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

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Abstract: *Botrylloides* species are important members of the fouling community colonizing artificial substrates in harbors and marinas. During monitoring in 2017–2020 of non-indigenous species in Korea, one colonial ascidian species was distinctly different from other native colonial ascidians, such as *B. violaceus* and *Botryllus schlosseri*, in South Korea. This species was identified as *B. diegensis*. DNA barcodes with mitochondrial COI were used to identify one-toned and two-toned colonies of *B. diegensis*. Intraspecific variations between Korean and other regions of *B. diegensis* from the NCBI ranged from 0.0% to 1.3%. The Korean *B. diegensis* was clearly distinct from other species of *Botrylloides* at 15.8–24.2%. In phylogenetic analysis results, Korean *B. diegensis* was established as a single clade with other regions of *B. diegensis* and was clearly distinct from Korean *B. violaceus*. After reviewing previous monitoring data, it was found that two-toned *B. diegensis* was already found in six harbors by July 2017. It has now spread into 14 harbors along the coastal line of South Korea. This means that *B. diegensis* might have been introduced to South Korea between 1999 and 2016.

Keywords: non-indigenous species; alien species; botryllids; DNA barcoding; COI

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1. Introduction

Introductions of non-indigenous species (NIS) have occurred at an increasing rate since the 20th century, showing increasing ranges and intensity of vectors [1]. However, identifying new or recently introduced NIS can be challenging if only traditional methods are used [2]. Many marine animal NIS in introduction hotspots (e.g., marinas and harbors) belong to taxonomic groups (especially colonial ascidians) that require substantial taxonomic expertise [3]. In this sense, the usefulness of a molecular barcoding approach has been well documented. Such an approach can be used to ascertain the presence of new NIS [4] to reveal false morphology-based NIS identification [5] and to determine the taxonomic status of previously unrecognized NIS [6]. An increasing number of studies have recommended the use of molecular tools to complement traditional methods (e.g., morphological taxonomic approach) for achieving reliable taxonomic identification of marine NIS [2,7,8], including those considered to be cryptic species, which have been widely reported for colonial ascidians [3,9–12].

Botrylloides and *Botryllus* (class Ascidiacea, order Stolidobranchia, family Styelidae) are ascidians belonging to a group of colonial species, of which 53 species have been described [13]. Among them, *Botrylloides* species are important members of the fouling community colonizing artificial substrates on the Pacific coast of the United States (for instance, in harbors and marinas) [14,15]. In Europe, one putatively native species, *B. leachii* (Savigny, 1816), has also been recognized, often showing coloration somewhat similar to the two-toned color pattern seen in *B. diegensis* [3]. One-toned *B. diegensis* has also been found to be misidentified as *B. violaceus* in the NCBI database. Recently, rearrangement of mitochondrial COI data of each species has been accomplished [3]. In Korea, the marine NIS research program was initiated by the Ministry of Oceans and Fisheries in 2008. Many ascidians inhabit many harbors in South Korea. Among them, a number of non-indigenous ascidians have been newly reported via this research program [16,17]. However, these new reports were focused on solitary ascidians. The identification of colonial ascidians, such as species identification in the field, remains a challenging task.

Thus, the objectives of this study were the following: (i) to identify botryllids ascidians in South Korea based on DNA barcoding, (ii) to provide mitochondrial COI data for *B. diegensis* from South Korea.

2. Materials and Methods

2.1. Sample Collection and Identification

Samples were collected from 11 May 2020 to 15 May 2020 in 14 harbors along the coastal line of South Korea (Figure 1, Table 1). All samples were taken from acrylic plates designed for monitoring non-indigenous and harmful organisms. The dimensions of the acrylic plates were 30 × 30 cm^2 with a thickness of 5 mm. Each plate was connected with polypropylene rope and the distance between each plate was 20 cm. A monitoring set was composed of 10 acrylic plates, and the first acrylic plate was situated 1 m below the surface of the water. The plates were installed from July 2017 to October 2020. Colonies were photographed with a digital camera (TG-5, Olympus, Tokyo, Japan) and labeled before the sample collection. We collected the sample from a colony of botryllids (0.5 × 0.5 cm^2) on a settlement plate. The samples were preserved immediately with an ethyl alcohol solution (>95%). They were then assigned voucher numbers (SYA200501–SYA200556), and stored in the Marine Biological Resource Institute, Sahmyook University, Korea. The collected samples used for DNA barcoding were identified based on their zooid morphological features from Tokioka [18] and Rho [19] under microscopes.

