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RESEARCH

Diet analysis of *Clithon retropictum* in south coast of Korea using metabarcoding

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Background: This study focused on the diet of *Clithon retropictum*, level II endangered species in Korea. Since the development of brackish water zones has led to a decline in the population of this species, to obtain information on the ecology of *C. retropictum* required for its conservation and restoration. To investigate the actual preys of *C. retropictum* in south coast of Korea, we conducted high-throughput sequencing and metabarcoding techniques to extract DNA from gut contents and periphyton in their habitats.
Results: Total 118 taxonomic groups were detected from periphyton samples. 116 were Chromista and Cyanobacteria dominated in the most samples. In gut contents samples, 98 taxonomic groups were detected. Similar to the results of periphyton, 96 were Chromista and Cyanobacteria dominated in the most samples. In the principal component analysis based on the presence/absence of taxonomic groups, gut content composition showed more clustered patterns corresponding to their habitats. Bryophyta was the most crucial taxonomic group explaining the difference between periphyton and gut contents compositions of *C. retropictum*.
Conclusions: Our finding suggests that *C. retropictum* may not randomly consume epilithic algae but instead, likely to supplement their diet with Bryophyta.

Keywords: brackish water zone, *Clithon retropictum*, diet analysis, gut contents, metabarcoding

Introduction

The *Clithon retropictum* belongs to the order Cycloneritida, family Neritidae and genus Clithon (genus Clithon Montfort, 1810) and is known to be the only species in the genus in South Korea. It has a distinct distribution pattern along the southern coast, Jeju Island, and few areas of the East Sea coast. Habitats for *C. retropictum* populations are located mainly in brackish water zones, where they typically occupy very narrow ranges of 1 to 20 m for their activities (Han et al. 2021). Brackish water zones are highly dynamic environments. While species diversity in these areas is limited, species living in these conditions make up a significant proportion of the unique biodiversity (Cognetti and Maltagliati 2000). Due to development of coastal areas, including brackish water zones, the habitat of the *C. retropictum* has undergone significant changes, leading to a decline in the population of this species. As a result, the Ministry of Environment designated the *C. retropictum* as a Level II endangered species, in 1998.

Research has been conducted on the habitat characteristics of the *C. retropictum* across the country, population characteristics based on environmental factors (Han et al. 2021), and estimation of occupancy probability for potential habitats (Park et al. 2022). Most of the studies on the *C. retropictum* have focused on its habitat and distribution, while research on its food sources has been largely lacking, except for few stable isotope studies (Antonio et al. 2010a, 2010b).

Since the concept of modern DNA barcoding using cytochrome *c* oxidase I was introduced in 2003 (Hebert et al. 2003), research utilizing DNA barcoding has gained momentum in various fields. Since microalgae, which play a crucial role in aquatic ecosystems and are frequently used as bioindicators, consist of diverse taxonomic groups and are challenging to identify morphologically. Consequently, DNA barcoding research in the field of microalgae, driven by the advancement of high-throughput sequencing tech-

nology, has become increasingly active (Celikkol-Aydin et al. 2016; Kang et al. 2018; Kim et al. 2023; Kowalska et al. 2019).

In this study, we aim to investigate the potential food sources within the unique environment of brackish water zone, through high-throughput sequencing and metabarcoding techniques. Through the analysis of gut contents, we attempt to uncover the taxonomic identities of the actual preys consumed to gain insights on its trophic relationships.

Materials and Methods

Sample collection

Samples of *C. retropictum* and periphyton were collected from July to August 2022 at seven brackish water zone located in the south coast of South Korea (Fig. 1; CB: Masanhappo-gu, Changwon-si, Gyeongsangnam-do, CG: Seongsan-gu, Changwon-si, Gyeongsangnam-do, CY: Masanhappo-gu, Changwon-si, Gyeongsangnam-do, GD: Samsan-myeon, Goseong-gun, Gyeongsangnam-do, GY: Geoje-myeon, Geoje-si, Gyeongsangnam-do, SS: Yonghyeon-myeon, Sacheon-si, Gyeongsangnam-do, TD: Dosan-myeon, Tongyeong-si, Gyeongsangnam-do). We randomly selected three large cobbles and removed periphyton on the surface except under 25 cm^2 square plate using disposable toothbrush. Remained periphyton under square plate was scraped off with new disposable toothbrush and flushed into sterilized 50 mL conical tube with distilled water. Collected periphyton samples were diluted with distilled water to total volume of 500 mL, and then 100 mL of aliquot was filtered through a 0.8 μm membrane filter (Whatman, Maidstone, United Kingdom) and transported to the laboratory on dry ice. Three individuals of *C. retropictum* in each site were randomly collected and transported to the laboratory on portable car fridge. Transported samples were stored at −80°C until further DNA extraction and dissection. The sample collection permit for level II endangered species was obtained through authorization of Nakdong River Basin Environment Office.

