



Biodiversity and spatial distribution of ascidian using environmental DNA metabarcoding

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ABSTRACT

Monitoring studies are necessary to understand the biodiversity of marine ecosystems and are useful for identifying and managing rare or invasive species. Because monitoring has traditionally relied only on visual surveys (e.g., trapping, netting, electrofishing, and SCUBA diving) with limited time and physical resources, environmental DNA (eDNA) analysis is being applied as an efficient monitoring method. This study compared whether the eDNA metabarcoding technique can replace the traditional visual survey in an ascidian fauna study. We designed ascidian-specific primers and identified a clear gap (3.75%) by barcoding gap analysis. Then, we collected seawater samples for eDNA analysis during the summer (August–September) of 2021 at three sites (Mokpo, Yeosu, and Uljin) in South Korea. In the survey sites of this study, 25 species were observed through literature and visual survey, among which 9 species were detected by metabarcoding and 16 species were not detected. On the other hand, 10 species were detected only by metabarcoding, and one of them was identified as *Pyura mirabilis*, an unrecorded species in South Korea. This study succeeded in detecting cryptic or rare species with one seawater collection, which can be used to determine their unexplored habitat. Therefore, we conclude that monitoring using eDNA is more efficient than visual surveys for detecting rare or cryptic ascidian species. We also suggest that, when combined with traditional monitoring methods, it could be a tool to complement ascidian fauna studies.

1. Introduction

The basis of ecological and biodiversity studies is the knowledge of the species existing in that ecosystem. It is essential to understand and monitor the biodiversity of ecosystems, which depends not only on the functional characteristics of each species but also on the interactions among those species. Therefore, continuous monitoring should be conducted (Mermillod-Blondin et al., 2005). Moreover, monitoring studies are necessary for effective ecosystem management, including the detection of invasive species (Epanchin-Niell et al., 2012; Brown et al., 2016) and conservation of ecological communities (Campbell et al., 2002; Heyde et al., 2020). Monitoring studies have traditionally relied on visual surveys, with species identification based on morphology. Time and physical resources are limited with these traditional survey methods (e.g., trapping, netting, electrofishing, and SCUBA).

Furthermore, these methods can sometimes cause stress or mortality in organisms and damage habitats, leading to biased study results. Furthermore, because the method of morphological identification differs depending on the taxa, it is difficult to rely on morphology for wide-range monitoring. Along with the demand for efficient monitoring, environmental DNA (eDNA) analysis has been applied to complement existing traditional survey methods (Thomsen and Willerslev, 2015; Adams et al., 2019).

Environmental DNA metabarcoding analysis is a method of sequencing barcode regions from DNA extracted from environmental samples, including sediments, water, seawater, and air (Bohmann et al., 2014; Alberdi et al., 2018). Metabarcoding of eDNA is increasingly being applied to determine higher levels of taxonomic composition in aquatic and terrestrial environments (Valentini et al., 2016; Kelly et al., 2017) and can determine the presence and abundance of organisms (Foote

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et al., 2012; Andruszkiewicz et al., 2017). Moreover, water sampling is relatively easy, and eDNA has excellent temporal and spatial efficiency for obtaining biodiversity data making it suitable for monitoring various marine organisms, including vertebrates and invertebrates (Thomsen and Willerslev, 2015; Andruszkiewicz et al., 2017; Heyde et al., 2020). Ascidians belong to the dominant group of marine benthic invertebrates (Jackson, 1977) and are often observed covering rocks and artificial structures from intertidal and shallow subtidal zones to deep seas (Lambert, 2005; Shenkar and Swalla, 2011). Because these species affect ecosystems globally due to biofouling issues and the rapid spread of invasive species, biodiversity and continuous monitoring are required (Lambert, 2009; Stefaniak, 2009; Li et al., 2019). Despite the importance of this monitoring, ascidians have continued to be investigated and classified traditionally. To advance the ecological investigation and management of ascidians, eDNA metabarcoding is required. However, to date, there have been no metabarcoding studies on ascidians, only studies to analyze the diversity of marine fish, zooplankton, microbes, etc., or to classify marine organisms in a specific area at the class level (Lallias et al., 2015; Bucklin et al., 2016; Miya, 2022).

Here, to examine the ascidian fauna of South Korea, we referred to reference studies such as survey records and the literature and in order to design ascidian-specific primers to be used for eDNA metabarcoding, various base sequences of ascidians registered in NCBI GenBank were used. Moreover, we compared the taxonomic composition of ascidians via eDNA analysis with a traditional faunal study in three sea areas (Yellow Sea, Korea Strait, and East Sea). This is the first study to survey the ascidian fauna using eDNA and comparing eDNA metabarcoding results with a traditional survey.

