Quantitative Monitoring and Spatial Detection of Ascidians Using Environmental DNA

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Research Overview

◆ **Background and necessity**
- Preventing economic losses to invasive ascidian, conserving biodiversity, etc.

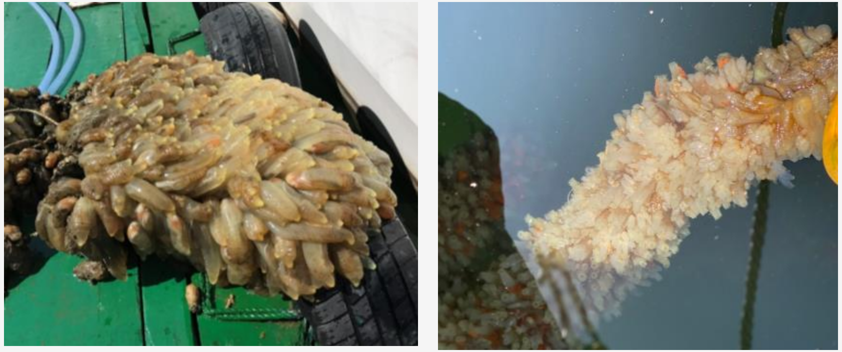
◆ **Objectives**
- Comprehensive understanding of the ecology and distribution of invasive ascidian

Chapter 01 ○ Quantitative assessment using qPCR and comparison with visual survey

Chapter 02 ○ Spatial distribution and diversity detection using metabarcoding

01 - Target Species : Ascidians (Ascidiacea)

- Ascidians (Tunicate, sea squirt)
  - Biofouling organism
  - Notorious marine invader
  - Influence on biodiversity due to dominance
- Ecologically important species
- *Ciona robusta* : Legally recognized and managed as an ‘Marine ecosystem disturbing species’



***Ciona robusta* (solitary)**

***Didemnum vexillum* (colonial)**

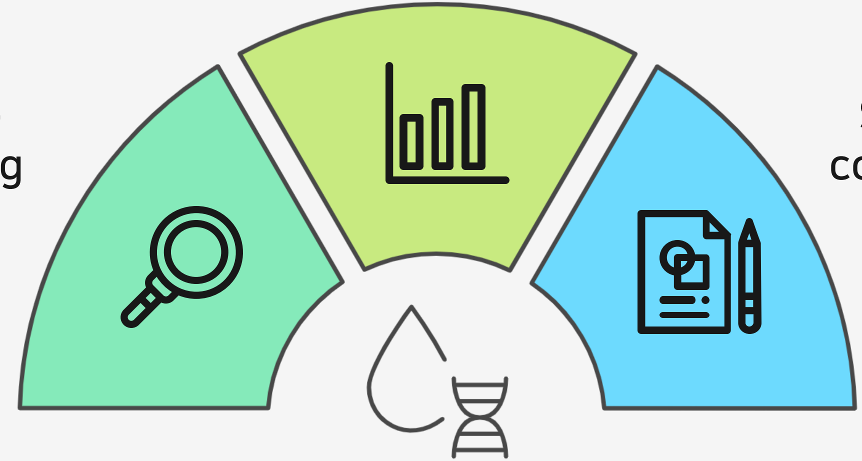
01

Quantitative assessment using qPCR and comparison with visual survey

01 - Introduction

◆ **Objectives**

- Validate that quantitative surveys using environmental DNA can complement visual surveys



Pattern analysis

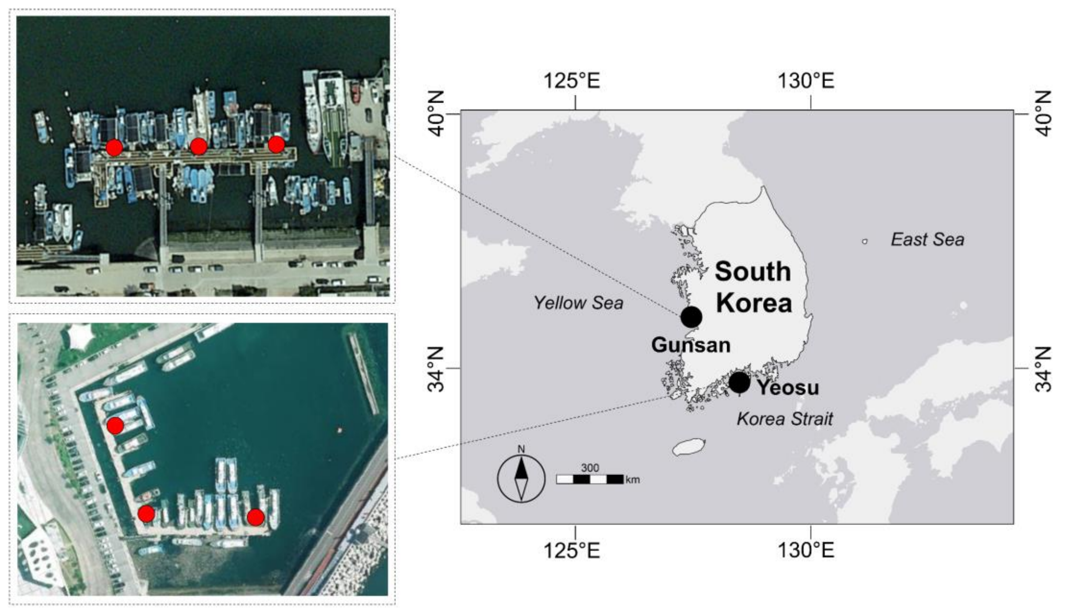
Effective monitoring

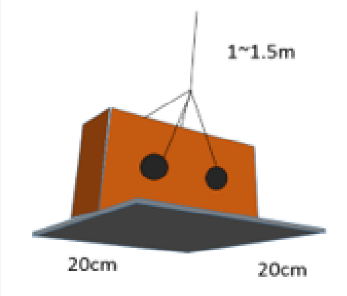
Significant comparability

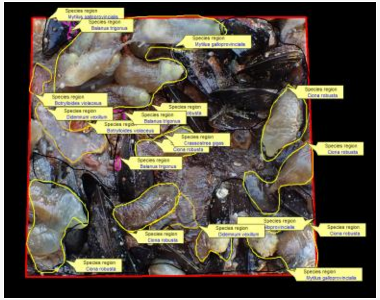
1) Validation of species-specific primers and eDNA quantitative data

2) Comparison of visual survey and eDNA quantitative data for two ascidians species

01 - Materials and methods







- Install artificial substrate (acrylic; 20x20cm) at a depth of 1–1.5m
- Three–month intervals from May 2020 to August 2021