Dissection of *Clithon retropictum*

We dissected *C. retropictum* to determine the actual diets of *C. retropictum* through gut content analysis. Stored *C. retropictum* samples were defrosted in 4°C refrigerator overnight. Forceps and dissecting scissors were used for removing shell of *C. retropictum* and separate guts from body mass. Separated guts were stored in −20°C until DNA extraction.

DNA extraction, paired-end library preparation and sequencing

Periphyton DNA was extracted using the Exgene™ Cell SV (Geneall, Seoul, Korea) following the manufacturer's protocol. To facilitate DNA extraction, the membranes were cut using scissors before extraction. Guts from *C. retropictum* were homogenized using mixer mill (MM200; Retsch, Haan, Germany), and DNA was extracted using the Exgene™ Stool DNA mini (Geneall) following the manufacturer's protocol.

For construct the paired-end libraries, we conducted two-step tailed PCR approach (Miya et al. 2015). Using the p23SrV_f1/p23SrV_r1 primer pair (GGA CAG AAA GAC CCT ATG AA/ TCA GCC TGT TAT CCC TAG AG, Sherwood and Presting 2007) with overhang adapter sequence targeting 23S rDNA plastid region. For multiplexing of

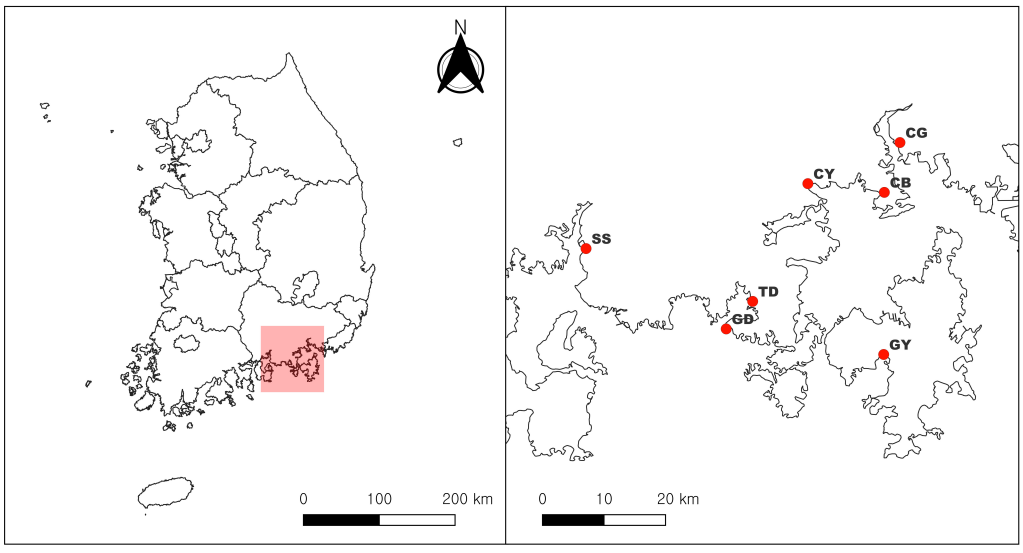

Fig. 1 Map of sampling sites in this study. CB: Masanhappo-gu, Changwon-si, Gyeongsangnam-do; CG: Seongsan-gu, Changwon-si, Gyeongsangnam-do; CY: Masanhappo-gu, Changwon-si, Gyeongsangnam-do; GD: Samsan-myeon, Goseong-gun, Gyeongsangnam-do; GY: Geoje-myeon, Geoje-si, Gyeongsangnam-do; SS: Yonghyeon-myeon, Sacheon-si, Gyeongsangnam-do; TD: Dosan-myeon, Tongyeong-si, Gyeongsangnam-do.