2. Materials and methods

2.1. Study sites and reference study

This study was conducted by selecting survey sites located in three sea areas (the Yellow Sea, Korea Strait, and East Sea) on the Korean Peninsula. The survey sites were Bieung harbor (38°26'53.06"N, 128°27'46.04"E) located in Mokpo City (hereafter Mokpo), Odong harbor (38°26'53.06"N, 128°27'46.04"E) located in Yeosu City

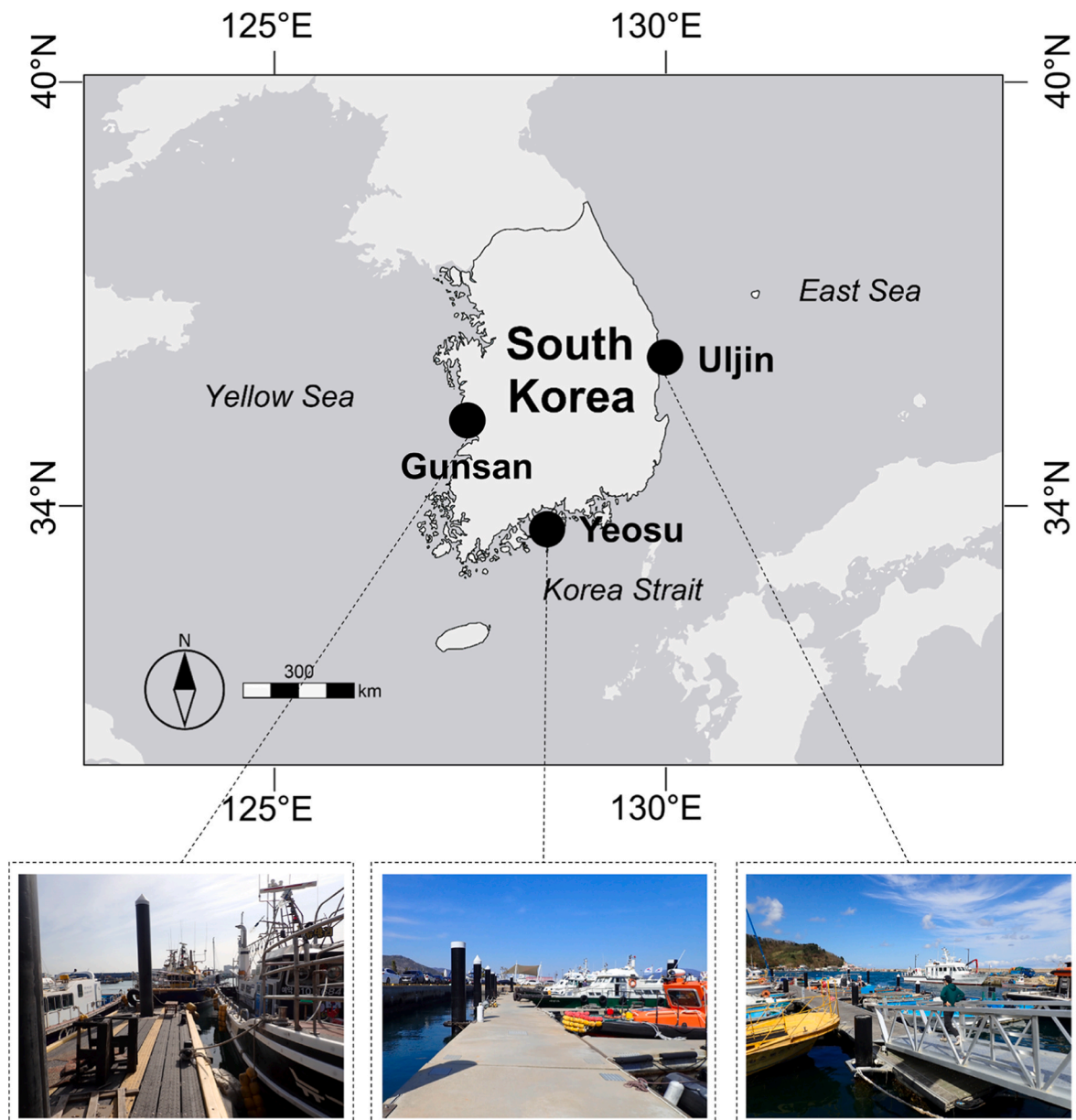


Fig. 1. Map showing the location of the three survey sites in the southern part of the South Korea and foreground photo of each survey site.

(hereafter Yeosu), and Osan harbor (38°26'53.06"N, 128°27'46.04"E) located in Uljin City (hereafter Uljin). Mokpo is in the Yellow Sea area affected by the Korean Coastal Current; Yeosu is in the Korean Strait area affected by the Tsushima Warm Current; and Uljin is in the East Sea area affected by the North Korean Coastal Current (Fig. 1). To minimize the effect of seawater collection time on eDNA abundance in season-based gaps, all seawater samples were collected during the same season (summer) from August–September 2021. For the comparison of ascidian diversity, the depth of seawater collection was 0.5–1.0 m depending on the habitat characteristics of the ascidian in each harbor.

For reference, we collected documentary data, including the status of specimens registered in national research institutes, species-recording research papers, and encyclopedias. To compare ascidian diversity measured in a traditional visual survey versus the eDNA metabarcoding technique, we gathered ascidian distribution information from an encyclopedia and various literature from 1971 to 2021 (Rho, 1971, 1977, 1995; Pyo et al., 2012; Lee and Shin, 2014, 2021). We also investigated the ascidian specimen collection status from the National Institute of Biological Resources (<https://species.nibr.go.kr>) and the Korean Natural History Research Information System (NARIS, <https://www.naris.go.kr/main.do>). The classification status and taxonomic information of the species were identified based on the currently accepted nomenclature in the World Register of Marine Species (WoRMS; <https://www.marinespecies.org>; accessed May 2021).