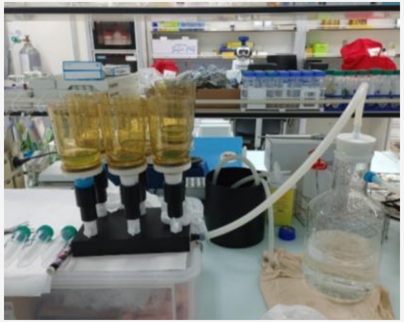
01 - Materials and methods

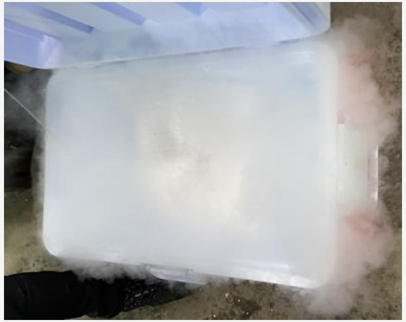
eDNA Sampling

Points: 3 points per site
Collection: 3L seawater
Filtration: 1L filtered (1.2μm filter)

DNA extraction and PCR

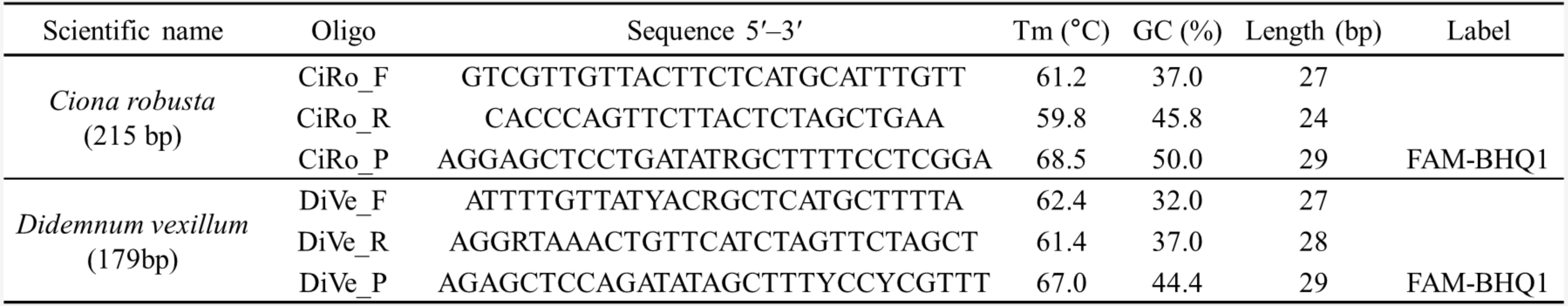
Storage: -70°C until analysis
Extraction: DNeasy Blood and Tissue Kits





01 – Results and discussion

species-specific primers and TaqMan probes for qPCR

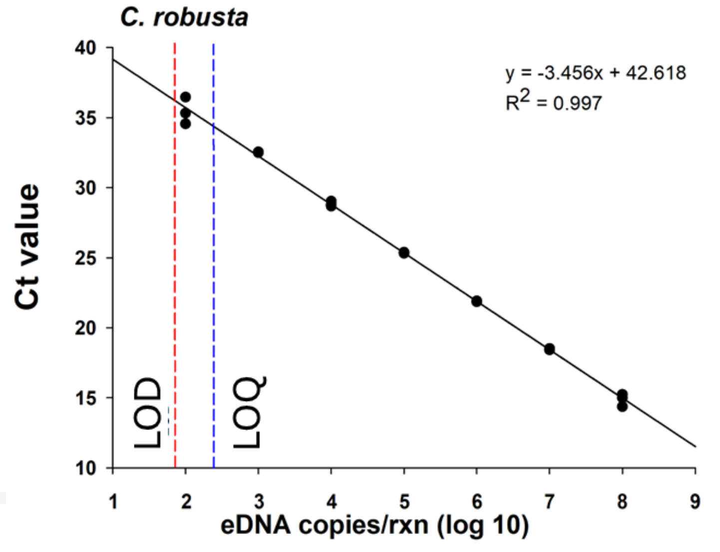


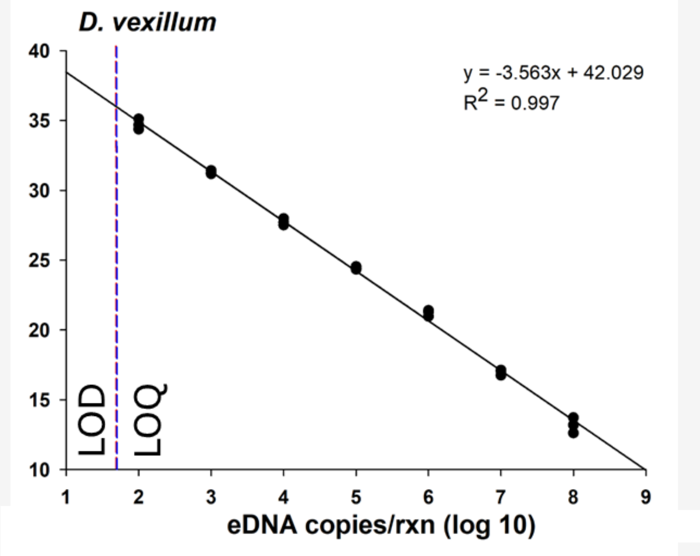
Scientific name	Oligo	Sequence 5′–3′	Tm (°C)	GC (%)	Length (bp)	Label
Ciona robusta (215 bp)	CiRo_F	GTCGTTGTTACTTCTCATGCATTTGTT	61.2	37.0	27	
	CiRo_R	CACCCAGTTCTTACTCTAGCTGAA	59.8	45.8	24	
	CiRo_P	AGGAGCTCCTGATATRGCTTTTCCTCGGA	68.5	50.0	29	FAM-BHQ1
Didemnum vexillum (179bp)	DiVe_F	ATTTTGTTATYACRGCTCATGCTTTTA	62.4	32.0	27	
	DiVe_R	AGGRTAAACTGTTCATCTAGTTCTAGCT	61.4	37.0	28	
	DiVe_P	AGAGCTCCAGATATAGCTTTYCCYCGTTT	67.0	44.4	29	FAM-BHQ1

- Designed from 2,650 multiple sequence alignments of 277 ascidians (NCBI)
- Perform 3 replicates of qPCR including no-template control (NTC)
- Calculated the limit of detection (LOD) and limit of quantitation (LOQ)

01 – Results and discussion

- LOD/LOQ calculate using the method suggested by Klymus et al., (2020)





- *C. robusta* (Ct value and copies/rxn)
  - – LOD : **36.2** / 1.89
  - – LOQ : **34.3** / 2.46
- *D. vexillum (Ct value and copies/rxn)*
  - – LOD : **35.5** / 1.89
  - – LOQ : **35.5** / 1.89