DNA from periphyton samples, we used a primer pair with fixed tag.

The first PCR conducted using AccuPower® HotStart PCR PreMix (Bioneer, Daejeon, Korea). Touchdown PCR was conducted to prevent amplification of unintended sequences. The Amplification conditions were as follows: initial denaturation at 95°C for 2 minutes, followed by 40 cycles of 94°C for 30 seconds, 66°C (temperature decreased 0.5°C every cycle until temperature of 58°C was reached) for 30 seconds, 72°C for 30 seconds, and a final extension at 72°C for 10 minutes (Sherwood et al. 2008). PCR products were purified using AccuPrep® PCR/Gel Purification Kit (Bioneer) and, quantified using BioPhotometer® 6131 (Eppendorf, Hamburg, Germany). We distinguish periphyton sequencing results from gut contents ones using g-prefix to site symbols.

The second PCR and Miseq sequencing were performed by commercial sequencing service company (Macrogen, Seoul, Korea).

Bioinformatics analysis and statistical analysis

The raw sequences were analyzed using Qiime2 (version 2023.2; Bolyen et al. 2019). Multiplexed samples were demultiplexed using cutadapt plugin (Martin 2011). Amplicon sequence variants (ASVs) were generated followed by quality filtering, denoising and chimera removal using DADA2 (Callahan et al. 2016). Taxonomic classification was performed using the q2-feature-classifier plugin (Bokulich et al. 2018) trained using μgreen-db r1.1 (Djemiel et al. 2020). Statistical analyses were performed using R (version 4.3.0).

Results

Results of the bioinformatics analysis

During the bioinformatics analysis using Qiime2, reads that did not meet the criteria were removed at each step. On average, a total of 11.31%, 27.53%, and 61.58% of input reads were eliminated during the quality filtering, denoising-merging and chimera removal steps, respectively (Table S1).

Detected sequences from periphyton from habitats of *Clithon retropictum*

After taxonomic assignment of observed ASVs after DADA2 analysis using q2-feature-classifier plugin from periphyton samples, we obtained 118 taxa including. One hundred sixteen Chromista taxa, and one each taxon from plantae and unassigned taxa. Most abundant taxa were detected in the phylum Cyanobacteria, Chlorophyta and Bacillariophyta. Similarly, these three phyla were dominant in terms of read number (Table 1).

We compared compositions of taxa in terms of habitats and found that composition of abundant taxa varies by habitats. Cyanobacteria were the most dominant taxon in most site. However, Chlorophyta were the most dominant in CG, followed by Cyanobacteria and Bacillariophyta (Fig. 2).

Sequences of unassigned Chromista detected at all sampling sites. Unassigned Chromista shows the fourth highest percentage of total read number, at about 7.5%.

Detected sequences from gut contents of *Clithon retropictum*

After taxonomic assignment of observed ASVs from gut contents of *C. retropictum*, we identified 98 groups including, 96 Chromista, 2 Plantae and 1 unassigned taxon. Similar to the results of periphyton, most abundant taxonomic groups detected were in the order of prevalence, Cyanobacteria, Chlorophyta and Bacillariophyta. The unassigned Chromista had the third highest group in terms of read count, after Cyanobacteria and Chlorophyta, outnumbering Bacillariophyta. Two taxonomic groups detected as

Table 1 List of detected taxonomic groups in periphyton samples from habitat of *Clithon retropictum* (n = 7)

Kingdom	Phylum	No. of taxonomic groups	No. of reads	Relative read abundance (%)
Chromista	Bacillariophyta	12	38,209	11.412
	Charophyta	4	289	0.086
	Chlorophyta	25	99,874	29.830
	Cryptophyta	5	105	0.031
	Cyanobacteria	57	166,324	49.677
	Euglenozoa	1	12	0.004
	Miozoa	3	3,173	0.948
	Ochrophyta	5	1,629	0.487
	Rhodophyta	3	39	0.012
	Unknown	1	25,084	7.492
Plantae	Unknown	1	31	0.009
Unassigned	-	1	41	0.012
Total	-	118	334,810	100

Taxonomic assignment was performed based on amplicon sequence variants.