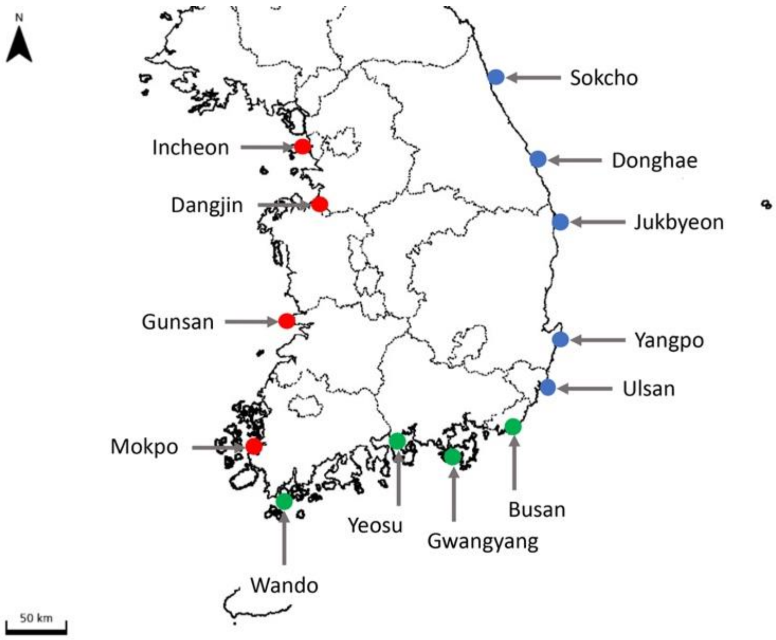

Figure 1. Sampling localities in this study. Locality marks were filled in different colors by region: blue—East Sea; green—Korea Strait; red—Yellow Sea.

Table 1. Sampling localities and environmental information of 14 sampling sites for this study.

	Locality	Region	GIS Coordinates	Survey Date (2020)	Water Temp. (°C)	Salinity (psu)	pH
1	Incheon	Yellow Sea	37.460556 N, 126.625278 E	11 May	14.9	30.1	8.02
2	Dangjin		36.986944 N, 126.746111 E	11 May	15.0	30.6	8.19
3	Gunsan		35.935833 N, 126.516667 E	11 May	15.5	31.3	8.25
4	Mokpo		34.783861 N, 126.389222 E	12 May	15.7	29.7	8.08
5	Wando	Korea Strait	34.317354 N, 126.753546 E	12 May	15.1	33.5	8.09
6	Yeosu		34.717166 N, 127.749114 E	12 May	17.2	32.9	8.18
7	Gwangyang		34.908611 N, 127.726111 E	12 May	18.2	31.5	8.09
8	Tongyeong		34.827222 N, 128.389222 E	13 May	16.3	34.0	8.03
9	Busan		35.099722 N, 129.755635 E	13 May	17.1	34.5	8.07
10	Ulsan	East Sea	35.511111 N, 129.385833 E	13 May	18.0	33.8	8.07
11	Yangpo		35.877818 N, 129.519892 E	14 May	13.7	34.4	8.09
12	Jukbyeon		37.055556 N, 129.419444 E	14 May	14.9	34.1	8.10
13	Donghae		37.498889 N, 129.134356 E	14 May	14.5	34.3	8.16
14	Sokcho		38.210444 N, 128.596249 E	15 May	15.7	34.1	8.12

2.2. DNA Extraction and Amplification of DNA Barcoding Region

Total genomic DNA was extracted from a single zooid in a colony using a DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Partial sequences of COI were amplified using two primer pairs as follows: LCO1490-HCO2198 [20] and dinF-Nux1R [21]. All genomic DNA samples were stored at −72 °C until use. Polymerase chain reaction was performed with a total reaction volume of 20.0 µL, including AccuPower PCR PreMix and Master Mix (Bioneer, Seoul, Korea), 1.0 µL of each primer (10 mM), and 0.3 µL of DNA template (>50 ng/µL), with the following thermal cycling conditions: one cycle at 94 °C for 3 min, 35 cycles of 94 °C for 30 s, 50 °C for 45 s, 72 °C for 60 s, and a final extension step at 72 °C for 7 min. The PCR products were directly sequenced with the forward and reverse primers used for amplification (Cosmogenetech, Seoul, Korea). The assemblies and alignments of sequencing results were performed using Geneious v. 11.1.5 (Biomatters, Auckland, New Zealand).

