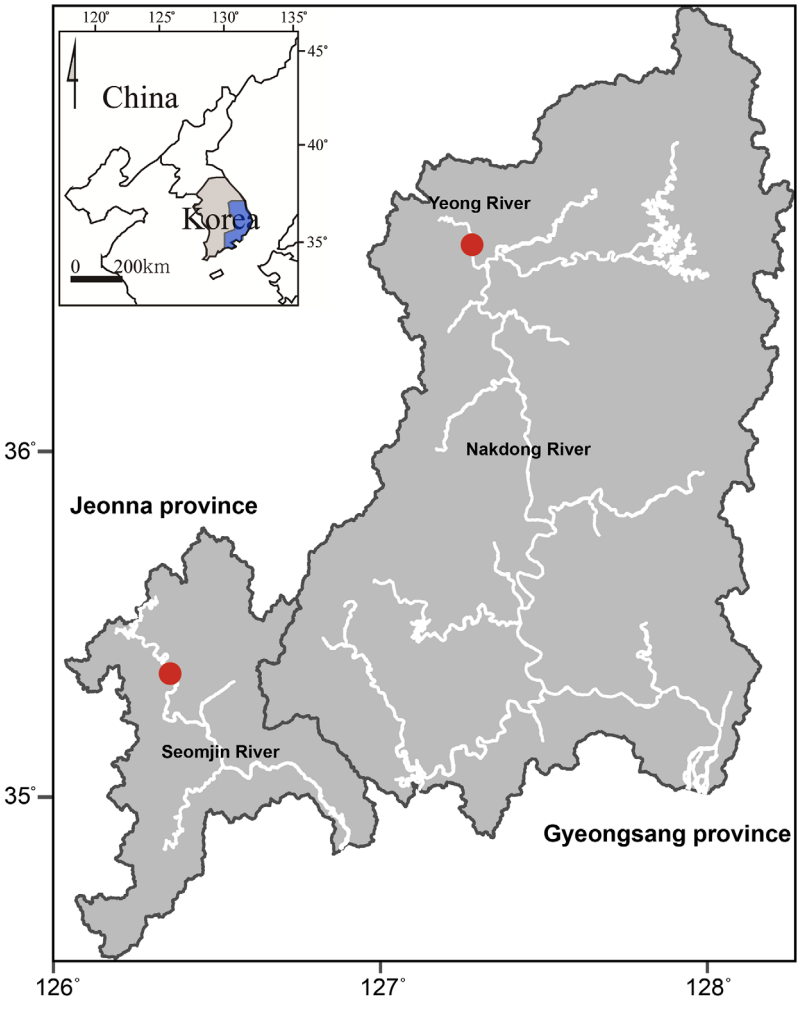

Fig. 1. Sampling sites (red dots) for adult specimens of *Acheilognathus majusculus* collected in the Nakdong and Seomjin rivers in South Korea

Table 1. Sampling information of 34 fecal samples from *Acheilognathus majusculus* collected in the Nakdong and Seomjin rivers in South Korea

Sampling site	Sample code	GPS coordinates	Sampling date	Sampling season	No. specimens
Nakdong River	NSu	36° 35′ 31″ N, 128° 12′ 47″ E	19 Aug 2020	Summer	8
	NFa		18 Nov 2020	Fall	8
	NSp		20 Apr 2021	Spring	10
Seomjin River	SSp	35° 25′ 19″ N, 127° 13′ 25″ E	22 Apr 2021	Spring	8

trace and eliminate the predator DNA in the data filtering step. A piece of pelvic fin was excised from 14 specimens of *A. majusculus* to extract genomic DNA according to Asahida et al. (1996). The quality was checked using a NanoDrop One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific). The partial 18S ribosomal DNA (18S rDNA) (ca. 1800 bp) of the specimens was amplified using extracted genomic DNA as template, a pair of primers, i.e. RYf2-EUK-18S-0001f (5′-AAC CTG GTT GAT CCT GCC AGT-3′) and YRr2-EUK-18S-1774r (5′-GAT CCT TCY GCA GGT TCA CCT AC-3′), and AccuPower® PCR Master Mix (Bioneer) according to the manufacturer's instructions. The amplification was performed using the ProFlex PCR System (Thermo Fisher Scientific) with the following conditions: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 2 min, and a final extension step at 72°C for 5 min. The PCR product was purified using the AccuPrep® PCR Purification Kit (Bioneer) and subjected to Sanger sequencing (Macrogen).

2.4. Library construction and NGS sequencing

In this study, the 18S rDNA V9 region was selected to target all major eukaryotic lineages contained in the fecal samples. The suitability of this region for uncovering eukaryotic diversity has been reviewed in many previous studies (Hadziavdic et al. 2014, Albaina et al. 2016). A set of eukaryotic forward (NGS-18S-1626f: 5′-GTA CAC ACC GCC CGT-3′; Lane 1991) and reverse (NGS-18S-1778r: 5′-TGA TCC TTC YGC AGG TTC AC-3′; Moon-van der Staay et al. 2001) primers with Illumina overhang adapters were selected for this DNA metabarcoding analysis. This set of primers have been verified for determining the eukaryotic community of invertebrate diets in several previous studies (Khaw et al. 2020, Tran et al. 2022, 2023).

Construction of Illumina libraries for 34 fecal samples from adult *A. majusculus* followed the 2-step PCR method described in the Illumina protocol for 16S metagenomic sequencing library preparation (Part #15044223 Rev. B, Illumina) with minor modification. In the first-stage amplification, pre-ordered fusion primers (Illumina overhang adapter 1 + NGS-18S-1626f and Illumina overhang adapter 2 + NGS-18S-1778r) were used to amplify the 18S rDNA V9 region. The amplification was performed in 3 replicates for each sample using AccuPower® *Pfu* PCR Master Mix (Bioneer) in a ProFlex™ PCR System with the following cycling conditions: initial denaturation at 94°C for 2 min, 35 cycles at 94°C for 15 s, 55°C for 15 s, and 72°C for 15 s, followed by a final step at 72°C for 5 min. All samples included negative controls to eliminate any additional contamination. The PCR products were purified using the AccuPrep® PCR Purification Kit and subsequently assessed by electrophoresis in a 1.5% agarose gel stained with ChamelGreen™ I. The purified triplicate PCR products from each sample were pooled together into 1 tube for the later steps.