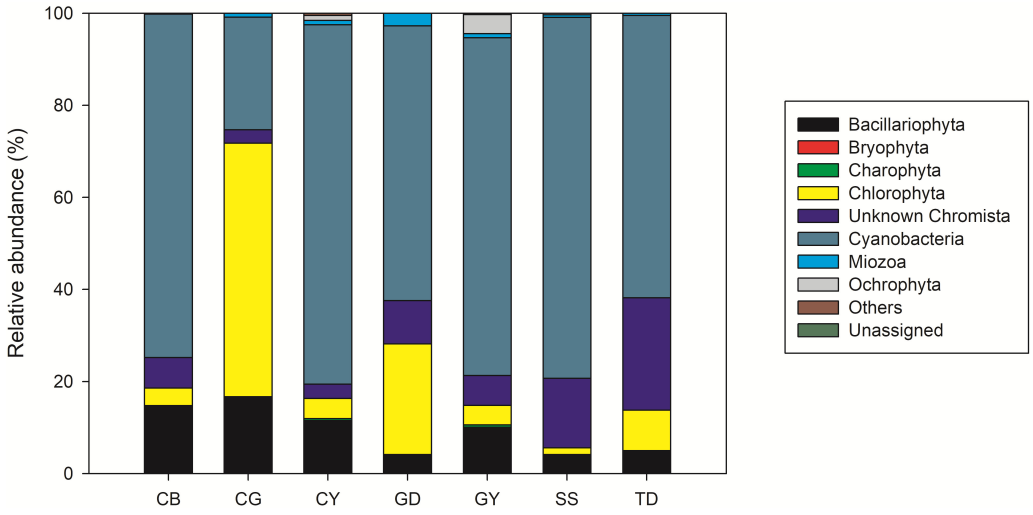

Fig. 2 Relative abundance of detected taxonomic groups in different *Clithon retropictum* habitat. CB: Masanhappo-gu, Changwon-si, Gyeongsangnam-do; CG: Seongsan-gu, Changwon-si, Gyeongsangnam-do; CY: Masanhappo-gu, Changwon-si, Gyeongsangnam-do; GD: Samsan-myeon, Goseong-gun, Gyeongsangnam-do; GY: Geoje-myeon, Geoje-si, Gyeongsangnam-do; SS: Yonghyeon-myeon, Sacheon-si, Gyeongsangnam-do; TD: Dosan-myeon, Tongyeong-si, Gyeongsangnam-do.

Table 2 List of detected taxonomic groups from gut contents of *Clithon retropictum* (n = 21)

Kingdom	Phylum	No. of taxa	No. of reads	Relative read abundance (%)
Chromista	Bacillariophyta	8	95,993	6.412
	Charophyta	2	1,415	0.095
	Chlorophyta	18	228,940	15.292
	Cryptophyta	3	756	0.050
	Cyanobacteria	55	928,085	61.990
	Euglenozoa	4	1,399	0.093
	Miozoa	3	12,438	0.831
	Ochrophyta	1	38,106	2.545
	Unknown	1	114,589	7.654
Plantae	Bryophyta	1	52,399	3.500
	Unknown	1	1,194	0.080
Unassigned	-	1	21,850	1.459
Total	-	98	1,497,164	100

Taxonomic assignment was performed based on amplicon sequence variants.

Plantae. Bryophyta was a frequent sequence, being detected in 16 out of 21 samples and accounting for up to 17.837% (gGY1) of the total number of reads detected in the sample (Table 2, Fig. 3).

The principal component analysis (PCA) scores revealed that the gut content composition of gTD1 and gTD2, both of which are predominantly dominated by Chlorophyta, exhibited distinct characteristics compared to other individuals, while the gut contents of other individuals appeared to form weaker associations according to their habitats. In the PCA results based on the presence/absence of taxonomic groups, it became evident that the gut content composition of individuals from different habitats, except for the CY site, showed similar patterns corresponding to their respective habitats (Fig. 4).