2.3. DNA Barcoding Data Analysis

All COI sequences obtained in this study were deposited in GenBank. The accession numbers are shown in Table 2. Genetic distances and phylogenetic relationships of Korean *B. diegensis* with *B. diegensis* from other regions (12 localities of 6 countries; Supplementary Table S1) and 11 other species of *Botrylloides* and *Botryllus schlosseri* were investigated. All data, except for Korean botryllids, were obtained from the NCBI. The best-fit model of nucleotide substitution for the COI dataset was selected using Modeltest v. 2.1.1 [22] with the Akaike Information Criterion (AIC) for maximum likelihood (ML). The ML tree was constructed using PhyML 3.0 [23] under the TrN + I + G model and 1000 replicate bootstrapping for the COI dataset. Bayesian inference (BI) was performed using 1,000,000 generations of Markov Chain Monte Carlo chains. One in every 1000 generations was sampled. The initial 250 generations were discarded as burn-in. All processes were executed with MrBayes 3.2.6 [24] under the TrN + I + G model. *Botryllus schlosseri* was determined to belong to the *Botrylloides* group in the ML and BI analyses. Pairwise distances were calculated using the Kimura 2-parameter model (K2P) [25] in MEGA 7.0 [26].

Table 2. GenBank accession number and sequencing information of Korean *Botrylloides diegensis* and *Botryllus schlosseri* used in this study.

Species	Collecting Sites	GenBank Accession No.	Sequence Length (bp)	Primers *	Color of Colony **
Botrylloides diegensis	Incheon	MW579604	672	1	Light brown, dark brown
	Gunsan	MW579609	672	1	Brown
		MW579615	672	1	Light brown, dark brown
	Yeosu	MW579611	672	1	White, dark purple
		MW579612	672	1	Dark purple
		MW579613	672	1	Light brown, dark brown
	Tongyeong	MW579620	867	2	Light brown, dark brown
	Ulsan	MW579610	867	2	Light brown
		MW579617	672	1	Dark purple
		MW579618	672	1	Light brown
	Yangpo	MW579605	672	1	Yellow, dark purple
		MW579606	672	1	Lemon, purple
		MW579607	672	1	Lemon, dark purple
		MW579619	672	1	Brown, dark brown
	Donghae	MW579615	672	1	Light brown, brown
	Sokcho	MW579616	867	2	Dark brown
Botryllus schlosseri	Incheon	MW584324	856	2	Purple
	Gunsan	MW584320	856	2	Dark purple
		MW584321	856	2	Light brown
		MW584322	856	2	Brown
		MW584323	856	2	Brown
		MW584327	856	2	Purple
	Yeosu	MW584326	856	2	Dark purple
	Ulsan	MW584325	856	2	Dark purple with yellow line
	Donghae	MW584319	856	2	Dark purple
	Sokcho	MW584328	856	2	Dark purple

* Used primer pairs were marked as the following: (1) LCO1490-HCO2198, and (2) dinF-Nux1R. ** All color photographs are presented in Supplementary Figure S1.

3. Results

3.1. DNA Barcoding Analysis for B. diegensis from South Korea and Other Colonial Ascidians

This study presents the first report of *Botrylloides diegensis* in South Korea. It was not clearly identifiable from *B. violaceus* or *Botryllus schlosseri* in the field survey (Figures 2 and 3, Supplementary Figure S1). Thus, we needed to compare it with more species of botryllids using DNA barcoding. We obtained 16 and 10 partial COI sequences of Korean *B. diegensis* and *B. schlosseri* at 672 bp and 858 bp, respectively (Table 2). We calculated the pairwise distances based on 396 bp sequences of COI genes of 11 species of *Botrylloides* and *Botryllus schlosseri* (Table 3, Supplementary Table S1).