In the second-stage amplification, dual Illumina index adapters were attached to the pooled PCR products from the first-stage PCR amplification using the Nextera XT Index V2 Kit (Illumina). The amplification was performed in a ProFlex™ PCR System (Thermo Fisher Scientific) using 2× KAPA HiFi HotStart ReadyMix (KAPA Biosystems) under the following conditions: initial denaturation at 95°C for 3 min, 12 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and a final step at 72°C for 5 min. The size and quantity of PCR-enriched fragments were checked using a DNA 1000 chip on an Agilent Technologies 2100 Bioanalyzer and qPCR, respectively, according to the Illumina qPCR Quantification Protocol Guide. The libraries were subjected to paired-end (2 × 300 bp) sequencing on the Illumina MiSeq™ system (Macrogen).

2.5. Bioinformatics and taxonomy assignment

After converting the base call binary into FASTQ using the Illumina package bcl2fasq, the demultiplexed raw sequences were processed through a series of bioinformatics steps to obtain the final refined data for downstream analysis. FastQC v0.11.9 was used to perform a quality control check on the raw sequencing data (Andrews 2010). Sequences of Illumina dual indices, adaptors, and primers were trimmed using fastp v0.23.1 (Chen et al. 2018). Representative amplicon sequence variants (ASVs) with read lengths ranging from 100 to 300 bp were generated after pair-end merging (-fastq_mergepairs), ASV clustering with 99% similarity threshold (-id 0.99 -centroids), and sequencing error and chimera removal (-unoise3) using Usearch (Edgar 2010). The ASV table was created by assigning paired reads to representative ASVs and any counts that passed a similarity threshold of 97% using Usearch (-usearch_global -id 0.97).

The ASV table, consisting of representative sequences and their associated counts per sample, was subjected to a filtering procedure to produce final

valid data. Sequences with low count (<10) and low relative read count (<0.01%) compared to the total count of all samples were removed in the abundance filtering steps. The passed ASVs were taxonomically assigned by searching the nucleotide ('nt') database v5 (downloaded April 25, 2023) using BLAST+ v2.12.0 with default setting (Camacho et al. 2009) in the GenBank database of the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov/). The searches excluded uncultured/environmental sample sequences to remove taxonomically ambiguous matches in the database. The top hit with >80% identity and taxonomic identification to at least the genus level was selected, whereas ASVs with 'no hit' or a hits identified as Bacteria and Archaea or organelles (mitochondria and chloroplasts) of Eukaryota were discarded.

For tracing predator DNA, the 18S rDNA sequences newly obtained from the pelvic fins of 14 specimens of *A. majusculus* were manually queried against the GenBank database using an online BLASTn tool with default parameter (accessed April 25, 2023). As the 18S rDNA sequences of *A. majusculus* were lacking in the database, the top hit, *Hemibarbus labeo* (GenBank accession number MH843153), consistently showing >97% similarity in all of the specimens (Table S2), was used to compare with the taxonomic assignment of all ASVs to detect predator DNA. Consequently, filtration of the predator DNA was carried out by discarding all ASVs assigned as *H. labeo*. The resulting ASV table was used for alpha- and beta-diversity analyses of dietary compositions. For enhancing the reliability of the taxonomic identification, additional constraints were applied by setting the relative count threshold to 0.1%. In addition, ASV sequences with query coverages of 100% were classified at species and genus levels only if they aligned precisely to the GenBank database at over 99% identity, while over 97% identity was only applied for higher taxonomic ranks (i.e. family, order, class, or phylum).

2.6. Data analysis

Individual-rarefaction curves based on the read abundance data from the retained ASVs were generated using the Paleontological Statistics Software Package (PAST) v.4.04 (Hammer et al. 2001). Alpha-diversity indices, i.e. species richness, Simpson's ($1 - D$), Shannon-Wiener (H'), and Chao1, were measured using PAST v.4.04 and visualized using the R package 'ggplot2' (Wickham 2016, R Core Team 2022). The Kruskal-Wallis test in SPSS v.20 (IBM) was used to assess the group difference of alpha-diversity indices.

After filtering procedures, counts assigned to valid ASV sequences per samples were converted to relative read abundance (RRA, %) and frequency of occurrence (FOO, %) according to Deagle et al. (2019). Stacked bar graphs of the primary prey contained in each fecal sample based on the RRA and FOO data were generated to compare prey community within and among groups at the phylum level. Heat maps of primary prey species were generated based on RRA and FOO data.

For beta-diversity analyses, a Bray-Curtis similarity matrix based on read abundance data was calculated for downstream multivariate analysis. Presence of any hierarchical clustering among samples was observed through an unweighted pair group method with an arithmetic mean (UPGMA) dendrogram. Non-metric multidimensional scaling (NMDS) unconstrained ordination analyses were carried out to identify changes in the dietary community structure among groups of samples. In addition, canonical analysis of principal coordinates (CAP) constrained ordination was carried out to visualize the community structure of the pre-defined groups, which was difficult to distinguish due to the data cloud having an unconstrained ordination (Anderson & Willis 2003). Permutation tests of the CAP analysis (permutations [perm.] = 999) were used to test for differences among the groups in multivariate space, the strength of the association through canonical correlations, and the proportion of allocations that were misclassified (misclassification error). A permutational multivariate analysis of variance (PERMANOVA) was used (perm. = 999) to test differences among *a priori* defined sample groups. The difference in average rank dissimilarity within pairs of sample groups was also tested (perm. = 999). All the measurements and graph visualization for beta-diversity analyses were performed using the Plymouth Routines in Multivariate Ecological Research software v.7 (Primer-e).