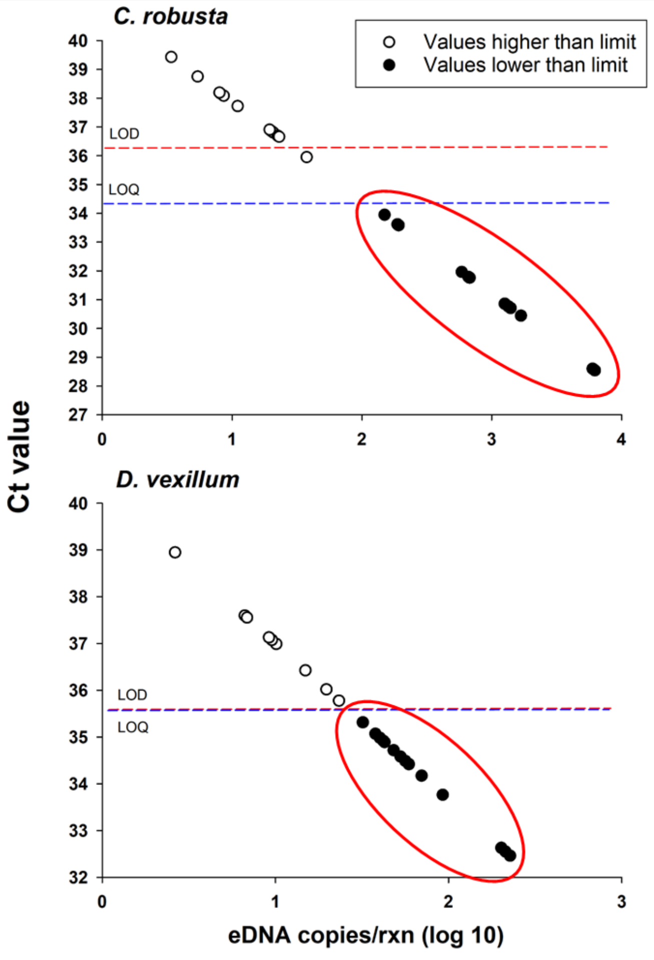
01 – Results and discussion

- 2 sites × 5 survey × 3 replication = 30 sample

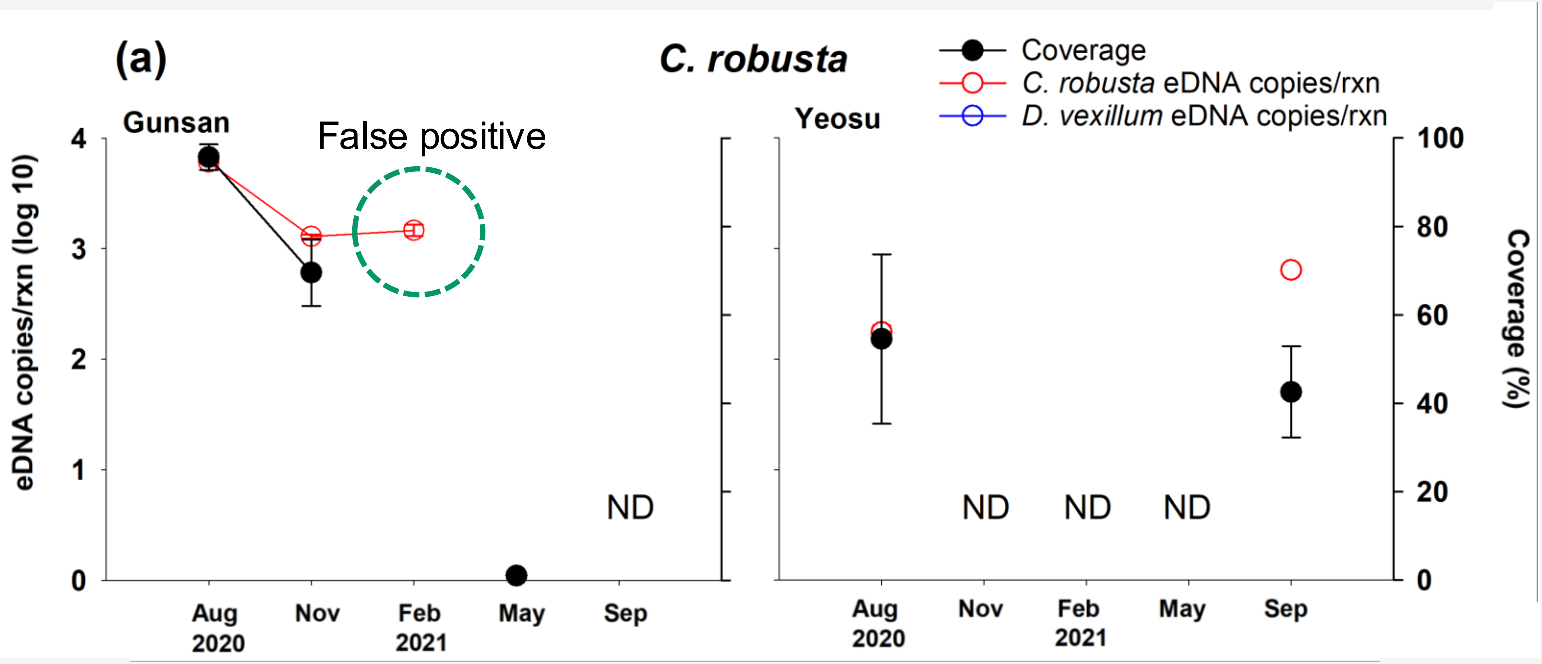
- *C. robusta* : **15 eDNA** sample analyzed (total)
  - **Gunsan : 9 / Yeosu : 6**
  - Undetected : 3
  - Out of range :12

- *D. vexillum* : **15 eDNA** sample analyzed (total)
  - **Gunsan : 11 / Yeosu : 4**
  - Undetected : 6
  - Out of range : 9