The intraspecific variation range of the Korean *B. diegensis* group was 0.0–1.3%, with a mean of 0.4% (Supplementary Table S1). The intraspecific variation between Korean and other regions of *B. diegensis* from NCBI was 0.0% to 1.3%. Variations for other regions were 0.0–1.0% (Supplementary Table S1). Intraspecific variations in the Korean group seemed to be higher than those in other regions. The mean variation of the Korean group was 0.4%, which was slightly higher than that for other regions group at 0.2% (Table 3). The interspecific variation between Korean *B. diegensis* and other species of *Botrylloides* was 15.8–24.2% (Table 3). The intraspecific variation of other *Botrylloides* species, except for *B. diegensis*, was 0.0–1.3%, similar to the intraspecific variation of *B. diegensis* in this study (Supplementary Table S1). Additionally, the phylogenetic trees of ML and BI show the same results (Figure 4). All species of *Botrylloides* were distinct from *B. schlosseri*, an outgroup (Figure 4). *Botrylloides diegensis* formed a single clade with Korean *B. diegensis* and *B. diegensis* from GenBank (Figure 4). This *B. diegensis* clade showed a clear, single clade, although several localities data were included: 20 localities in 9 countries (Figure 4, Supplementary Table S1). The posterior probability support values for several resolved nodes were >0.8, although some bootstrapping support values in the ML tree were not well supported (<70) in the clade of *Botrylloides* (Figure 4).

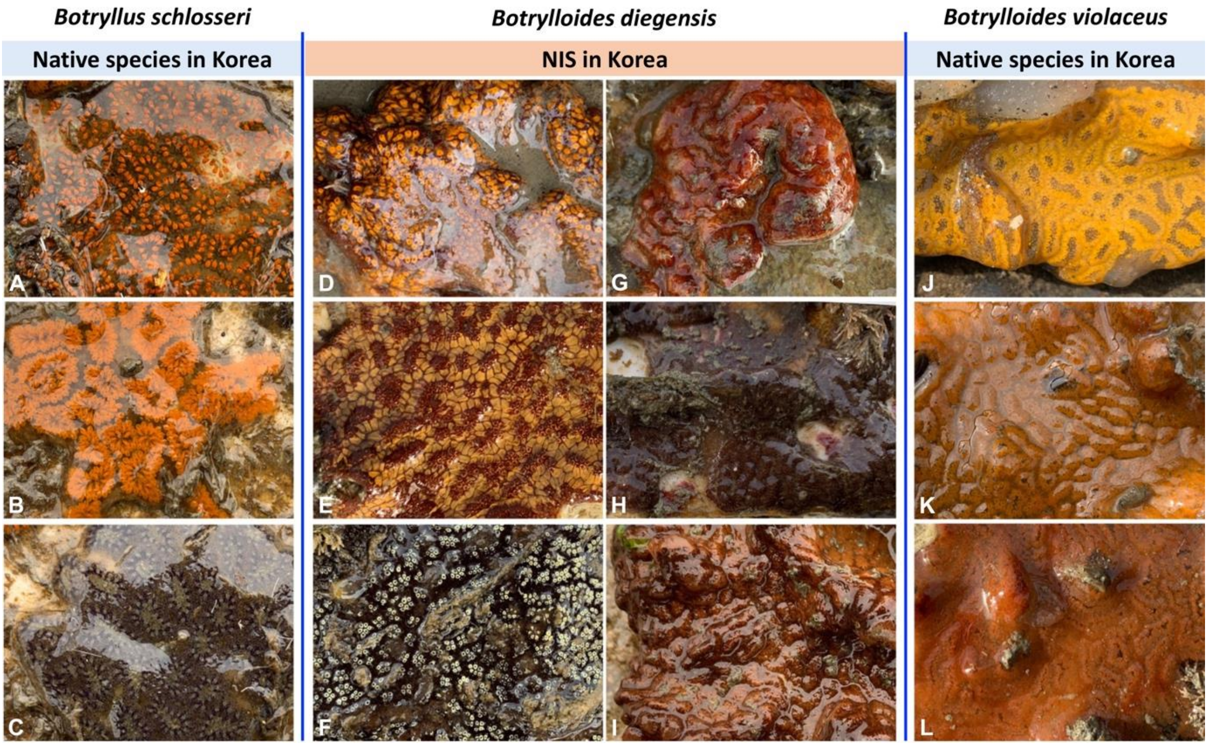

Figure 2. Various color and morphotypes of *Botryllus schlosseri* (**A**–**C**), *Botrylloides diegensis* (**D**–**I**), and *B. violaceus* (**J**–**L**) in South Korea.