3. RESULTS

3.1. NGS sequencing

All libraries from the 34 fecal samples from adult *Acheilognathus majusculus* collected in different seasons (summer, fall, and spring) and locations (the Nakdong and Seomjin rivers) were successfully sequenced using the Miseq™ system (Illumina), generating a total of 2608373 reads. After the quality

control and filtering procedure, a total of 1 642 037 reads of 210 ASVs were retained. The rarefaction curves showed a discrepancy in sequencing depth from the samples collected in summer as opposed to spring and fall. However, all samples approached the asymptote, indicating that they were sufficient to capture the dietary diversity of all samples (Fig. 2). The pairwise Kruskal-Wallis test showed that the read abundance of summer samples from the Nakdong River was significantly lower than that of fall ($p = 0.003$) and spring samples from the Nakdong River ($p = 0.002$), and spring samples from the Seomjin River ($p = 0.009$).

3.2. Dietary diversity

Alpha-diversity indices, i.e. species richness, Simpson's ($1 - D$), Shannon-Wiener (H'), and Chao1, of the 34 fecal samples from adult *A. majusculus* showed differences in the dietary diversity across sampling seasons and locations (Fig. 3). The species richness in summer was significantly lower compared to fall and spring in the Nakdong River ($p < 0.001$ and $p = 0.002$ for the species richness and Chao1 indices, respectively). No differences in species richness were detected in spring in the Seomjin River compared to fall and spring in the Nakdong River. Both Simpson's and the Shannon-Wiener indices had a similar trend, indicating a greater diversity in spring in the Nakdong River compared to those in summer ($p = 0.002$ for both indices) in the Nakdong River and in spring ($p = 0.013$ and $p = 0.043$) in the Seomjin River. However, no significant differences were observed between fall in the Nakdong River and the other seasons and river. Overall, a high dietary diversity (mean Simpson's index > 0.70) was found in fecal samples from the Nakdong and Seomjin rivers in summer, fall, and spring.

3.3. Dietary composition and seasonal variation

Taxonomic assignment analysis successfully identified 10 phyla, 16 classes, 23 orders, and 29 families of primary prey from the 34 fecal samples from *A. majusculus*. When more stringent criteria were applied to increase the reliability of species and genus identification, 29 genera and 26 species were classified.

Overall, the relative abundance of the 10 phyla based on the FOO (Fig. 4B) displayed a consistent pattern, with green algae (Chlorophyta) and diatoms

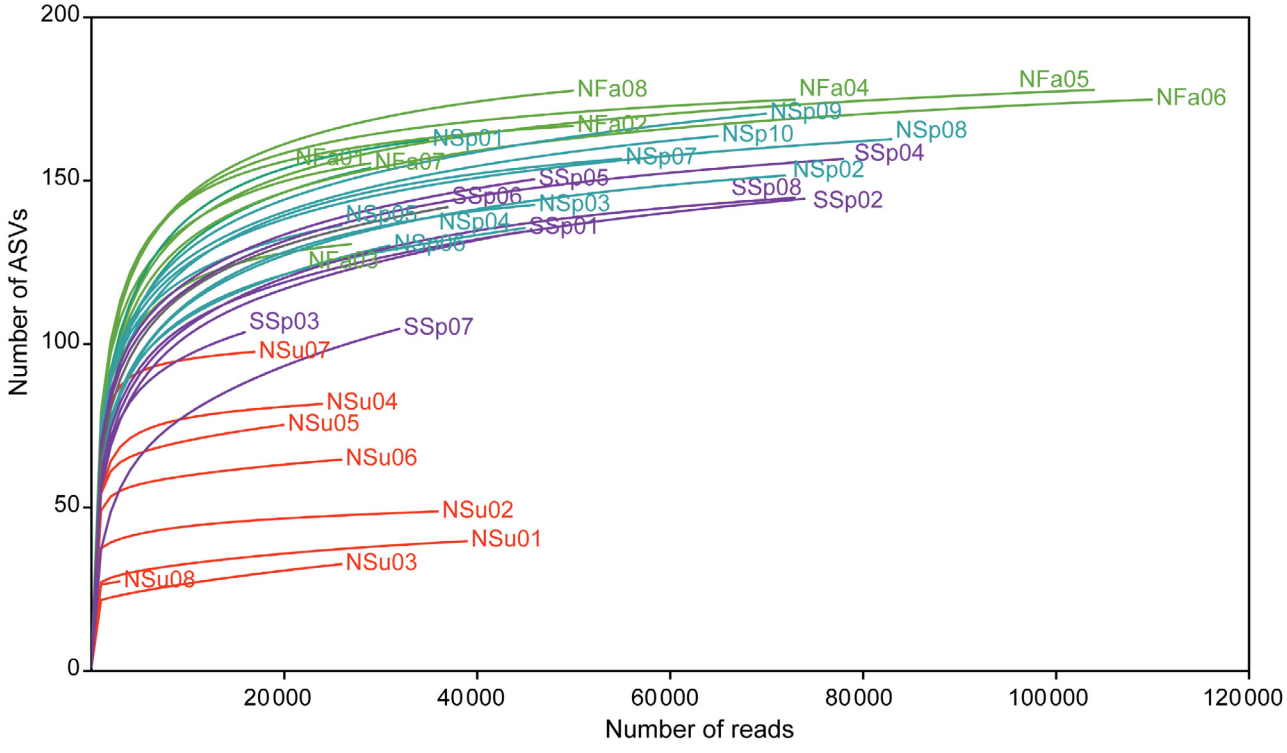

Fig. 2. Species richness (the number of amplicon sequence variants [ASVs]) of the fecal samples from adult *Acheilognathus majusculus* collected in summer (NSu), fall (NFa), and spring (NSp) in the Nakdong River and in spring (SSp) in the Seomjin River in South Korea