2.2. Seawater sampling and isolation of eDNA

Seawater samples were collected from each site using sterilized bottles (Sterile LDPE Water Sample Bottle, Daihan Scientific Co., Korea) and stored at 4 °C until filtration within 4 h. Seawater was sampled (2.5 L) four times at intervals of 10 m from each survey site to cover the entire floating dock area. These four 2.5 L samples were combined into a total of 10 L, and then 1 L was filtered through a 1.2 µm mixed cellulose ester membrane (Merck Millipore Corp., Billerica, MA, USA). Before filtration, the seawater was sieved through a 500 µm stainless mesh to avoid non-biotic component clogs, such as sand, mud, and plastic particles. All filtration equipment used for eDNA sampling was cleaned using 10% bleach in every eDNA sampling and seawater filtration step to avoid cross-contamination. After filtration, the filter membranes were immediately stored in an icebox with dry ice (−20 °C) and transported from the field sites to the laboratory. It did not exceed the 24 h, including transportation, until isolation of eDNA from filtered membranes.

DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) was used for both genomic DNA and eDNA isolation. Genomic DNA and eDNA were isolated from alcohol-fixed gonad tissue and seawater-filtered membranes, respectively. The kit instructions were followed, except for adding additional buffers (180 µL of buffer ATL and 20 µL of proteinase K) during the lysis and elution steps. To reduce the effects of PCR inhibitors during amplification, diethyl pyrocarbonate (DEPC)-treated water (Thermo Fisher Scientific, Cleveland, USA) was used instead of AE buffer. All eDNA were stored at −20 °C until further analysis.

2.3. Development of ascidian-specific primer set

We designed an ascidian-specific primer set using the ascidian molecular metabarcoding region and mitochondrial cytochrome *c* oxidase subunit I (COI) sequences for molecular species identification. To determine the conserved nucleotide regions that specifically bind to ascidian while excluding other taxa, COI sequences of various taxonomic groups, such as barnacle, hydrozoan, sponge, bryozoan, echinodermata, and bivalve, which share habitats with ascidian, were downloaded from the National Center for Biotechnology Information (NCBI) GenBank (<https://www.ncbi.nlm.nih.gov/>). For reliability and accuracy of sequence information, COI sequences of taxonomic groups other than ascidians were obtained from complete mitochondrial genome data. The

COI sequences of non-target taxa included 274 species, 38 genera, 20 orders, 12 classes, and 8 phyla, including benthic invertebrates (Table S1). In the case of ascidians, to obtain as much COI data as possible, we collected all sequences regardless of molecular type, such as partial and mitochondrial genomes, and a total of 3669 sequences were gathered.

A homogeneous sequence database was obtained by aligning the entire nucleotide sequence, and regions suitable for primer-binding were identified. Primers were designed with short nucleotide regions (197 bp) capable of specifically binding ascidians in the forward and reverse directions in the COI region. Subsequently, to test the effectiveness of the primer using *in silico* analysis, we reassembled the COI dataset (2922 sequences), excluding non-ascidian sequences, non-overlapping, too short, and non-amplifying sites in alignment status. All validation steps of the primer set were performed using Geneious Prime version 2021.2.2 (Biomatters Ltd., Auckland, New Zealand).

2.4. Barcoding gap analysis

Appropriate intra- and interspecific variations are the key criteria for species identification. Thus, eligible barcodes require that interspecific genetic variation be significantly greater than intraspecific genetic variation. The sufficient distance between these two variations (intra- and interspecific) is known as the barcoding gap; ideally, the two variations should not overlap. In this study, we used our primer set for accurate molecular identification as a mini barcode marker. We computed the barcoding gaps with R, version 4.0.2 (R core team, 2013) using the “BarcodingR” package (Zhang et al., 2017; <https://cran.r-project.org/web/packages/BarcodingR/index.html>) and calculated the genetic distance using the K80 model. Barcoding gap analysis was performed using a dataset of 2357 sequences and 198 species, excluding non-identified data from that used for *in silico* analysis.

2.5. PCR amplification, library preparation and next-generation sequencing (NGS)

To verify the specificity of the ascidian-specific primers, we conducted PCR amplification using genomic DNA from 20 species belonging to the classes Gymnolaemata, Crinoidea, Asteroidea, Thecostraca, Echinoidea, and Ascidiacea (Table S2). Specimens were deposited at the National Marine Biodiversity Institute of Korea (Seocheon, Korea). Genomic DNA was extracted using the QIAGEN DNeasy Blood and Tissue Kit. The designed ascidian-specific primers were amplified using the AccuPower PCR PreMix (BIONEER, Daejeon, South Korea). The total volume (20 µL) for the PCR analysis contained AccuPower PCR PreMix, 1.5 µL of template DNA, 1 µL of each primer (10 pM/µL), and 16.5 µL DEPC-treated water. The reaction conditions were as follows: initial denaturation at 95 °C for 5 min, 40 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 10 min. Two µL of the PCR product was separated by electrophoresis on a 1% agarose gel stained with a staining solution (BIONEER), and loaded together with a 100-bp DNA ladder (Takara, Tokyo, Japan) to confirm the size.