Comparing periphyton and gut content of *Clithon retropictum*

The comparison analysis of periphyton and gut content, subjected to MiSeq analysis from different habitats, was conducted based on the presence or absence of detected taxonomic groups. PCA analysis revealed a distinct separation between gut contents and periphyton samples (Fig. 5). To discern the factors contributing to this difference, we conducted an orthogonal partial least squares discriminant analysis (OPLS-DA). The most crucial taxonomic group identified from OPLS-DA and S-Plot was Bryophyta (Fig. 6). This group was absent in periphyton samples but was detected in the gut contents of all individuals except for gCB1, gCB2, gCY2, gGD2, and gTD1, resulting in its detection in 16 out of 21 gut content samples (Table 3).

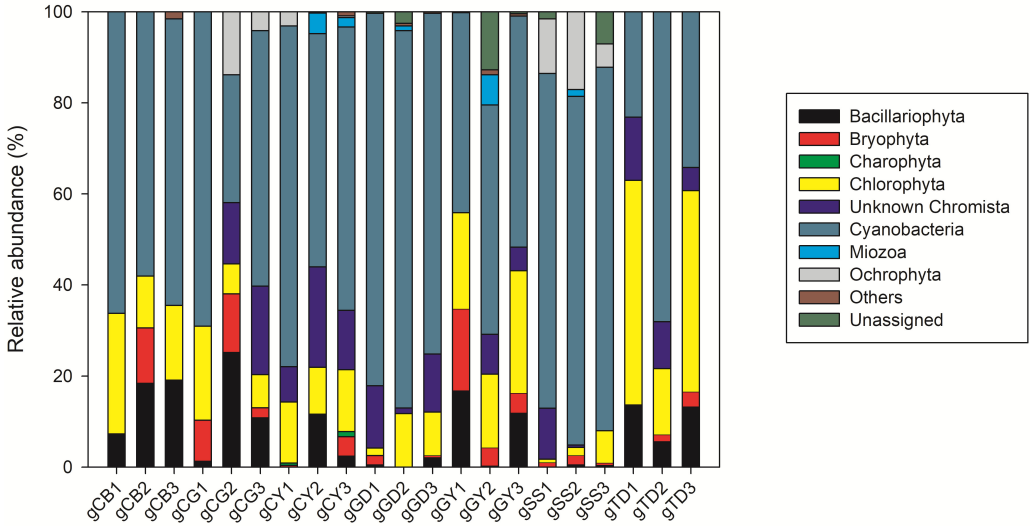

Fig. 3 Relative abundance of detected taxonomic group from gut content of *Clithon retropictum* at phylum level (unknown Chromista and unassigned groups are displayed at the lowest taxonomic level) (Cryptophyta, Euglenozoa, Rhodophyta, and unknown Plantae were grouped as Others). CB: Masanhappo-gu, Changwon-si, Gyeongsangnam-do; CG: Seongsan-gu, Changwon-si, Gyeongsangnam-do; CY: Masanhappo-gu, Changwon-si, Gyeongsangnam-do; GD: Samsan-myeon, Goseong-gun, Gyeongsangnam-do; GY: Geoje-myeon, Geoje-si, Gyeongsangnam-do; SS: Yonghyeon-myeon, Sacheon-si, Gyeongsangnam-do; TD: Dosan-myeon, Tongyeong-si, Gyeongsangnam-do.