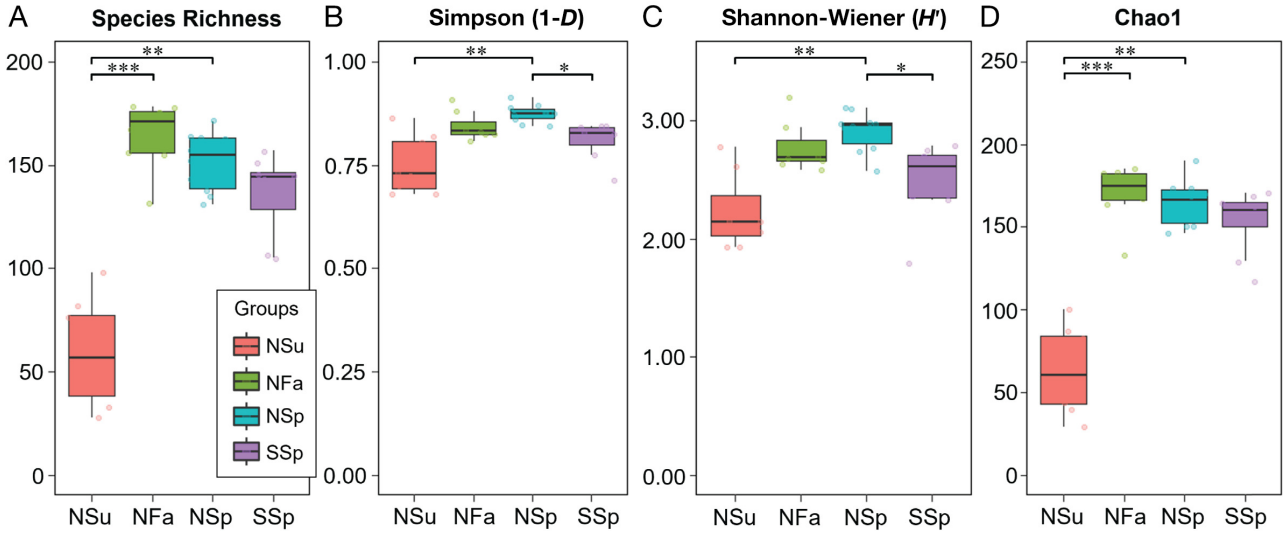

Fig. 3. Alpha diversity indices, including (A) species richness, (B) Simpson's (1-*D*), (C) Shannon-Wiener (*H'*), and (D) Chao1, of fecal samples from *Acheilognathus majusculus* collected in summer (NSu), fall (NFa), and spring (NSp) in the Nakdong River, and in spring (SSp) in the Seomjin River in South Korea. Boxes represent the interquartile range (IQR) between the first and third quartiles (25th and 75th percentiles, respectively), and the horizontal line inside the box defines the median. Whiskers represent the lowest and highest values within 1.5 times the IQR from the first and third quartiles, respectively. (circles) Outliers that are greater than 1.5 times and less than 3 times the IQR. *p < 0.05; **p < 0.01; ***p < 0.001 (Kruskal-Wallis test)

(Bacillariophyta) predominantly present in all fecal samples, ranging from 5.56 to 43.59% and 30.00 to 66.67%, respectively. However, according to the RRA (Fig. 4A), the fecal samples from the Nakdong River in fall and spring, and from the Seomjin River in spring, exhibited a markedly different composition compared to the summer samples from the Nakdong River. The RRA results showed that green algae (42.89–86.99%) and diatoms (7.90–55.00%) were predominantly present in all samples from the Nakdong River in fall and spring, as well as from the Seomjin River in spring (except for NFa03 and SSp07). Diatoms (41.01–83.74%) were the dominant group in 5 summer fecal samples (NSu01, NSu04 to NSu07) from the Nakdong River, while fungi (Ascomycota) (46.17–58.68%) composed a high percentage in samples NSu02, Nsu03, and Nsu08. Additionally, green algae were found in low proportions (1.28–9.46%) in all summer samples from the Nakdong River.

According to the RRA data, parasitic protists of the class Ichthyosporea (Opisthokonta incertae sedis), ciliates (Ciliophora), photosynthetic microalgae, eustigmatophytes (Ochrophyta), and xanthophytes (Ochrophyta incertae sedis) at the phylum levels were exclusively detected in fall and spring in the Nakdong River, and in spring in the Seomjin River, respectively, whereas these phyla appeared in all mentioned seasons in both rivers according to the FOO data. Fungi were found in varying percentages (2.78–9.09%) during summer in the Nakdong River, while few to none were found during the other seasons in the Nakdong and Seomjin rivers. Flatworms (Platyhelminthes) were commonly detected in most samples during fall and spring in both rivers. However, in summer in the Nakdong River, they were found in only 2 out of 8 samples. Based on the FOO, land plants (Streptophyta) were detected in all summer samples, with FOO values ranging from 3.23 to 15.39%. However, based on the RRA, land plants were found in all summer samples with values ranging from 1.39 to 20.49%, with few to none detected in the other seasons.

A heatmap visualizing the RRA and FOO of dietary ASVs at the species level of *A. majusculus* fecal samples is provided in Fig. 5. Among 26 identified species, 17 (65%) were assigned to diatoms and green algae that were detected in most of the fecal samples (except for *Monoraphidium subclavatum* and *Ulothrix mucosa*, not found in summer in the Nakdong River) based on the FOO. However, the distribution pattern of diatoms and green algae differed based on the RRA. Among the diatoms, higher RRA values were obtained for *Cyclotella cryptica*, *Fragilaria nanana*, and *Gomphonema parvulum* in summer, fall, and spring in the Nakdong River, and for *Sellaphora cap-*