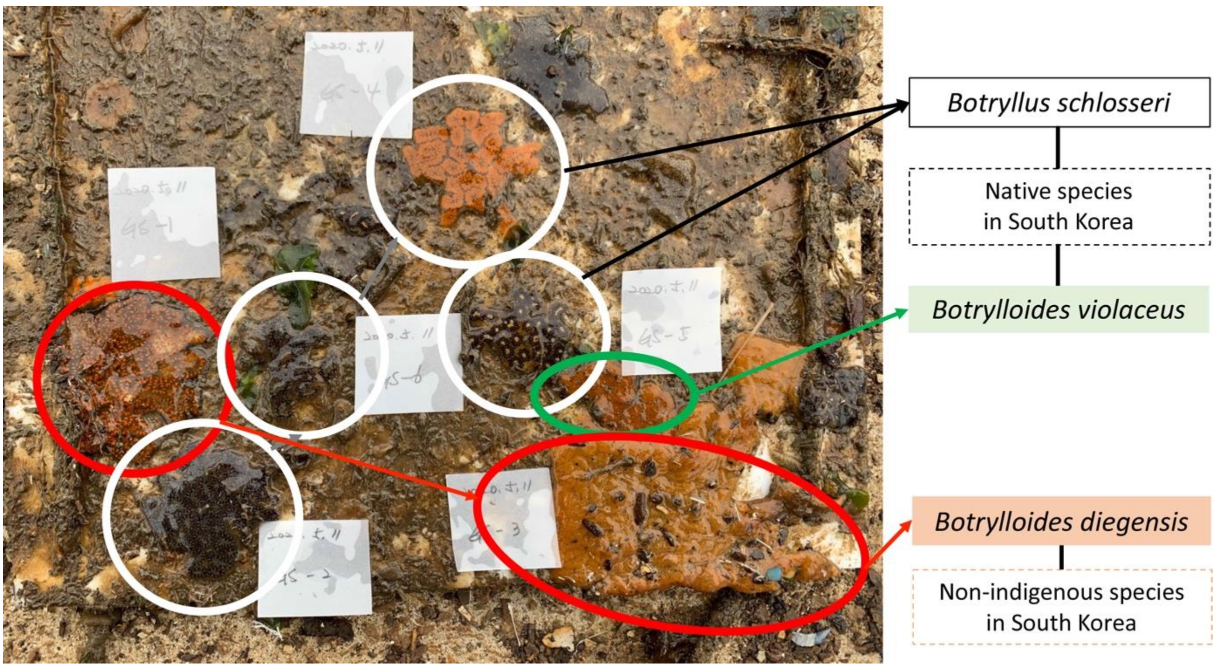

Figure 3. Colonies of *Botrylloides diegensis*, *B. violaceus*, and *Botryllus schlosseri* in a settlement plate (acrylic resin) of Gunsan harbor (11 May 2020).