- Only values within the limits are used for analysis

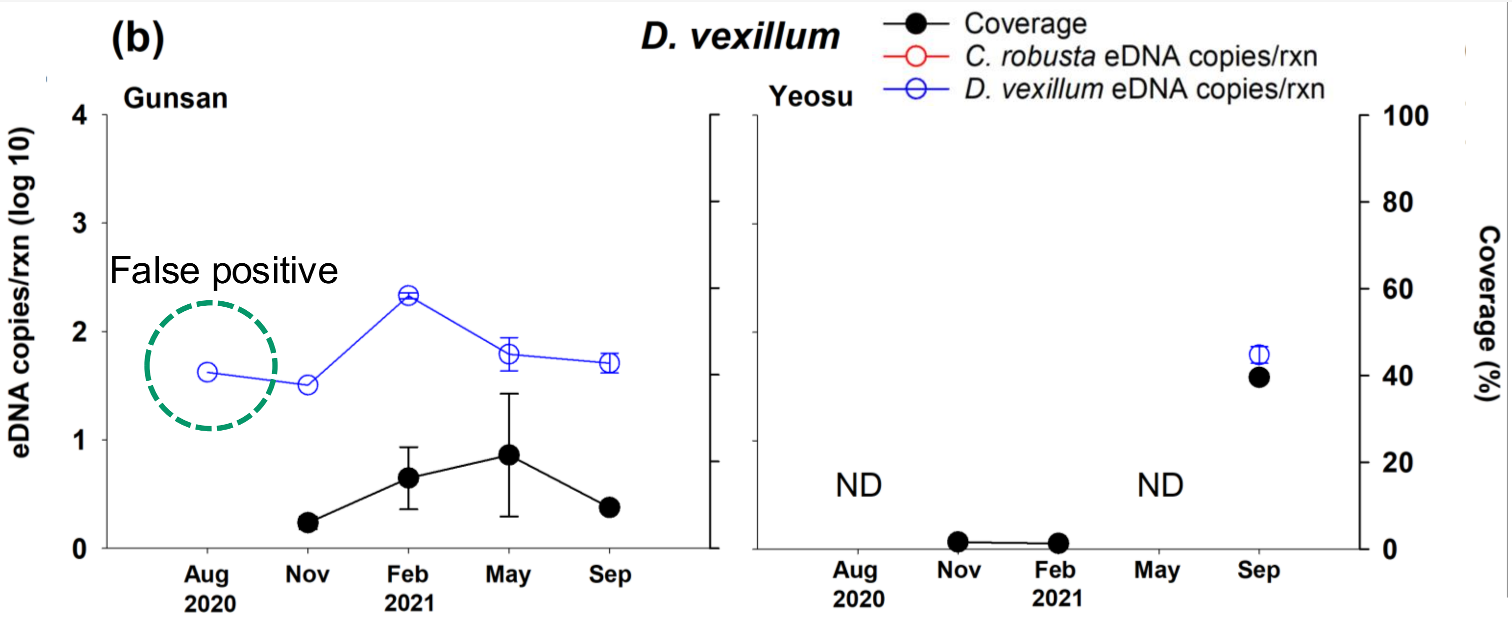


01 – Results and discussion



- DNA preservation caused by low winter temperatures

01 – Results and discussion



- Effects of *D. vexillum* in existing habitats (optimal growth temperature 14–18 °C)

01 – Results and discussion

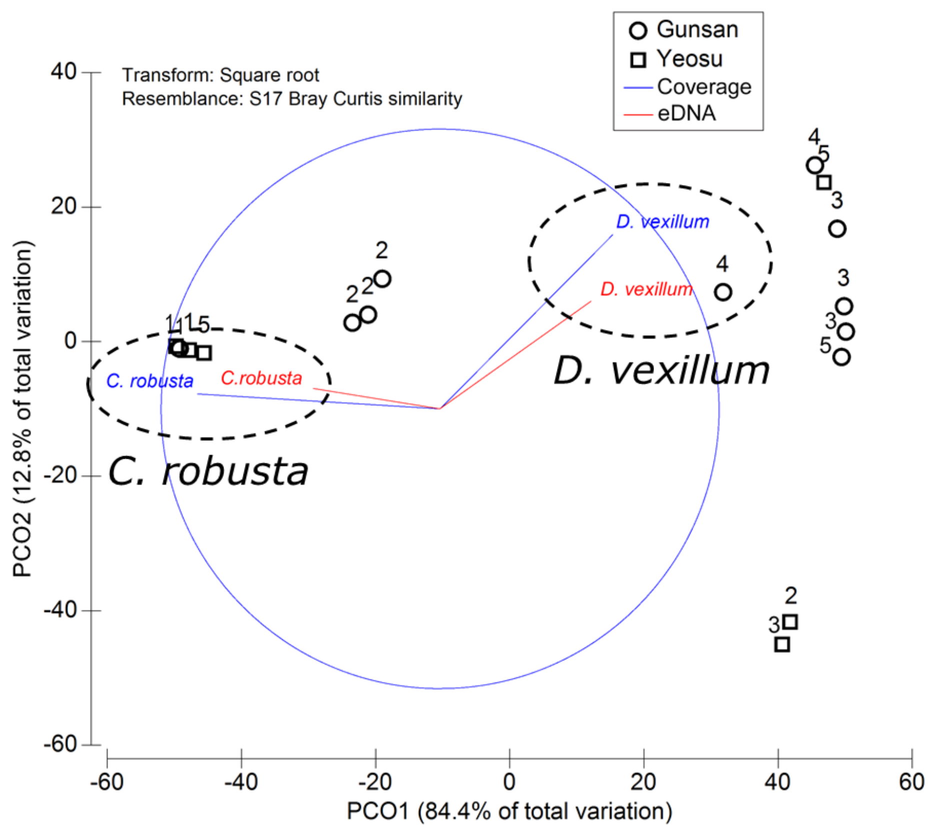
Water temperature (°C) information observed at Gunsan and Yeosu in February 2021

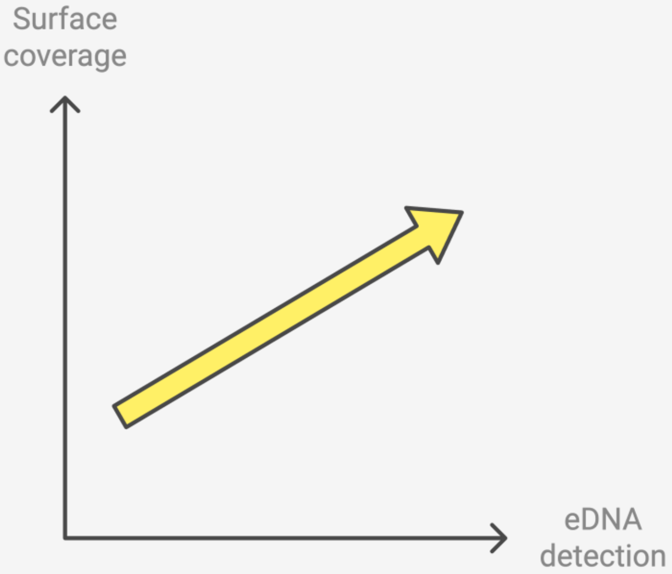
Site	Minimum	Maximum	Average	SD
Gunsan	2.90	5.34	4.11	0.47
Yeosu	6.40	8.66	7.36	0.49