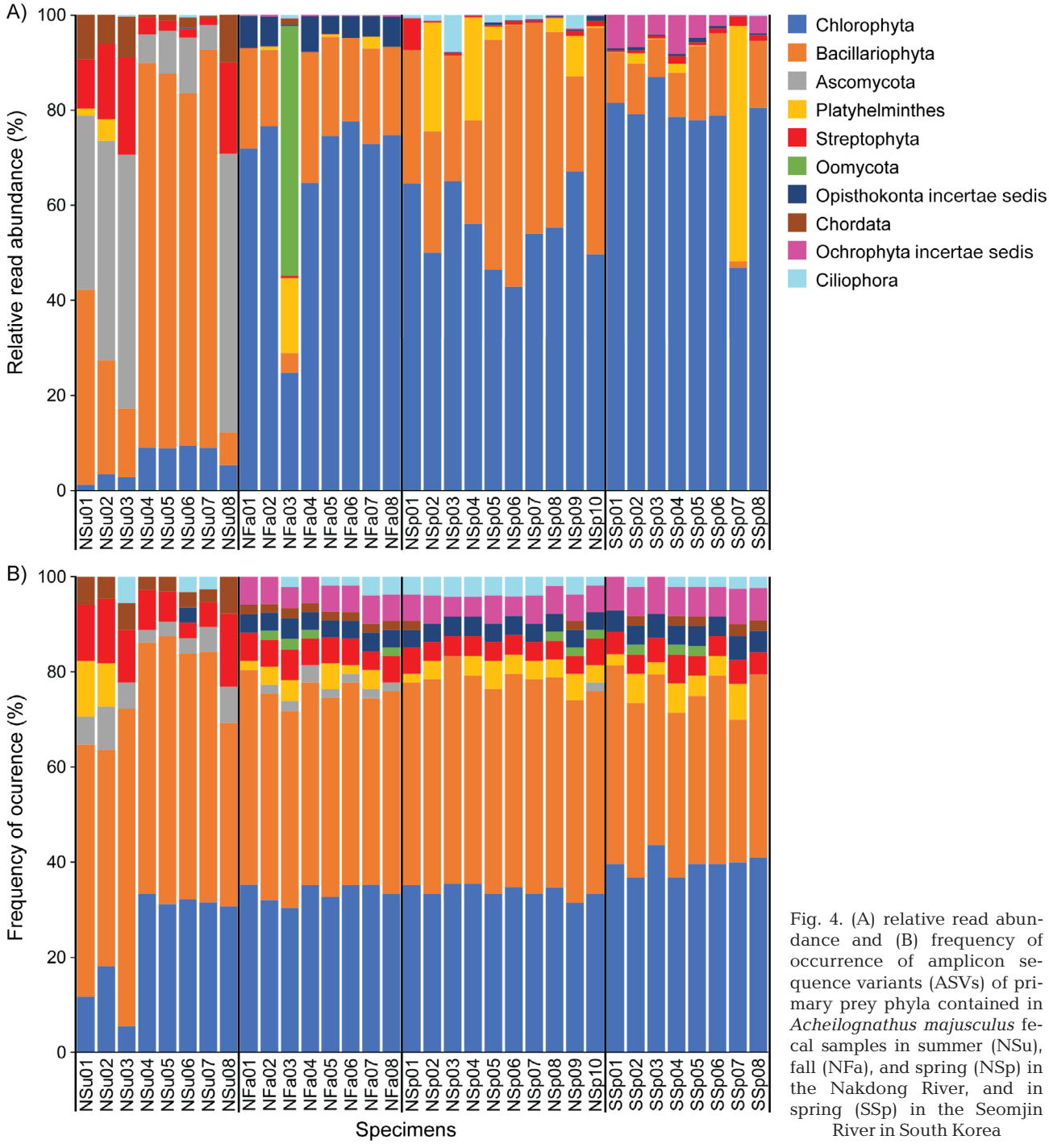

Fig. 4. (A) relative read abundance and (B) frequency of occurrence of amplicon sequence variants (ASVs) of primary prey phyla contained in *Acheilognathus majusculus* fecal samples in summer (NSu), fall (NFa), and spring (NSp) in the Nakdong River, and in spring (SSp) in the Seomjin River in South Korea

itata and *Sellaphora* cf. *seminulum* in fall and spring in the Nakdong River. Fecal samples from the Seomjin River had low RRA values for diatom species compared to samples from the Nakdong River, except for *Cyclotella cryptica* (27%) and *Synedra berolinensis* (20%). *Melosira varians* (86% RRA) and *Ulnaria acus* (93% RRA) were detected in the Nakdong River in summer and fall, respectively. Among the green algae, nearly all the species were detected in all fecal samples (except for *Monoraphidium subclavatum* and *Ulothrix mucosa*, not found in summer in the Nakdong River) based on the FOO. However, the highest RRA values for *Desmodesmus communis*, *Monoraphidium subclavatum*, *Pseudopediastrum boryanum*, *Scenedesmus armatus*, *Stauridium privum*, *Tetradesmus obliquus*, and *Ulothrix mucosa* were recovered in

Phylum_Species	RRA (%)				FOO (%)			
Ascomycota_*Candida parapsilosis*	100	0	0	0	100	50	0	0
Ascomycota_*Digitopodium cannae*	99	1	0	0	25	50	10	0
Bacillariophyta_*Cyclotella cryptica*	13	36	24	27	63	100	90	100
Bacillariophyta_*Fragilaria nanana*	16	48	35	1	75	100	100	75
Bacillariophyta_*Gomphonema parvulum*	52	23	17	7	88	100	100	100
Bacillariophyta_*Melosira varians*	86	1	12	1	100	100	100	88
Bacillariophyta_*Sellaphora capitata*	6	28	63	4	88	100	100	100
Bacillariophyta_*Sellaphora* cf. *seminulum*	6	47	43	4	100	100	100	100
Bacillariophyta_*Synedra berolinensis*	1	10	69	20	50	100	100	100
Bacillariophyta_*Ulnaria acus*	5	93	2	0	63	100	80	13
Chlorophyta_*Chlamydomonas incerta*	99	0	0	0	75	63	30	38
Chlorophyta_*Cloniophora spicata*	15	0	0	85	63	38	10	50
Chlorophyta_*Desmodesmus communis*	2	23	31	44	38	100	100	100
Chlorophyta_*Monoraphidium subclavatum*	0	60	22	18	0	100	100	100
Chlorophyta_*Pseudopediastrum boryanum*	8	4	83	5	50	100	100	100
Chlorophyta_*Scenedesmus armatus*	1	26	27	45	88	100	100	100
Chlorophyta_*Stauridium privum*	4	15	78	4	38	88	100	100
Chlorophyta_*Tetradesmus obliquus*	0	34	34	31	63	100	100	100
Chlorophyta_*Ulothrix mucosa*	0	49	36	15	0	88	100	88
Chordata_*Pan paniscus*	96	4	0	0	100	100	20	63
Ciliophora_*Chilodonella hexasticha*	7	1	93	0	38	38	100	0
Ciliophora_*Trichodinella myakkae*	0	4	92	4	0	50	80	75
Ochrophyta incertae sedis_*Nannochloropsis oculata*	0	3	4	93	0	100	100	100
Oomycota_*Pythium terrestris*	0	100	0	0	0	50	30	38
Streptophyta_*Agoseris glauca*	99	0	0	0	100	88	20	13
Streptophyta_*Humulus lupulus*	6	1	77	15	88	100	100	100
	NSu (Summer)	**NFa** (Fall)	**NSp** (Spring)	**SSp** (Spring)	**NSu** (Summer)	**NFa** (Fall)	**NSp** (Spring)	**SSp** (Spring)