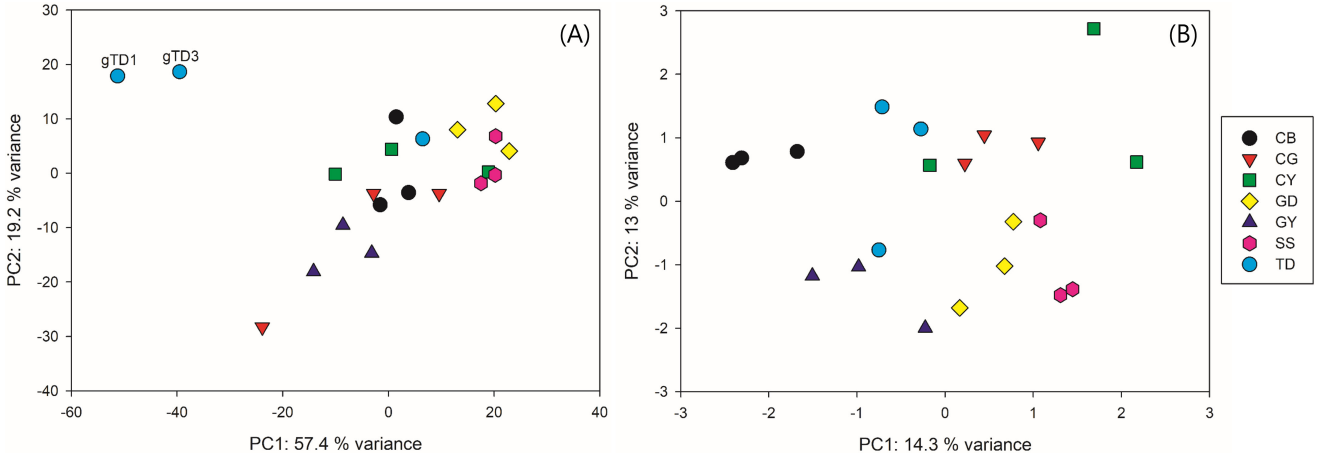

Fig. 4 PCA results of detected taxonomic groups from *Clithon retropictum* gut contents (A) based on the read relative abundance and (B) presence/absence of taxonomic groups. Taxonomic groups have been assigned, down to lowest taxonomic level based on ASVs. CB: Masanhappo-gu, Changwon-si, Gyeongsangnam-do; CG: Seongsan-gu, Changwon-si, Gyeongsangnam-do; CY: Masanhappo-gu, Changwon-si, Gyeongsangnam-do; GD: Samsan-myeon, Goseong-gun, Gyeongsangnam-do; GY: Geoje-myeon, Geoje-si, Gyeongsangnam-do; SS: Yonghyeon-myeon, Sacheon-si, Gyeongsangnam-do; TD: Dosan-myeon, Tongyeong-si, Gyeongsangnam-do; PCA: principal component analysis; ASV: amplicon sequence variant.

Diversity of potential and actual prey of *Clithon retropictum*

The Shannon diversity of periphyton samples ranged from 1.36 to 2.39, while gut content samples ranged from 1.56 to 2.75. Among the gut content samples, the samples from CG site showed a relatively high variation with a standard deviation of 0.56, while the rest of the sites showed relatively low variation in diversity index with standard deviation of 0.06 to 0.22. To compare the Shannon diversity index of potential and actual prey of *C. retropictum*, regression analysis was conducted. No significant association was detected between the Shannon diversity of potential and actual prey (Fig. S1, $R^2 = 0.0554$).

Discussion

In this study, we investigated the potential food source and actual diet of *C. retropictum* using high-throughput sequencing and metabarcoding techniques (Figs. 2 and 3, Tables 1, 2). Due to the characterization of *C. retropictum* as feeding on epilithic algae (Antonio et al. 2010a) and the difficulty in morphological classification of benthic algae, DNA based analysis was conducted. To selectively amplify

only the DNA of algae in the sample, touchdown PCR with primer pair targeting plastid 23S DNA was used (Sherwood et al. 2008). In the ASVs detected after Miseq sequencing and bioinformatics analysis on the Qiime2 platform, more than 90% (periphyton: 99.978%; gut content: 94.962%) of the reads were belonging to Chromista including Cyanobacteria, Chlorophyta, and Bacillariophyta were amplified as intended, suggesting that the primers and touchdown PCR technique used are suitable as a tool to study benthic algal composition. However, taxonomic groups belonging to Plantae were also detected. This is believed to be due to the primer pair used targeting the 23S rDNA plastid region, resulting in the amplification of sequences from plant lineages with relative to Chlorophyta.

Brackish water zones have very unusual biota because the daily fluctuations in salinity due to the tidal rhythm acts as a very effective barrier for many species, especially freshwater organisms that are not tolerant of salt water (den Hartog 1967; Cognetti and Maltagliati 2000). The community structure of benthic algae varies depending on the type of substrate (Kim et al. 2009), but the composition of benthic algae in brackish water zone has not been available so far. The results of DNA metabarcoding of benthic algae in this study show that benthic algae in the brackish water zone were mainly composed of Cyanobacteria, Bacillariophyta and Chlorophyta (Fig. 2). Interestingly, the periphyton sample from the CG site was dominated by Chlorophyta, which is likely due to the dominance of Ulvophyceae