Gunsan in January

Gunsan in August

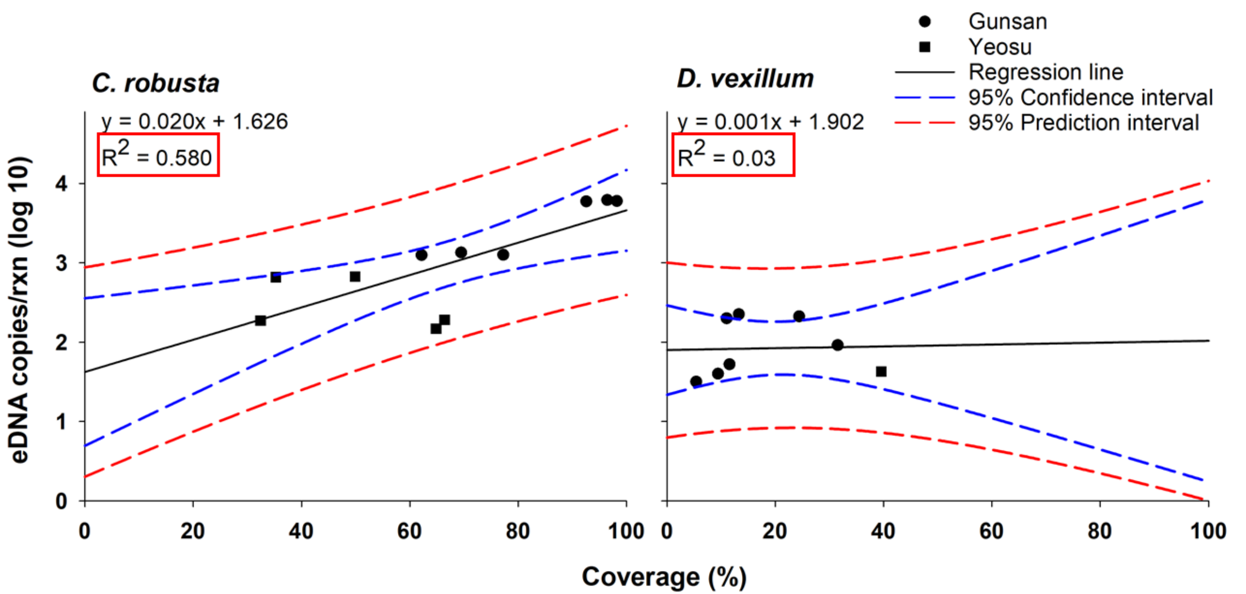
01 – Results and discussion





- **eDNA detection** adequately indicates **high coverage**

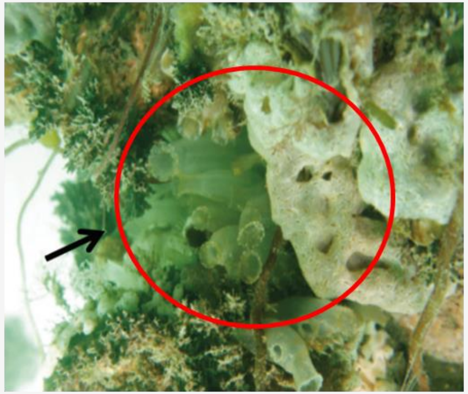
01 – Results and discussion



- *C. robusta* are significantly correlated
- *D. vexillum* are non–significantly correlated

01 – Discussion

C. robusta
Solitary ascidian



D. vexillum
Colonial ascidian

- Difference in siphon area between *C. robusta* and *D. vexillum*
- *C. robusta* : 5.3–13 cm / *D. vexillum* : 1–2 mm
- The presence of filter–feeding species **reduces the amount of eDNA detection** from other species in the co–habitat (Kwong et al., 2021)

01 – Conclusion

Key Findings by Species

Ciona robusta: Significant correlation with visual surveys

Didemnum vexillum: Better suited for habitat/distribution studies

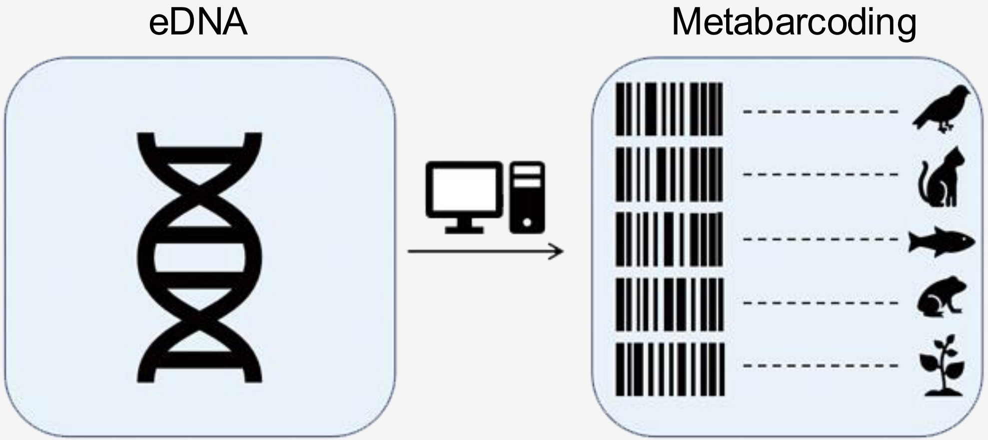
Quantitative comparison

False−positives and −negatives require methodological enhancements

Ciona robusta

Potential to complement/replace quantitative data in visual survey

Further studies are needed on quantitative detection in a factor−controlled laboratory environment.



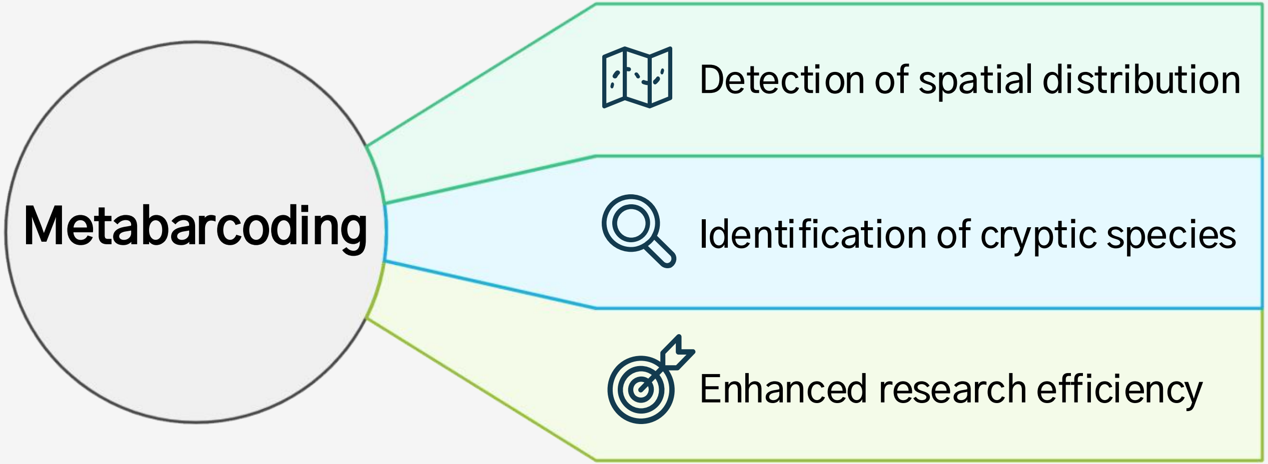
02

Spatial distribution and diversity detection using metabarcoding

02 - Introduction

◆ Objectives

- Complement conventional monitoring by detecting species across different marine regions