Fig. 5. Relative read abundance (RRA; white: 0% to dark blue: 100%) and frequency of occurrence (FOO; white: 0% to dark orange: 100%) of dietary amplicon sequence variants (ASVs) at the species level of *Acheilognathus majusculus* samples collected in summer (NSu), fall (NFa), and spring (NSp) in the Nakdong River, and in spring (SSp) in the Seomjin River in South Korea

fall and spring in the Nakdong River and in spring in the Seomjin River, as opposed to in summer in the Nakdong River. *Chlamydomonas incerta* (99% RRA) and *Cloniophora spicata* (85% RRA) were largely found in summer in the Nakdong River and in spring in the Seomjin River, respectively. According to the RRA, the majority of the fungi *Candida parapsilosis* and *Digitopodium cannae*, the vertebrate *Pan paniscus*, and the land plant *Agoseris glauca* were exclusively discovered in summer in the Nakdong River. Likewise, 2 species of ciliates (*Chilodonella hexasticha* and *Trichodinella myakkae*) predominated in spring in the Nakdong River, whereas the photosynthetic eustigmatophyte *Nannochloropsis oculata* and the fungus (Oomycota) *Pythium terrestris* were exclusively counted in spring in the Seomjin River and in fall in the Nakdong River, respectively, based on the RRA. The land plant *Humulus lupulus* was identified in most of the samples with a large RRA (77%) in spring in the Nakdong River.

3.4. Dietary composition structure

The UPGMA dendrogram showed a close relationship between dietary compositions in fall and spring in the Nakdong River, as well as in spring in the Seomjin River (Fig. 6). Conversely, summer samples from the Nakdong River formed a distinct cluster, a result supported by the NMDS (Fig. 7A) and PERMANOVA ($F_{3,30}$ = 12.001; p = 0.001) (Table 2). Furthermore, the dendrogram also demonstrated that samples within a group were clustered together.

Using 4 pre-defined groups of fecal samples, i.e. from the Nakdong River in summer, fall, and spring, and the Seomjin River in spring, the CAP analysis (Fig. 7B) effectively classified samples into 4 groups distinguishable from one another with a 100% allocation success rate (Table 2). This classification showed a strong association strength, i.e. the first and second squared canonical correlation, δ^2 = 0.982 and 0.923, respectively. The separation of

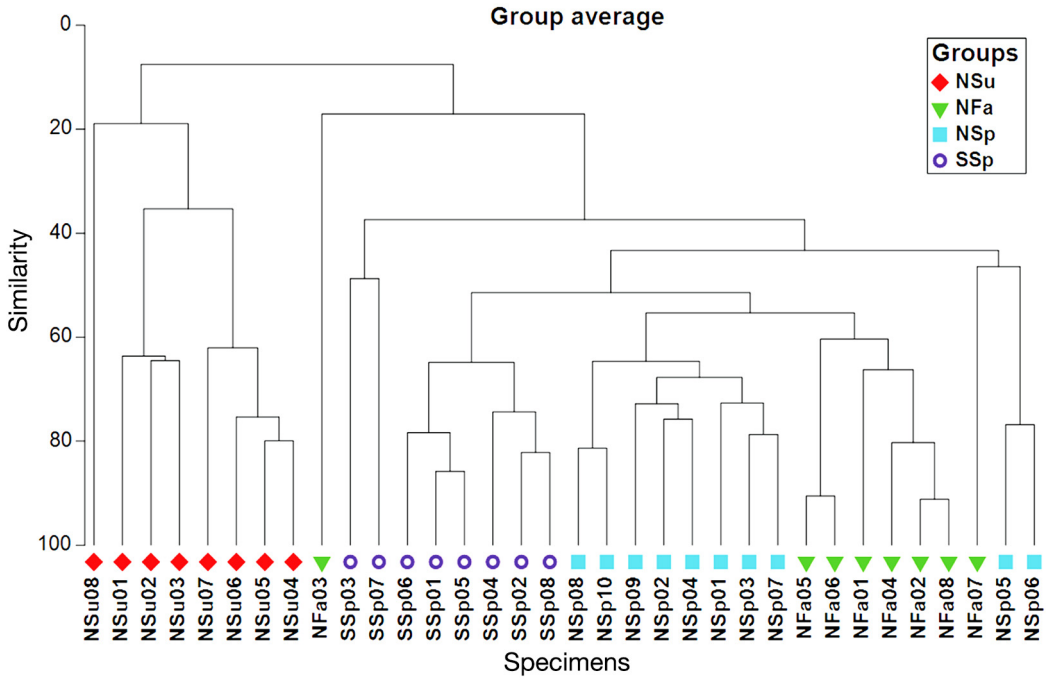

Fig. 6. Unweighted-pair group method with arithmetic mean (UPGMA) dendrograms based on Bray-Curtis similarities of fecal samples from *Acheilognathus majusculus* collected in summer (NSu), fall (NFa), and spring (NSp) in the Nakdong River, and in spring (SSp) in the Seomjin River in South Korea