The ascidian-specific primer set we developed was used to amplify the ascidian fauna by PCR and was sequenced using the NGS method. The amplicon libraries preparation was performed on the MiSeq® Platform (Illumina, CA, USA) using the Herculase II Fusion DNA Polymerase (Agilent, Waldbronn, Germany) and Nextera XT Index Kit v2 (Illumina, CA, USA). PCR amplicons were purified with AMPure XP beads (Beckman Coulter, CA, USA), quantified with KAPA HiFi HotStart ReadyMix (Kapa Biosystems, MA, USA), amplified for an additional eight cycles to add Nextera indices and adaptors (Illumina, CA, USA). Amplicon size distribution was verified using a TapeStation D1000 (Agilent Technologies, CA, USA). Paired-end sequencing was performed on MiSeq® Platform using a 2 × 300 base pair cycle MiSeq Reagent Kit V2 (Illumina, CA, USA), following the standard Illumina sequencing

protocol (G&C Bio Co, Ltd, Daejeon, South Korea).

2.6. Data analysis

According to the NGS analysis results, we trimmed the low-quality data and deleted the adaptor sequence using Trim_Galore v0.6.1 (<https://github.com/felixkrueger/TrimGalore>). Next, the paired-end sequenced reads were assembled by BBmerge from the BBmap package v38.90 (Bushnell, 2014). We sorted the reads that were amplified with the primers we used, and the reads were clustered based on the identified values to determine the representative sequence from each cluster using CD-HIT (v. 4.8.1) (Fu et al., 2012). In addition, molecular operational taxonomic units (MOTU; “clusters”) were produced by grouping reads with 99% similarity as a cutoff using UCLUST (Edgar, 2010). For taxonomic classification of the produced clusters, BLASTn (Altschul et al., 1990) was used based on the NCBI GenBank, and only clusters with coverage and similarity of $\geq 90\%$ were used for subsequent analysis. Taxonomic assignment was accepted only when there was a percentage identity between query and reference reads of $>90\%$ and query coverage of $>90\%$.

Phylogenetic tree analysis was performed using 185 sequences of 25 species, whose distribution was confirmed in South Korea among the datasets used for barcoding gap analysis (2357 sequences). A model test was performed using the PAUP* plugin (Swofford, 2002) of Geneious Prime to estimate the optimal model for phylogenetic tree construction, and a maximum likelihood (ML) analysis was performed with 1000 bootstrap replicates based on the HKY85 model using the PhyML plugin (Guindon et al., 2010).

3. Results

3.1. Primer set validation

We designed class-specific primers to detect ascidians based on 658 bp of COI, known as the DNA barcode region (Table 1). *In silico* PCR was performed on 2922 ascidian sequences to confirm that the coverage of the designed primer pairs was consistent. Geneious Prime software (v. 21.2.2) was used to simulate the virtual amplification of a gene, and all benthic invertebrate sequences were obtained from GenBank and screened for primer-binding positions. We used the primer test-related function provided by Geneious Prime, which allowed for up to four mismatches in the primer-binding region and one mismatch at the 3' end, and 90.7% (2651/2922 sequences) of the ascidian sequences were amplified. We compared the sequence of the primer pair with that of a non-target species retrieved from the NCBI BLAST nucleotide database. We also confirmed that the primer region hybridized to the targeted portion of the mitochondrial COI gene region. To examine whether the primers were effective, PCR analysis was conducted on DNA samples from 20 specimens consisting of various marine invertebrates, including ascidians (Table S2). The target nucleotide regions of our primer set were successfully amplified and sequenced in all ascidian specimens but not in non-target specimens.

After determining the specific primer specificity and quality, we tested the distribution of genetic distance within the amplified sequence using the primers designed in this study according to the barcoding gap analysis. Owing to the barcoding gap analysis performed on the nucleotide sequences of 198 ascidian species, a clear barcoding gap (3.75%) was observed for the ascidian primer. The intraspecific and interspecific genetic distances ranged from 0 to 6.25% and 10–90%, respectively

Table 1

Sequence list and information of ascidian-specific primers designed in this study.

Primer label	Sequence (5'–3')	Tm (°C)	Amplicon size (bp)	Target gene
AscCOLF	CCTGATATGGCNTTYCCHCG	57.3	197	Mitochondrial COI
AscCOIR	GCTAAATGHAHAHGAATAATWGC	51.7		

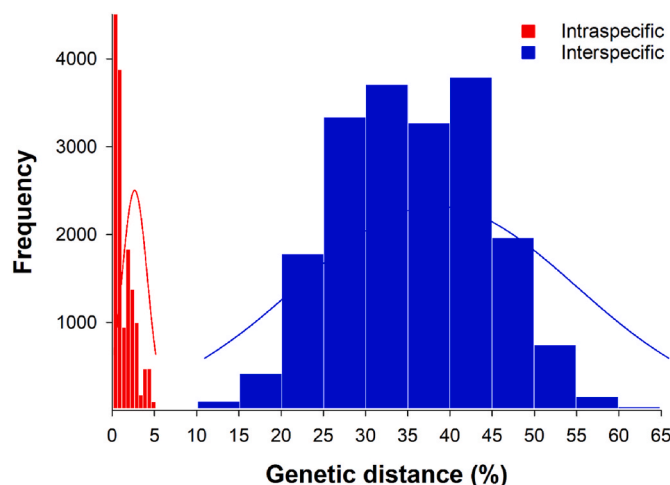


Fig. 2. Frequency histogram showing intraspecific (red) and interspecific (blue) variation by DNA barcoding gap analysis based on 197 bp of COI sequences within 198 ascidian species.