Metabarcoding

- Detection of spatial distribution
- Identification of cryptic species
- Enhanced research efficiency

1) Design and validation of ascidian-specific primers for species-level detection
2) Comparison between existing distribution data and eDNA metabarcoding results

02 - Materials and methods

Sequence and distribution dataset

Category	Data Source	Detail	Reference year
Sequence data	NCBI GenBank	Non-ascidian: 35 species Ascidian: 198 species **Total: 3,369 sequences**	–
Distribution data	Previous studies and literature records	173 Specimen	1971–2021
	MBRIS / NIBR / NARIS	62 Specimen	1998–2021

Rho, (1971), (1977), (1995); Pyo et al. (2012); Lee and Shin, (2014), (2021)

- Seawater was collected in August–September 2020 (summer)
- Sequence data for primer design and distribution data for comparison

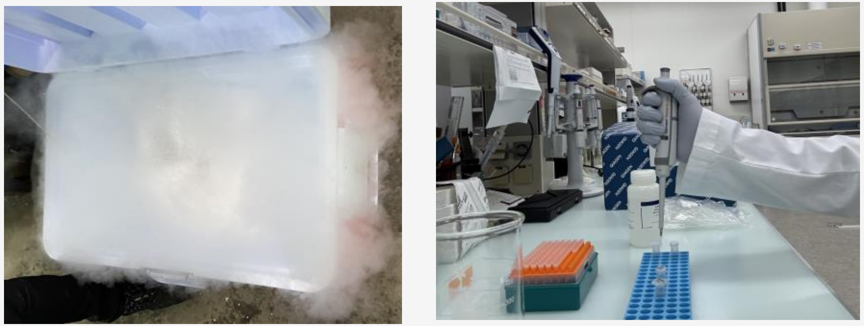
02 - Materials and methods

eDNA Sampling

Points: 3 points per site
Collection: 3L seawater
Filtration: 1L filtered (1.2μm filter)

DNA extraction and PCR

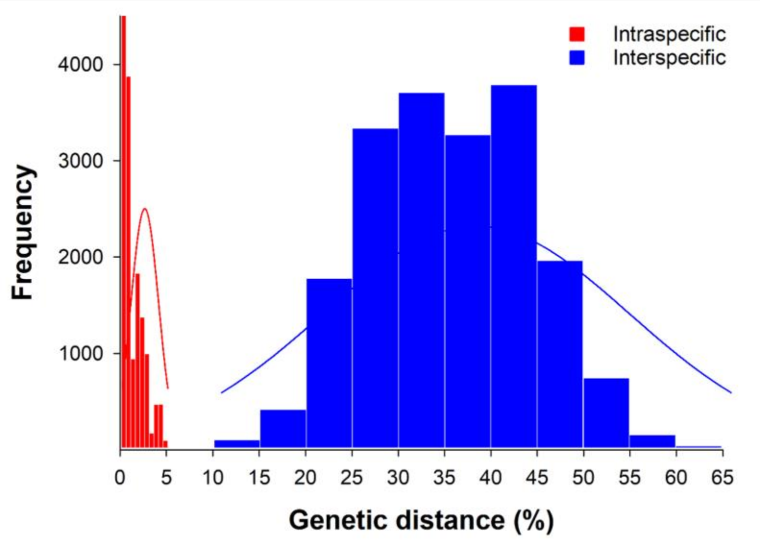
Storage: -70°C until analysis
Extraction: DNeasy Blood and Tissue Kits



02 – Results and discussion

Ascidian-specific primers

Primer label	Sequence (5′–3′)	Tm (°C)	Amplicon size (bp)	Target gene
AscCOI_F	CCTGATATGGCNTTYCCHCG	57.3	197	Mitochondrial COI
AscCOI_R	GCTAAATGHAAHGAAAAAATWGC	51.7		



- *In silico :* primer binding **2,651/2,922 (90.7%)**
- *In vitro* : successfully amplified and sequenced in all ascidian specimens (**10**)
- Barcoding gap result (198 ascidian species)
  - Intraspecific : **0-6.25%**
  - Interspecific : **10-90%**
  - Clear gap : **3.75%**

02 – Results and discussion

Summary of NGS results

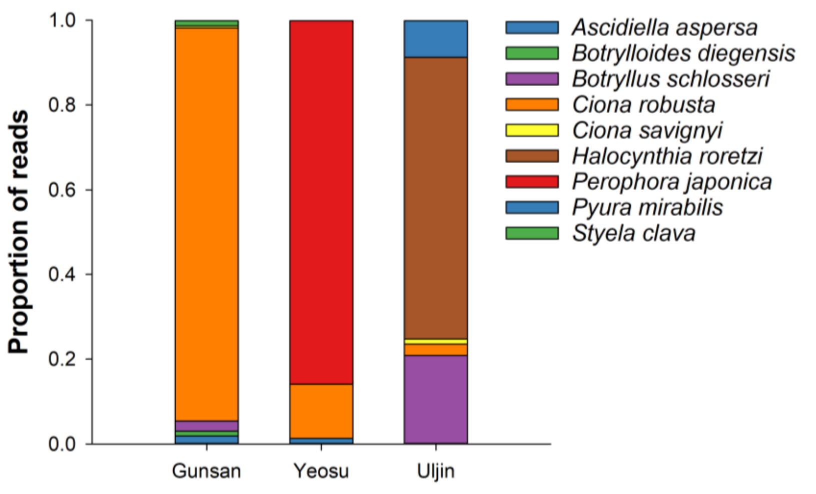
Site	Reads	Read bases (bp)	Clusters (OUT)	Clusters bases (bp)	Species richness
Gunsan	241,826	72,789,626	31,284	5,980,515	6
Yeosu	112,418	21,478,747	28,498	5,418,617	3
Uljin	149,068	44,869,468	6,618	1,221,087	5
Total	**503,312**	139,137,841	**66,400**	12,620,219	**9**

- A total of 9 species of ascidians were detected
- Species with the highest number of leads