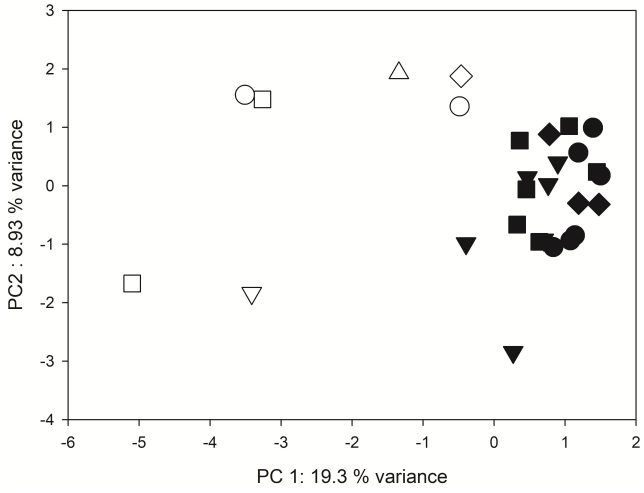

Fig. 5 PCA results of detected taxonomic groups from gut contents and periphyton, based on presence/absence of taxonomic groups. Open circles indicate periphyton while closed circles indicate gut contents from *Clithon retropictum*. PCA: principal component analysis.

Table 3 Mean and standard deviation of the number of reads from Bryophyta

Sampling site	Number of reads based on the origin of sequence	
	Periphyton	Gut contents
CB	ND	1,942.666
CG	ND	4,999 ± 3,308.799
CY	ND	1,203
GD	ND	597 ± 780.351
GY	ND	6,809 ± 4,588.635
SS	ND	805 ± 435.632
TD	ND	1,110.667 ± 983.469

Values are presented as number only or mean ± standard deviation.
CB: Masanhappo-gu, Changwon-si, Gyeongsangnam-do; CG: Seongsan-gu, Changwon-si, Gyeongsangnam-do; CY: Masanhappo-gu, Changwon-si, Gyeongsangnam-do; GD: Samsan-myeon, Goseong-gun, Gyeongsangnam-do; GY: Geoje-myeon, Geoje-si, Gyeongsangnam-do; SS: Yonghyeon-myeon, Sacheon-si, Gyeongsangnam-do; TD: Dosan-myeon, Tongyeong-si, Gyeongsangnam-do; ND: not detected.