(Fig. 2 and S1). This primer had sufficient minimum interspecific and maximum intraspecific genetic distance ratios (>10 ; Hebert et al., 2003b) and thus met the criterion for a DNA barcode.

3.2. Raw sequencing results

In total, 503,312 raw reads were obtained from a single run on an Illumina MiSeq platform comprising three sites. After merging paired end reads, fastq quality filtering, and identification of tags and adapters, 148,974 target reads remained. Target reads with at least 99% homology were clustered for further analysis. In Gunsan, 31,284 clusters were generated, of which 3843 (12.28%) were ascidian. In Yeosu, 28,498 clusters were generated, with 1272 (4.46%) ascidian clusters. In Uljin, 6618 clusters and 146 (2.20%) ascidian clusters were generated (Table 2). Overall, five families, seven genera, and ten species were identified in the ascidian clusters from the three sites: six species in Gunsan, three species in Yeosu, and five species in Uljin (Fig. 4). The species with the highest number of ascidian clusters was *Ciona robusta* (3484 clusters), which had the highest percentage in Gunsan. The next highest percentages were from *Perophora japonica* (952 clusters) and *Botryllus schlosseri* (207 clusters) in Yeosu and Uljin, respectively (Fig. 3).

Table 2

Summary of NGS results obtained from PCR using ascidian-specific primers. Clusters = MOTU.

Site	Total reads	Target reads	Clusters	Ascidian clusters	Ascidian clusters (%)	Species richness
Gunsan	241,826	113,858	31,284	3843	12.28	6
Yeosu	112,418	28,498	28,498	1272	4.46	3
Uljin	149,068	6618	6618	146	2.20	5
Total	503,312	148,974	66,400	5261	7.92	9

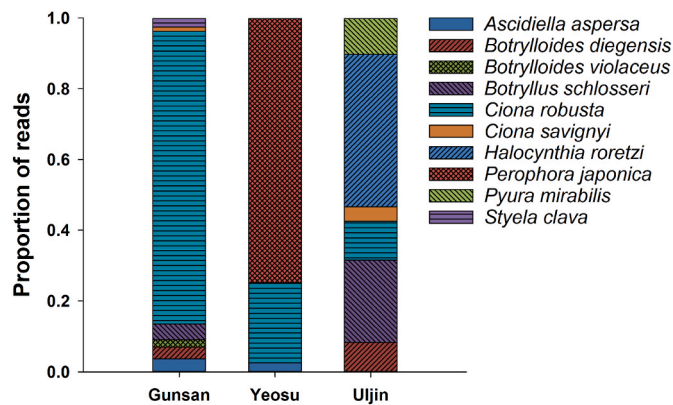


Fig. 3. Proportion of leads to ascidians in each surveyed site.

3.3. Detection of cryptic species

We analyzed the reference sequence of GenBank and the metabarcoding results using ascidian-specific primers to identify cryptic species that were not observed in previous studies. A total of 53 species were observed in the three regions by visual survey using the traditional method, but only 25 had COI sequence information available in GenBank (Table S1). Owing to the phylogenetic tree analysis, 10 cryptic species were included (Fig. 4). These species showed a genetic difference of more than 6.2% from the reference sequence, including nine species that were not identified at the species level and one species identified by the reference sequence. The unidentified species were four Cionidae, two Styela, three Ascidiidae, and one ascidian species; it was difficult to ascertain a genus or family. One species identified was *Pyura mirabilis*, which was remains unreported in South Korea (Fig. 5).

3.4. Comparison of visual survey and eDNA metabarcoding

We observed a difference in results when comparing the visual survey and metabarcoding methods. The visual survey yielded 9 species in Gunsan, 19 species in Yeosu, and 19 species in Uljin. Using metabarcoding, 16 species were observed in Gunsan, 5 in Yeosu, and 6 in Uljin. In the visual survey results, the number of species in Yeosu and Uljin was higher than that in Gunsan, whereas the metabarcoding method yielded the opposite result. In addition, seven species were observed at all three sites by visual survey, but no species overlap among the three sites was detected using metabarcoding (Fig. S2). At all survey sites, 25 species were observed by visual survey, whereas 19 species appeared through metabarcoding, including cryptic species. In comparing results from the two survey methods, 9 species were detected by both, 16 were observed only in the visual survey, and 10 appeared only in metabarcoding. With metabarcoding, only one out of ten species was identified as *Pyura mirabilis*, and the other nine species were not identified.