– Gunsan : *Ciona robusta*

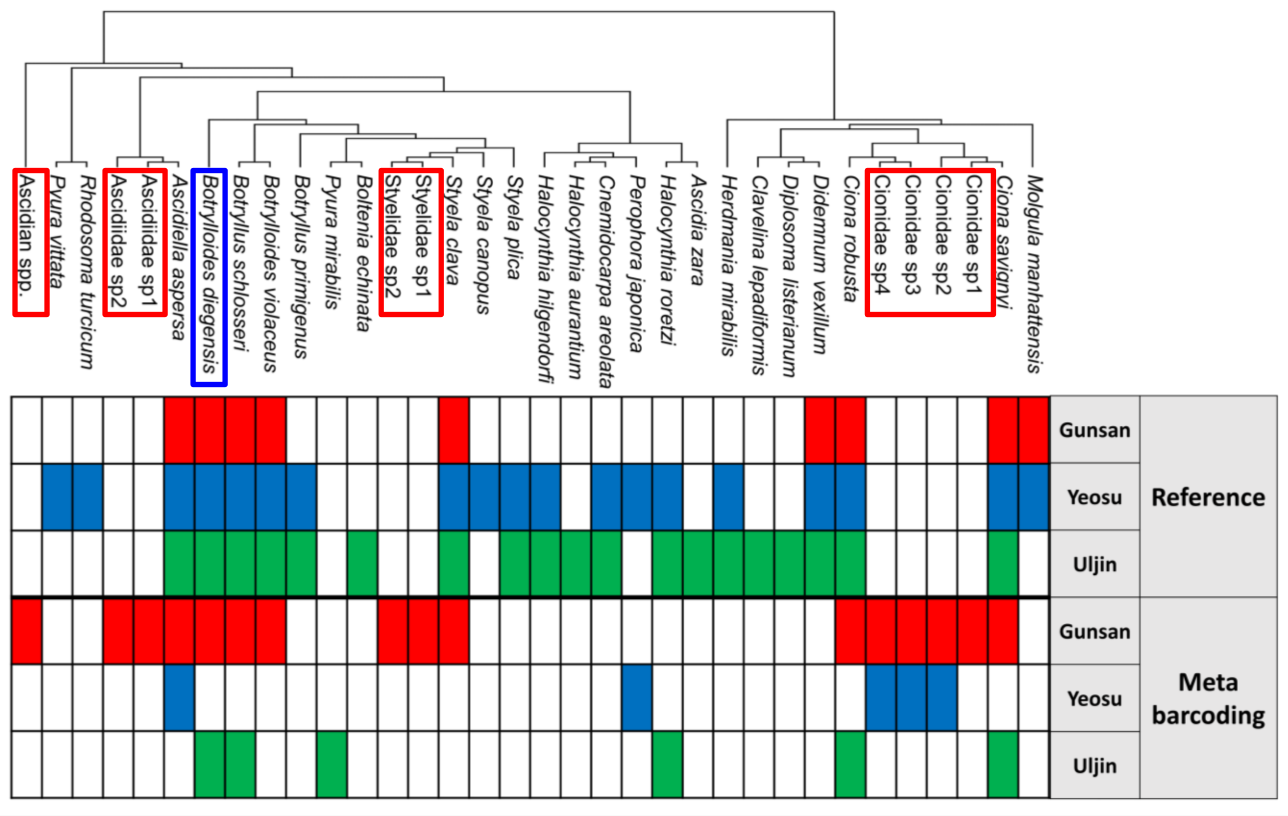
– Yeosu : *Perophora japonica*

– Uljin : *Halocynyhia roretzi*



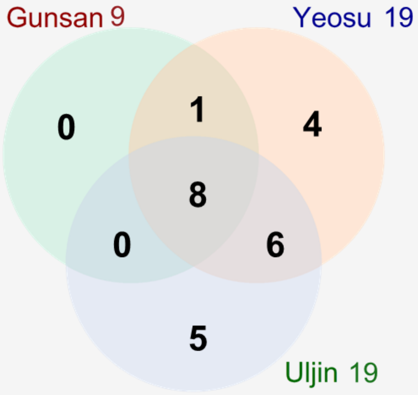
02 – Results and discussion

Phylogenetic tree (6.25%) based on NGS metabarcoding results

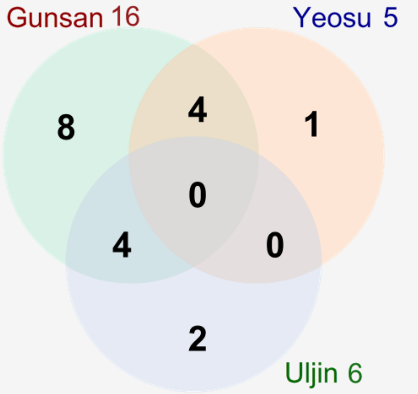


	Reference			Meta barcoding		
	Gunsan	Yeosu	Uljin	Gunsan	Yeosu	Uljin
Ascidian spp.				🟥		
Pyura vittata		🟦				
Rhodosoma turcicum		🟦				
Ascidiidae sp2				🟥		
Ascidiidae sp1				🟥		
Ascidiella aspersa	🟥	🟦	🟩	🟥	🟦	
Botrylloides diegensis	🟥	🟦	🟩	🟥		🟩
Botryllus schlosseri	🟥	🟦	🟩	🟥		🟩
Botrylloides violaceus	🟥	🟦	🟩	🟥		
Botryllus primigenus		🟦	🟩			
Pyura mirabilis						🟩
Boltenia echinata			🟩			
Styelidae sp2				🟥		
Styelidae sp1				🟥		
Styela clava	🟥	🟦	🟩	🟥		
Styela canopus		🟦				
Styela plica		🟦	🟩			
Halocynthia hilgendorfi		🟦	🟩			
Halocynthia aurantium			🟩			
Cnemidocarpa areolata		🟦	🟩			
Perophora japonica		🟦			🟦	
Halocynthia roretzi		🟦	🟩			🟩
Ascidia zara			🟩			
Herdmania mirabilis		🟦	🟩			
Clavelina lepadiformis			🟩			
Diplosoma listerianum			🟩			
Didemnum vexillum	🟥	🟦	🟩			
Ciona robusta	🟥	🟦	🟩	🟥		🟩
Cionidae sp4				🟥	🟦	
Cionidae sp3				🟥	🟦	
Cionidae sp2				🟥	🟦	
Cionidae sp1				🟥		
Ciona savignyi	🟥	🟦	🟩	🟥		🟩
Molgula manhattensis	🟥	🟦				

Reference 24



Metabarcoding 19

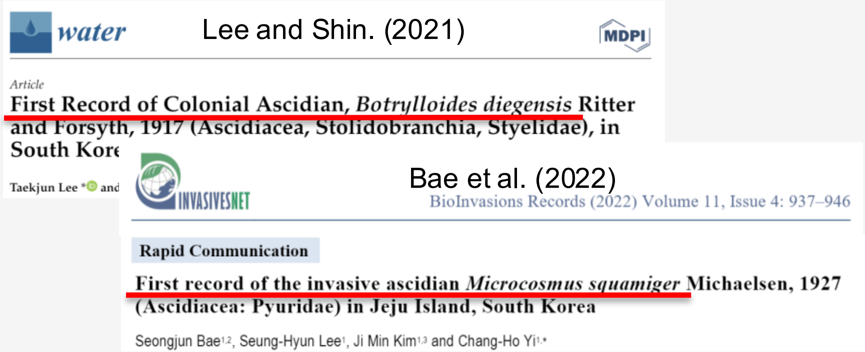


02 – Results and discussion

Difference in spatial detection

Visual survey	Metabarcoding
Long-term monitoring (50 years)	One-time survey
Multiple researchers	Single research team