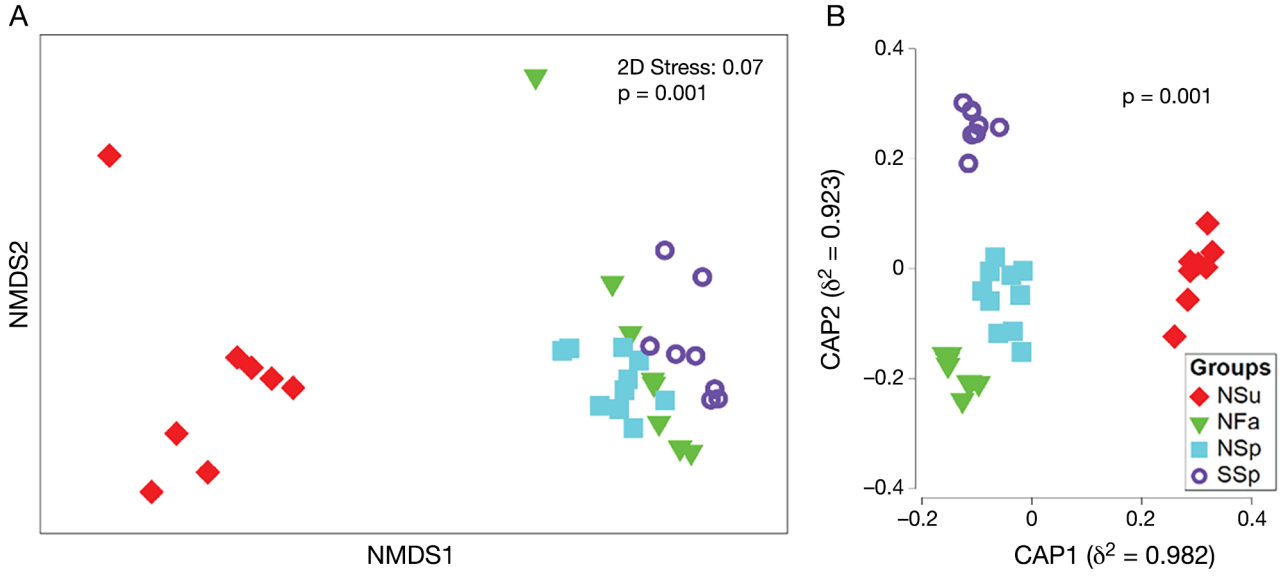

Fig. 7. (A) Non-metric multidimensional scaling (NMDS) and (B) canonical analysis of principal coordinates (CAP) ordination plots based on the Bray-Curtis distance of fecal samples from *Acheilognathus majusculus* collected in summer (NSu), fall (NFa), and spring (NSp) in the Nakdong River, and in spring (SSp) in the Seomjin River in South Korea. The stress level of the NMDS analysis and the statistical significance of differences in the PERMANOVA test (p = 0.001) and CAP analysis (p = 0.001) are indicated. δ^2: squared canonical correlation

dietary composition in fecal samples from *A. majusculus* into these 4 groups was supported by a significant CAP test (p = 0.001) and significant pair-wise tests (all p ≤ 0.002).

4. DISCUSSION

Knowledge of dietary habits and feeding resources is essential for designing effective management and

Table 2. (A) PERMANOVA and (B) post-hoc pairwise test and misclassification error of the CAP analysis for groups of fecal samples from *Acheilognathus majusculus* collected in summer (NSu), fall (NFa), and spring (NSp) in the Nakdong River, and in spring (SSp) in the Seomjin River in South Korea. Pseudo-*F*: *F*-value by permutation; p-values were obtained using 999 permutations

(A) Source	df	SS	MS	Pseudo-*F*	p-value (perm)
Groups	3	45189	15063	12.001	0.001
Residual	30	37653	1255.1		
Total	33	82842			
(B) Groups	NSu	NFa	NSp	SSp	
NSu	–	0	0	0	
NFa	0.001	–	0	0	
NSp	0.001	0.001	–	0	
SSp	0.001	0.002	0.001	–	

conservation plans of threatened or endangered wildlife (Pompanon et al. 2012, de Sousa et al. 2019, Sherley et al. 2020). Traditional methods for identifying the food sources of a species are often limited, particularly in the case of endangered predators, due to the challenges in sample collection and limitations (Tabassum et al. 2022). Fecal DNA metabarcoding serves as an optimal alternative, known for its non-invasiveness, speed, accuracy, and cost-effectiveness in providing dietary information for a target species (Ando et al. 2018). In this study, fecal DNA metabarcoding demonstrated its efficacy by revealing a diverse range of prey taxa in the diet of *Acheilognathus majusculus*, including taxa not previously identified, such as ciliates, vertebrates, and flatworms. This analysis facilitated the creation of a comprehensive prey list for *A. majusculus*, the majority of which were identified to the species level. Notably, this technique posed no harm to the endangered *A. majusculus*. These findings highlight the advantages and applicability of fecal DNA metabarcoding in elucidating the dietary composition of endangered species, such as *A. majusculus*.

With the examination of 34 fecal samples from adult *A. majusculus* collected across 3 seasons (summer, fall, and spring) at 2 distinct locations (the Nakdong and Seomjin rivers), the prevalence of photosynthetic taxa, i.e. green algae and diatoms, was observed in the majority of samples. These photosynthetic taxa are mostly attached microalgae or photosynthetic periphyton. Our findings suggest that *A. majusculus* primarily relies on attached microalgae as its main food source, aligning with previous studies on the dietary habits of bitterlings (Kim & Yang 1998, Koutrakis et al. 2003, Moreva et al. 2017). Most bitterlings, including *A. majusculus*, are predominantly herbivorous (National Institute of Biological Resources 2023). They often employ a spectrum of dietary strategies, ranging from specialists to generalists, to meet their nutritional needs (Nielsen & Matocq 2021). Generalist herbivores tend to maintain more diverse diets. Moreover, larger bitterlings are characterized as strong detritivorous species, consuming detritus and plant material (Przybylski & Zieba 2000). This could account for the presence of various other food taxa in the fecal samples of *A. majusculus*, such as ciliates, flatworms, green plants, vertebrates, and fungi, as demonstrated in this study. However, it is crucial to approach the conclusion that these taxa represent actual dietary preferences of *A. majusculus* cautiously, as these DNA traces might also come from opportunistic parasites or symbiont species, potentially contaminating the analysis. For instance, fungi, involved in the decomposition of complex carbohydrates and fibers, coexist in smaller numbers alongside other dietary components within the gut of various fish species (Siriyappagouder et al. 2018).