4. Discussion

4.1. Ascidian-specific primer

We considered the need for a rapid, sensitive, and cost-effective monitoring method for the ascidian fauna of South Korea. Fauna surveys using eDNA have temporal and spatial efficiency (Allan et al., 2021; Minamoto et al., 2017), and many studies on metabarcoding using eDNA have been conducted in the ocean (Thomsen et al., 2012; Jeunen et al., 2019; Suter et al., 2021). In previous studies, only certain taxa, such as fish, crustaceans, phytoplankton, and zooplankton, have been studied for metabarcoding. Fish and copepods for which relatively taxa-specific primer studies were actively conducted had species-level research

results (Miya et al., 2015; McDevitt et al., 2019; Fernández et al., 2021; Hirai et al., 2021), and studies on plankton had genus- or family level results, not species-level (Djurhuus et al., 2018; Wright et al., 2019). We conclude that accurate species-level detection is very important for understanding fauna. Therefore, in this study, we designed an ascidian-specific primer set that can detect the class Ascidiacea at the species level to establish the diversity of the ascidian fauna in South Korea.

The primer was designed at the COI barcoding region to be used for phylogenetic analysis, and the sequence for ascidians registered in the NCBI GenBank was used for accurate validation. However, due to misidentification and sequencing errors of nucleotide sequences registered in the NCBI GenBank, *in silico* specificity validation of primers remains a difficult issue. In the *in silico* results of this study, among the 2651 ascidian nucleotide sequences, only 90.7% were amplified (271 were not). It is likely that there are hypervariable regions and sequencing errors in the primer-hybridized sites of the COI barcode region, and the lack of these reference sequences negatively affects the resolution of the primers (Beaz-Hidalgo et al., 2015; Stavrou et al., 2018; Schenekar et al., 2020). Therefore, to increase the resolution of primers, data can be integrated from multiple primers and probe sets in multiple regions in metabarcoding-based biodiversity studies (Hajibabaei et al., 2012; Alberdi et al., 2018; Zhang et al., 2018). For example, Mifish primers, which are mainly used for fish metabarcoding studies, have an amplified region that is too short to identify tuna species; therefore, Mifish tuna was added (Miya et al., 2015). The disadvantage is that the molecular markers for detecting all fish species cannot distinguish between the specific phylogenetic groups. Therefore, to detect as many species as possible, several primers for each taxon are sometimes used (Liu and Zhang, 2021).

The ascidian COI region is also effective for molecular identification at the species level owing to its hypervariable interspecific variation. However, the hypervariability of this molecular marker may work as a limit of a molecular marker to detect all ascidians as a whole. However, because each primer has different species-specific amplifying sequence regions and different characteristics and utilities, it is difficult to detect all the sequences by the same detection rate or experiment. Therefore, we used a single primer set to reduce experimental variability.

Nevertheless, in our *in silico* PCR analysis, 271/2922 sequences could not be amplified from 19/102 species. These results demonstrate the limitations of using a single marker. However, our study expanded the scope of domestic ascidian fauna research using eDNA. In addition, this approach can be used to detect 34 species of ascidian in Korea within mixed DNA samples of various species, such as indigenous, unrecorded, and rare species, based on the same detection rate of a single primer set. However, we suggest using multiple primer sets to detect new species or obtain a more accurate species distribution. eDNA metabarcoding primers for biodiversity monitoring should be capable of detecting various species in the target taxa. The validation results of this study indicate that ascidian-specific primers can detect more than 90% of the ascidians registered in GenBank. Therefore, it is considered suitable for complementing ascidian monitoring studies using visual surveys.

4.2. Barcoding gap analysis

We used barcoding gap analysis to validate whether species discrimination was possible in the amplification region of the primers and to secure intra- and interspecific genetic distances in ascidians. The empirical threshold for species discrimination in commonly used COI genes is 1–3% (Ratnasingham and Hebert, 2007; Hebert et al., 2003a; b; Zhang and Bu, 2022). Therefore, in many previous studies, a threshold value (e.g., 2% and 3%) with unclear evidence has been frequently adopted and used (Collins and Cruickshank, 2014). When the threshold was relatively low, a species was divided into two or more species. By contrast, a relatively high threshold may merge two or more species together. Rather than using a fixed empirical threshold, an optimal