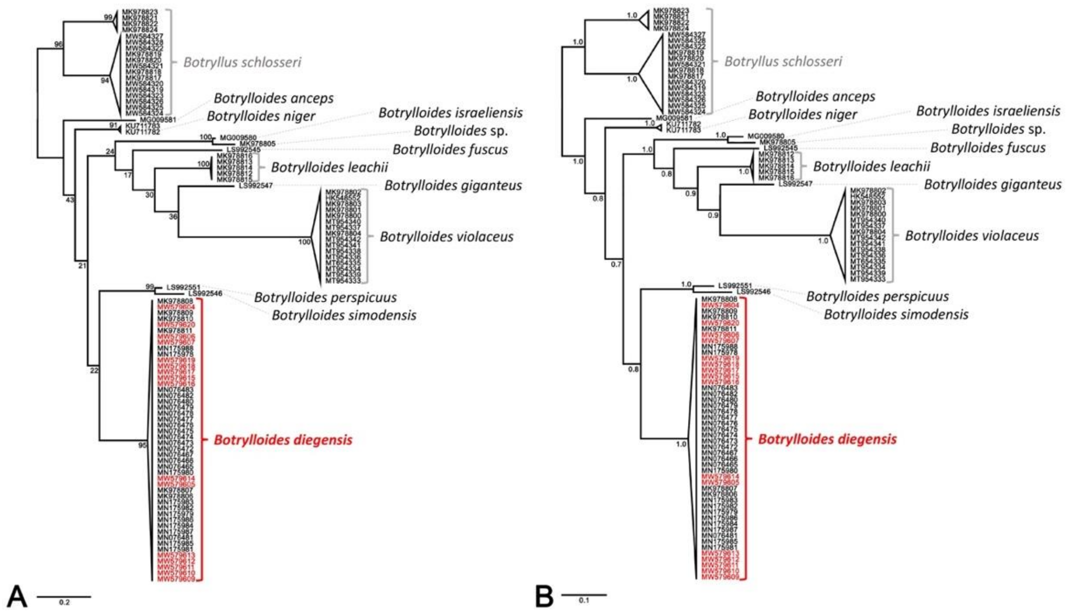

Figure 4. Phylogenetic trees constructed by the maximum likelihood (**A**) and Bayesian inference methods (**B**) for *B. diegensis*, other 10 *Botrylloides* species, and *B. schlosseri* (outgroup).

3.2. Distributions of B. diegensis and Other Similar Native Colonial Ascidians in South Korea

One-toned *Botrylloides diegensis* was quite similar to *B. violaceus* (Figures 2 and 3, Supplementary Figure S1). Thus, the existence of *B. diegensis* was not clearly recognized before this study. We carefully reexamined all settlement plate photographs and checked the distribution of two-toned *B. diegensis* (Table 4). From July 2017, two-toned *B. diegensis* appeared at six harbors (Table 4). It was newly observed in Incheon in January 2018 and appeared in Gwangyang and Dangjin in August 2018 and May 2020, respectively (Table 4). Two-toned *B. diegensis* and other botryllid species (*B. violaceus* and *B. schlosseri*) were observed in 12 of the 14 harbors, not including Busan and Yangpo (Figure 5). Among them, four harbors (Gunsan, Wando, Yeosu, and Ulsan) showed the existence of three botryllid species, including two-toned *B. diegensis* (Figure 5).

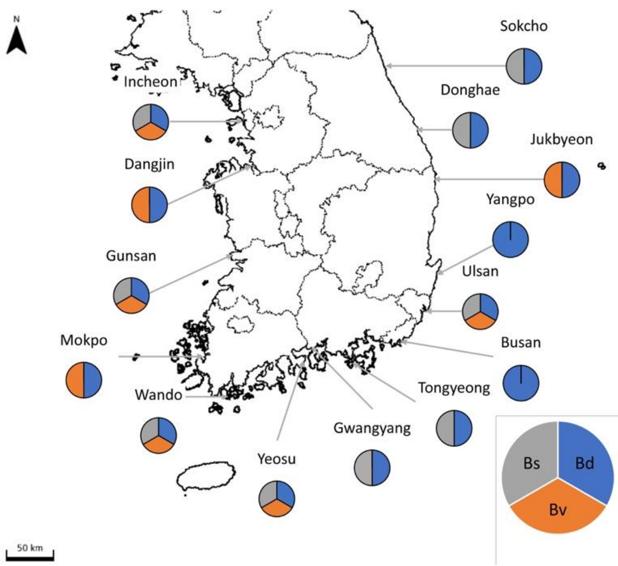

Figure 5. Pie charts showing the presence of three Botryllids in 14 harbors of South Korea. Bd—*Botrylloides diegensis*; Bs—*Botryllus schlosseri*; Bv—*Botrylloides violaceus*.

Table 3. Pairwise distances (%) for 11 species of *Botrylloides* and *Botryllus schlosseri* obtained from South Korea and GenBank based on the Kimura 2-parameter model.

Title	Species	*n* *	1	2	3	4	5	6	7	8	9	10	11	12	13
1	*B. diegensis* (Korea)	16	0.4												
2	*B. diegensis* (other regions) **	22	0.3	0.2											
3	*B. anceps*	1	15.9	16.0	NA										
4	*B. fuscus*	1	21.0	21.2	21.2	NA									
5	*B. giganteus*	1	18.8	18.9	22.2	21.9	NA								
6	*B. israeliense*	1	18.2	18.2	21.8	22.4	18.8	NA							
7	*B. leachii*	5	17.2	17.1	20.0	19.6	16.2	19.9	0.4						
8	*B. nigrum*	2	16.4	16.5	15.1	17.8	20.6	20.9	19.1	0.8					
9	*B. perspicuus*	1	15.8	16.0	18.4	18.9	23.1	20.3	20.3	16.5	NA				
10	*B. simodensis*	1	17.3	17.3	18.0	19.2	21.2	22.8	22.2	17.1	9.1	NA			
11	*B. violaceus*	16	24.2	24.4	22.1	24.8	22.7	25.8	22.5	21.6	23.4	23.4	1.3		
12	*Botrylloides* sp.	1	20.0	20.2	21.3	23.1	19.8	6.7	20.9	20.9	22.1	23.2	23.5	NA	
13	*Botryllus schlosseri*	18	20.0	20.0	18.2	20.9	21.2	23.2	22.3	18.3	19.2	19.5	24.4	22.9	6.5