Body measurements and sex determination were recorded for 18 *A. majusculus* samples collected in spring in the Seomjin and Nakdong rivers. Based on the FOO analysis, dietary composition within the same group exhibited a consistent pattern, regardless of body size or sex. The uniformity in dietary patterns among *A. majusculus* individuals was further corroborated by the clustering of the fecal samples from the same group in both the UPGMA dendrogram and the CAP analysis. These results suggest that *A. majusculus* likely occupies a specific trophic level within the ecosystem and maintains highly conserved feeding behaviors and ecological niches, resulting in a consistent diet among individuals within the same group.

In South Korea, more than 33 000 weirs have been installed, primarily for agricultural and water security purposes (National Fishway Information System, www.fishway.go.kr). These weirs have disrupted natural water flow, significantly altering the hydrological features, water quality, and freshwater algae populations of aquatic ecosystems, consequently leading to a decline in fish diversity (Cha et al. 2015, Kang et al. 2019). Particularly, the creation of lentic water conditions by these weirs has caused a shift in the species composition of attached algae towards species favoring stagnant conditions. Although the dietary compo-

sition diversity of fecal samples from *A. majusculus* in spring in the Seomjin River is slightly lower than of those in the Nakdong River, both locations display a similar pattern characterized by a substantial presence of green algae and diatoms. Nevertheless, continuous year-round monitoring of dietary composition and food availability at these sites, along with expanded sampling collection to include additional sites, is crucial for a comprehensive comparative analysis.

Differences in the FOO among different groups of fecal samples can be associated with prey occurrences (Deagle et al. 2019, Tran et al. 2022). In this study, high FOO values for green algae and diatoms were consistently found in the diet of *A. majusculus* across different seasons and locations, suggesting sufficient food availability for its survival in the sampling sites. However, the prey occurrences of the summer samples collected in the Nakdong River showed inconsistencies when compared to the other seasons. Low RRA values for green algae at the phylum level, with a high accumulation of fungi and land plants, were also found in summer samples. In addition, the summer samples consistently formed a distinct cluster in NMDS analysis and showed significant differences in PERMANOVA, indicating a similar dietary pattern across them. The Nakdong River experiences various factors impacting its ecological dynamics, such as monsoon rainfall during summer as well as artificial waterfront weirs and climate change (Lee et al. 2021, Jargal & An 2023). Jeong et al. (2011) demonstrated that rainfall, total dam discharge, and river flow significantly decrease chlorophyll *a* concentration in the midstream of the Nakdong River during summer. Kim et al. (2007) also recorded a low abundance of phytoplankton in August from 2005 to 2006 in the mid to lower parts of the river. These factors might contribute to the detection of low reads in the summer fecal samples from the Nakdong River. Moreover, the summer seasons in the Nakdong River are characterized by monsoonal climates and several typhoons with high precipitation occurring from July to August (Kang et al. 2019). This seasonal fluctuation and discharge of water significantly impact the ecosystem of the Nakdong River during summer, potentially resulting in a reduced diversity and availability of prey items for *A. majusculus*.

Understanding the dietary habits of *A. majusculus* provides crucial insights that can inform effective conservation and management strategies. The findings of this study underscore the need for targeted conservation actions to protect and restore aquatic habitats that support the primary food sources of this endangered species, such as green algae and diatoms. The presence of substantial stones in riverbeds, which provide shelter and reduce current speeds, is critical for survival of the species and should be a focus in habitat restoration efforts. In addition, the impact of climate change, weirs, monsoons, and other anthropogenic factors on the dietary composition of *A. majusculus* highlights the importance of mitigating these influences through sustainable water management practices. For instance, regulating the flow of water from weirs and dams, especially during critical breeding seasons, could help maintain the availability of essential food resources. Additionally, efforts to reduce water pollution and control invasive species can further enhance the quality and stability of the habitats.

Fecal DNA metabarcoding, while a valuable tool, does have limitations. Taxonomic assignment relies on matching sequences to the GenBank database, which is subject to various factors affecting reliability, including database completeness, query sequence length, and similarity threshold (Alberdi et al. 2019). However, these criteria vary widely among studies, lacking a consistent pipeline. Although our study collected a large number of samples, it lacked winter samples from the Nakdong River. Sampling *A. majusculus* during winter is challenging, as the species migrates to deeper habitats to endure the cold period. A more comprehensive characterization of *A. majusculus* diet would necessitate a larger sample size, expanded sites, and year-round sampling, particularly in the seasons when collecting samples is most challenging. Validating the reliability of our dietary information could involve assessing food availability at sampling sites through direct observation or employing DNA metabarcoding on water samples. Additionally, noting environmental factors like water temperature, pH, velocity, and discharge frequency could offer insights into the dietary preferences of *A. majusculus*.

The current study highlights that the endangered bitterling *A. majusculus* primarily consumes green algae and diatoms, alongside other aquatic organisms in much smaller proportions. Detailed taxonomic identification of these prey items to the species level was provided. Additionally, its feeding behavior appears consistent across body size and sex. Conservation measures must be taken to conserve these essential food sources to ensure sustained food availability for *A. majusculus*. The study provides evidence of temporal variation in diet, particularly in summer compared to the other seasons. This emphasizes the necessity for continuous year-round dietary monitoring, especially by extending sample collection into summer and winter seasons. The application of fecal DNA metabarcoding demonstrated in this study is

not only valuable for *A. majusculus*, but also holds promise for other threatened and endangered species. This non-invasive, accurate, and cost-effective method can be widely adopted to study the foraging ecology and food availability of various species, contributing to the development of effective conservation strategies across different ecosystems.

Data availability statement. The datasets are available from the corresponding author on reasonable request.

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