Category	Family	Recorded in Korea	Absence of sequence information (NCBI)
Cryptic species (red)	Cionidae sp.	2 species	-
	Styelidae sp.	19 species	15 species
	Ascidiidae sp.	5 species	2 species



Lee and Shin. (2021)

water

MDPI

Article

First Record of Colonial Ascidian, *Botrylloides diegensis* Ritter and Forsyth, 1917 (Ascidiacea, Stolidobranchia, Styelidae), in South Kore

Taekjun Lee * and

Bae et al. (2022)

INVASIVESNET

BioInvasions Records (2022) Volume 11, Issue 4: 937–946

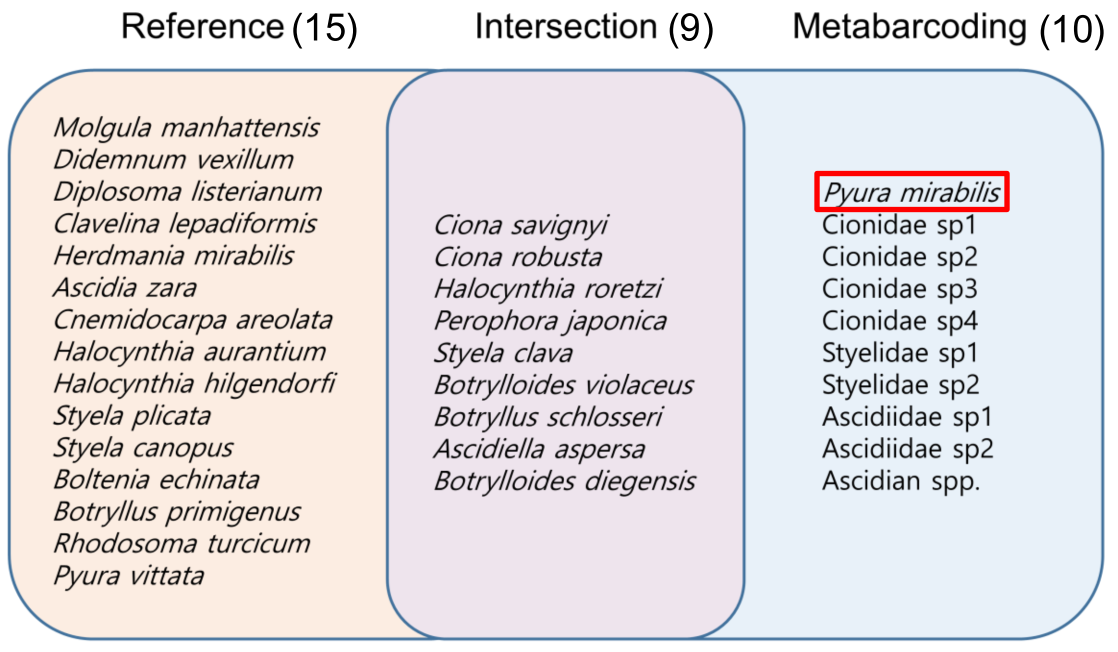
Rapid Communication

First record of the invasive ascidian *Microcosmus squamiger* Michaelsen, 1927 (Ascidiacea: Pyuridae) in Jeju Island, South Korea

Seongjun Bae[1,2], Seung-Hyun Lee[1], Ji Min Kim[1,3] and Chang-Ho Yi[1,*]

- Direct comparison is limited due to differences in the temporal range
- Absence of reference sequences in NCBI GenBank

02 – Results and discussion



Reference (15)

Molgula manhattensis
Didemnum vexillum
Diplosoma listerianum
Clavelina lepadiformis
Herdmania mirabilis
Ascidia zara
Cnemidocarpa areolata
Halocynthia aurantium
Halocynthia hilgendorfi
Styela plicata
Styela canopus
Boltenia echinata
Botryllus primigenus
Rhodosoma turcicum
Pyura vittata

Intersection (9)

Ciona savignyi
Ciona robusta
Halocynthia roretzi
Perophora japonica
Styela clava
Botrylloides violaceus
Botryllus schlosseri
Ascidiella aspersa
Botrylloides diegensis

Metabarcoding (10)

Pyura mirabilis
Cionidae sp1
Cionidae sp2
Cionidae sp3
Cionidae sp4
Styelidae sp1
Styelidae sp2
Ascidiidae sp1
Ascidiidae sp2
Ascidian spp.

- *Pyura mirabilis* has been reported in Japan continuously from 1979 to 2019 (Fuke, 1979; Hasegawa et al., 2019)
- The accuracy and quantity of GenBank reference sequences are important
- Need for complementary use of the two methods

02 – Conclusion

Key Findings by methods

Metabarcoding: single sampling enables extensive species detection

Visual Survey: obtain precise species identification and distribution data

Cryptic or rare species detection

10 cryptic species were newly detected

Complementary Approach

Increased temporal and spatial efficiency

Building and securing a sequence library of reference sequences is important

Thank you for your attention

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Reference

- Bae, S., Lee, S.-H., Kim, J., Yi, C.-H., 2022. First record of the invasive ascidian Microcosmus squamiger Michaelsen, 1927 (Ascidiacea: Pyuridae) in Jeju Island, South Korea. Bioinvasions Rec 11, 937–946. https://doi.org/10.3391/bir.2022.11.4.12
- Fuke, M.T., 1979. Studies on the coelomic cells of some Japanese ascidians. In: TheBulletin of the Marine Biological Station of Asamushi, vol. 16. Tohoku University,pp. 143–159.
- Hasegawa, N., Kajihara, H., Kajihara, H., 2019. A redescription of Syncarpacomposita(Ascidiacea, Stolidobranchia) with an inference of its phylogenetic position withinStyelidae. ZooKeys 857, 1–15. https://doi.org/10.3897/zookeys.857.32654.
- Kwong, S.L.T., Villacorta-Rath, C., Doyle, J., Uthicke, S., 2021. Quantifying shedding and degradation rates of environmental DNA (eDNA) from Pacific crown-of-thorns seastar (Acanthaster cf. solaris). Mar Biol 168, 85. https://doi.org/10.1007/s00227-021-03896-x
- Lee, T., Shin, S., 2021. First record of colonial ascidian, Botrylloides diegensis ritter andforsyth, 1917 (Ascidiacea, stolidobranchia, Styelidae), in South Korea. Water-sui 13,2164. https://doi.org/10.3390/w13162164.
- Valentine, P., 2009. Larval recruitment of the invasive colonial ascidian Didemnum vexillum, seasonal water temperatures in New England coastal and offshore waters, and implications for spread of the species. Aquat Invasions 4, 153–168. https://doi.org/10.3391/ai.2009.4.1.16