* The number of sequences for each species used in this analysis. ** All sequences of *B. diegensis* were obtained from Viard et al. (2019) and Nydam et al. (2021).

Table 4. Results of settlement plate monitoring of two-toned *B. diegensis* in 14 harbors of South Korea from July 2017 to October 2020.

Locality	GIS		2017						2018								2020			
	N	E	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Feb.	May	Jul.	Oct.
1. Incheon	37.460556	126.625278							+	+				+	+			+	+	+
2. Dangjin	36.986944	126.516667																+	+	+
3. Gunsan	35.935833	126.516667	+	+	+	+	+	+	+	+		+		+	+	+		+	+	+
4. Mokpo	34.783861	126.389222	+	+	+	+	+	+	+				+	+	+	+				
5. Wando	34.317354	126.753546		+			+	+	+	+		+	+	+	+	+	+	+	+	+
6. Yeosu	34.717166	127.749114	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+
7. Gwangyang	34.908611	127.726111														+	+	+		
8. Tongyeong	34.827222	128.389222	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9. Busan	35.099722	129.755635	+	+	+	+	+	+	+	+	+	+	+	+	+	+			+	
10. Ulsan	35.099722	129.755635				+	+	+	+	+	+	+	+	+	+	+	+	+	+	
11. Yangpo	35.511111	129.385833	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
12. Jukbyeon	35.877818	129.519892		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
13. Donghae	37.498889	129.134356		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
14. Sokcho	38.210444	128.596249			+	+	+	+				+	+	+	+	+		+	+	+

4. Discussion

Several widely distributed botryllids, including *B. diegensis*, have been misidentified, and the correct identification of these species is critical for understanding their biology and spread, as well as for detecting the spread of additional species [27]. Preliminary molecular analyses revealed that these one-toned color colonies included specimens attributable to *B. diegensis*. Thus, *B. diegensis* might be misidentified in the field as *B. violaceus* based on the criterion of possessing one-toned color rather than two-toned color [3]. In addition, one-toned color *B. diegensis* is morphologically very similar to *B. violaceus* in Korea. In this study, we selected the mitochondrial cytochrome *c* oxidase subunit 1 (COI) for the detection of one- and two-toned color *B. diegensis* in South Korea. The COI was identified as the marker of choice for species discrimination [28] and has been effectively used for detecting NIS [8,29] and botryllids [27,30]. As a result, we recognized the presence of *B. diegensis* in South Korea based on DNA barcoding analysis. Thus, we needed to know when and where this species first appeared. We reviewed the monitoring data from 2017–2019, focusing on two-toned colonies of *B. diegensis*. As a result, *B. diegensis* was found to be present in six harbors in July 2017. It has now spread to 14 harbors along the coastal line of South Korea. *Botrylloides diegensis* was not present in the Northwest Pacific region, including Korea and Japan [18,31,32], according to previous ascidian studies (~2020). Professor Rho, a great ascidian taxonomist in Korea, did not report this species either. Only two *Botrylloides*, *B. magnicoecum* and *B. violaceus*, have been reported by Rho [19,33–40]. However, in 2021, Nydam et al. [27] first reported *B. diegensis* in Japan and these specimens were collected in 2005–2009 in three localities of Japan. Thus, we supposed that *B. diegensis* was introduced to the Northwest Pacific region before 2006. *Botrylloiodes violaceus* and *B. diegensis* are both native to the North Pacific [3]. While the former is native to the Northwest Pacific, there is more uncertainty regarding the native range of the latter [41]. Although *B. diegensis* was originally described from the Northeast Pacific (southern California), it might have been introduced from the Indo-Pacific [31,42]. This remains unclear. The presence of *B. diegensis* was confirmed through this study, and therefore, the investigation of the introductory route of *B. diegensis* is urgently needed, and also investigate the ecological and economic impact from *B. diegensis* in South Korea.