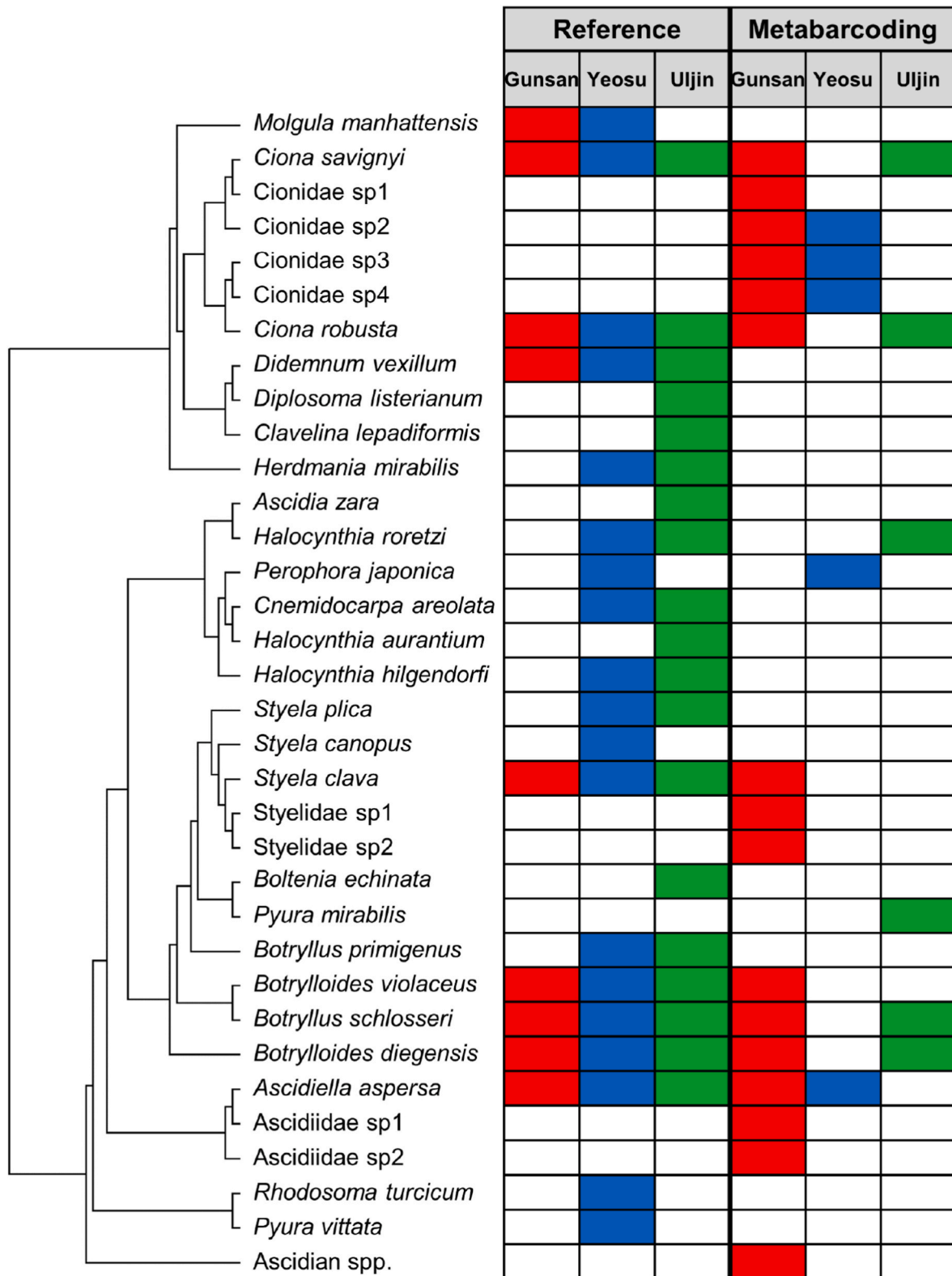


Fig. 4. Species-level taxonomic groups identified in reference studies, and eDNA metabarcoding analysis. Colored boxes indicate the species present in each of the survey sites (red: Gunsan; blue: Yeosu; green: Uljin); uncolored boxes indicate species absence. The ML tree was generated with 1000 bootstrap replicates based on the HKY85 model.

threshold should be calculated for the target taxon (Zhang and Bu, 2022). In this study, we calculated an optimal threshold using barcoding gap analysis. Gap analysis introduces errors in the accuracy of species classification when intra- and interspecies variations overlap. However,

in the present study, a clear gap was formed within and between species, suggesting that there was no error in the classification of ascidian species using the barcoding gap (Fig. 3).

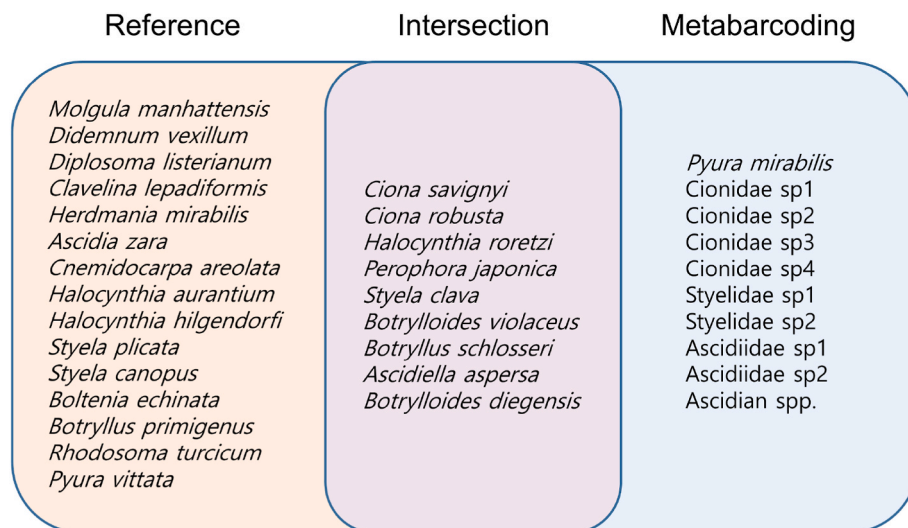


Fig. 5. Comparison of ascidian species lists from the reference studies and eDNA metabarcoding analysis. Scientific names are listed according to the maximum percent identity in the NCBI BLAST of MOTU sequences classified according to the DNA barcoding gap analysis result in this study.