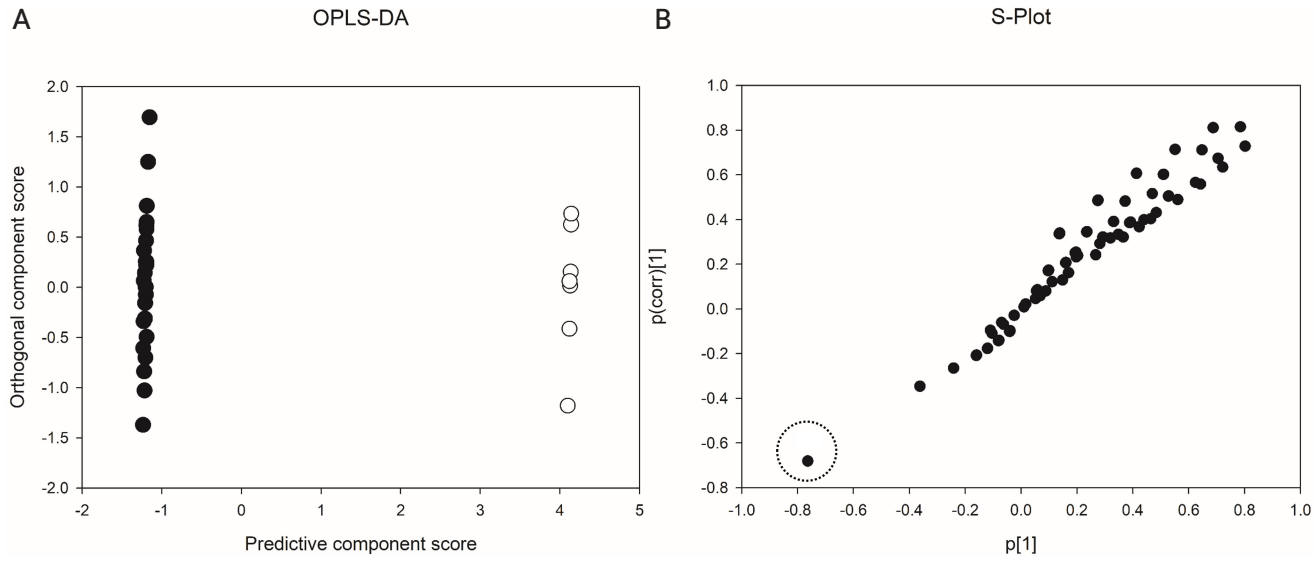

Fig. 6 OPLS-DA and S-plot results, based on presence/absence of taxonomic groups. (A) Scores from OPLS-DA. Open circles indicate taxonomic groups profile from gut contents of *Clithon retropictum* while closed circles indicate taxonomic groups profile from periphyton. (B) S-Plot of loadings (p[1]) and correlation loadings (p(corr)[1]). The closed circle in the dotted circle indicates important taxonomic group Bryophyta. OPLS-DA: Orthogonal partial least squares discriminant analysis.

(relative abundance of Ulvophyceae at each site. CY: 2.78, CB: 3.74, TD: 8.81, GY: 1.24, SS: 1.28, GD: 24.0, CG: 54.9%). This suggests that CG sites may be a more favorable environment for the growth of Ulvophyceae than other sites. However, since the sampling was done only once, we could not determine major environmental factors influence of the change in benthic algae composition. Previous studies utilizing stable isotope analysis had reported that *C. retropictum* selectively feeds on epilithic organic matter and only feed sediment organic matter in the absence of epilithic algae (Antonio et al. 2010a, 2010b). Similarly, in this study, Cyanobacteria, Chlorophyta, and Bacillariophyta, which are thought to be epilithic algae, had the highest percentages, supporting previous studies that *C. retropictum* selectively feeds primarily on epilithic organic matter.

We found that only up to the kingdom level (Chromista) the assigned reads represented a proportion of more than 7% (periphyton: 7.492%; gut content: 7.654%) of the total read number. This may be due to the limitations of the universal algae markers used (Kezlya et al. 2023) or the lack of sequence information for benthic algae living in the highly specific biota area of the brackish water zone.

Based on the presence/absence of taxonomic groups detected in the periphyton and gut content of *C. retropictum*. We found that Bryophyta was only detected in gut content samples. The mean relative read abundance of Bryophyta in the gut contents was 3.5%, which is a relatively low percentage, suggesting that *C. retropictum* feeds primarily on benthic algae, but the presence of Bryophyta that were not detected in the periphyton samples suggests that *C. retropictum* may have other food sources, such as sediments, in addition to epilithic algae. Furthermore, a comparison of the Shannon diversity index of detected ASVs in periphyton and gut content samples supports this idea, as the diversity index of periphyton sample was not significantly associated with the diversity index of gut content.

Conclusions

In summary, we conducted diet analysis of *C. retropictum*, a Level II endangered species, through molecular approaches. Our findings suggest that *C. retropictum* may not randomly consume epilithic algae but instead, likely to supplement their diet with Bryophyta and high-throughput sequencing was an efficient tool for detect algal DNA in brackish water zone. These results may serve as the base data for preservation of endangered species in brackish water zone. In further studies, fatty acid analysis for indicate food quality can provide data on the effects of dietary changes due to brackish water zone urbanization.

Supplementary Information

Supplementary information accompanies this paper at https://doi.org/10.5141/jee.23.084.

Table S1. Changes in the number of reads during the bioinformatics processing using Qiime2. **Fig. S1.** Interrelationship among Shannon diversity index of periphyton (potential prey) and gut content (actual prey).

Abbreviations

ASV: Amplicon sequence variant

PCA: Principal component analysis

OPLS-DA: Orthogonal partial least squares discriminant analysis

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Authors' contributions

SWH analyzed and interpreted data of all samples. YB conducted the collection of *C. retropictum*. SWH, DH, YB, EL, and SP contributed to the collection of periphyton. SWH was major contributor in writing and editing the manuscript. All authors were involved reviewing the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The dataset generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

Corresponding author Sangkyu Park has been Editor-in-Chief of *Journal of Ecology and Environment* since 2019. He was not involved in the review process of this article. Otherwise, no potential competing interest was reported with this article.

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