4.3. Comparison with visual survey (cryptic species)

In the metabarcoding results of the three survey sites, the ratio of clusters was high, in the order of *C. robusta*, *P. japonica* and *B. schlosseri* (Fig. 3). However, since there were no abundance data from previous studies, it was difficult to confirm whether the ratio of *C. robusta* among ascidians in the study sites was the highest, even in a visual survey. Therefore, this study focused on whether the species observed in the previous study could be detected by metabarcoding and whether cryptic species (not observed in the visual survey) could be detected by metabarcoding. In all surveyed sites, 53 species (100%) were observed to be distributed by visual survey (Rho, 1971, 1977, 1995; Pyo et al., 2012; Lee and Shin, 2014, 2021). Among them, nine species (16.9%) were detected through metabarcoding, indicating that only a relatively small number of species were detected (Figs. 4 and 5). However, since the number of species observed through visual surveys is the accumulation of observations by many researchers for approximately 50 years since 1971, it was considered that many species could not be detected with a single metabarcoding analysis. Furthermore, out of 53 species, only 25 species had COI sequence information available; therefore, it is possible that 28 species could not be identified even if they were amplified with NGS. Consequently, we predicted that more species would have been detected by metabarcoding if more nucleotide sequence information were available.

The main purpose of monitoring studies is to continuously monitor the distribution and spread of a species (Yoccoz et al., 2001). In particular, eDNA monitoring has the advantage of being more efficient in detecting unrecorded and cryptic species (Luca et al., 2021; Semmouri et al., 2021). In the metabarcoding results of this study, 10 cryptic ascidian species that were not observed in visual surveys were detected. Only two species of Cionidae (*Ciona robusta* and *Ciona savignyi*) have been reported in South Korea (Lee and Shin, 2014; Park et al., 2018; Yi and Kim, 2020; MABIK, 2021), and four additional cryptic species have been detected with metabarcoding. These four species are presumed to be closely related to the genus *Ciona* and are considered cryptic. In the case of Styelidae and Asciidiidae, nineteen and five species, respectively, have been reported in Korea (MABIK, 2021), but 15 species of Styelidae and one species of Asciidiidae did not have COI nucleotide sequence information in GenBank. Therefore, it is highly probable that the species could not be identified because of the lack of reference sequences (Fig. 4).

In the process of conducting eDNA metabarcoding study, a DNA reference library is essential to select MOTU from NGS raw data. In

particular, reference libraries of good quality and quantity are very important because they enable easy molecular identification during the BLAST process. Specifically, even if a high-quality NGS raw data is obtained, it is difficult to obtain sufficient MOTU for molecular identification without DNA sequences deposited in public sources such as Genbank (Elbrecht and Leese, 2017; Leite et al., 2020). In this study, there were also species for which molecular identification was impossible. However, various taxonomic DNA barcode sequences are continuously being shared in public resources on a global scale, and this activity will increase the quality and quantity of DNA reference libraries. Therefore, expansion of the DNA reference library will improve the resolution for molecular identification, and it is expected that species that were not molecularly identified in this study will be able to be identified in the future.

In metabarcoding studies performed on marine organisms, cryptic species have been detected only in some fish studies (Bessey et al., 2020). However, in this study, unrecorded or cryptic species were detected in ascidians, a taxon with relatively insufficient primer sequences. Although morphological identification was impossible, molecular identification detected *Pyura mirabilis*, which has not been reported in South Korea. *P. mirabilis* has been reported continuously from 1979 to 2019 in Japan (Fuke, 1979; Hirose et al., 1990; Hasegawa et al., 2019) and was likely introduced to South Korea, which is close to Japan. *Botrylloides diegensis* was first recorded in South Korea in 2020 (Lee and Shin, 2021) and in Japan in 2005 (Nydham et al., 2021). It is possible that it was introduced to South Korea before 2020, but it was not observed in the visual surveys before 2020. However, in this study, *B. diegensis* was detected in all three survey sites. Therefore, the ascidian-specific marker in the present study is a more suitable tool for detecting cryptic or unrecorded species than visual surveys.

5. Conclusion

The present study determined whether eDNA metabarcoding analysis based on COI regions could be used as a supplemental method for visual surveys to understand ascidian diversity. Metabarcoding analysis detected 10 cryptic species that could not be detected using visual approaches, including *Pyura mirabilis*, heretofore unreported in South Korea. Therefore, eDNA metabarcoding is advantageous for detecting rare or cryptic ascidian species. Moreover, we suggest that eDNA metabarcoding can be a more efficient and complementary tool for studying ascidian fauna when combined with traditional monitoring methods. However, since the molecular identification of the nine cryptic species

remains unresolved, library construction and barcoding studies are needed to secure the nucleotide sequences necessary for molecular identification.

CRedit author statement

Seongjun Bae: Conceptualization, Supervision, Methodology, Investigation, Formal analysis, Visualization, Writing – original draft. Philjae Kim: Conceptualization, Methodology, Formal analysis, Visualization, Writing – original draft. Chang-Ho Yi: Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.marenvres.2023.105893>.

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