5. Conclusions

Based on our DNA barcoding results, one- and two-toned color *B. diegensis* has spread along all coastal lines of South Korea. It was possibly introduced to South Korea between 1999 and 2016 based on field monitoring data and previous studies. Further studies are needed to analyze the specific route of its introduction to South Korea based on the population genetic studies and previous monitoring data.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/w13162164/s1. Table S1: Pairwise distances (%) within 11 species of *Botrylloides* and *Botryllus schlosseri* from South Korea and GenBank, based on the Kimura 2-parameter model. Figure S1: *Botrylloides diegensis*, *Botrylloides violaceus* and *Botryllus schlosseri* in South Korea.

Author Contributions: T.L. contrived the subject of the article, performed the sampling, experiments, literature review, and contributed to the writing of the paper. S.S. performed the project administration. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data supporting the findings of this study are available within the article.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Nunes, A.L.; Katsanevakis, S.; Zenetos, A.; Cardoso, A.C. Gateways to alien invasions in the European seas. *Aquat. Invasions* **2014**, *9*, 133–144. [CrossRef]
2. Darling, J.A.; Galil, B.S.; Carvalho, G.R.; Rius, M.; Viard, F.; Piraino, S. Recommendations for developing and applying genetic tools to assess and manage biological invasions in marine ecosystems. *Mar. Policy* **2017**, *85*, 54–64. [CrossRef]
3. Viard, F.; Roby, C.; Turon, X.; Bouchemousse, S.; Bishop, J. Cryptic diversity and database errors challenge non-indigenous species surveys: An illustration with *Botrylloides* spp. in the English channel and Mediterranean Sea. *Front. Mar. Sci.* **2019**, *6*, 615. [CrossRef]
4. Bishop, J.D.D.; Roby, C.; Yunnie, A.L.E.; Wood, C.A.; Leveque, L.; Turon, X.; Viard, F. The southern hemisphere ascidian *Asterocarpa humilis* is unrecognised but widely established in NW France and Great Britain. *Biol. Invasions* **2013**, *15*, 253–260. [CrossRef]
5. McGlashan, D.; Ponniah, M.; Cassey, P.; Viard, F. Clarifying marine invasions with molecular markers: An illustration based on mtDNA from mistaken calyptraeid gastropod identifications. *Biol. Invasions* **2008**, *10*, 51–57. [CrossRef]
6. Ordóñez, V.; Pascual, M.; Fernández-Tejedor, M.; Turon, X. When invasion biology meets taxonomy: *Clavelina oblonga* (Ascidiacea) is an old invader in the Mediterranean Sea. *Biol. Invasions* **2016**, *18*, 1203–1215. [CrossRef]
7. Comtet, T.; Sandionigi, A.; Viard, F.; Casiraghi, M. DNA (meta)barcoding of biological invasions: A powerful tool to elucidate invasion processes and help managing aliens. *Biol. Invasions* **2015**, *17*, 905–922. [CrossRef]
8. Dias, P.J.; Fotedar, S.; Munoz, J.; Hewitt, M.J.; Lukehurst, S.; Hourston, M.; Wellington, C.; Duggan, R.; Bridgwood, S.; Massam, M.; et al. Establishment of a taxonomic and molecular reference collection to support the identification of species regulated by the Western Australian Prevention List for Introduced Marine Pests. *Manag. Biol. Invasion* **2017**, *8*, 215–225. [CrossRef]
9. Smith, K.F.; Stefaniak, L.; Saito, Y.; Gemmill, C.E.C.; Cary, S.C.; Fidler, A.E. Increased inter-colony fusion rates are associated with reduced COI haplotype diversity in an invasive colonial ascidian *Didemnum vexillum*. *PLoS ONE* **2012**, *7*, e30473. [CrossRef]
10. Smith, K.F.; Abbott, C.L.; Saito, Y.; Fidler, A.E. Comparison of whole mitochondrial genome sequences from two clades of the invasive ascidian, *Didemnum vexillum*. *Mar. Genom.* **2015**, *19*, 75–83. [CrossRef]
11. Stefaniak, L.; Zhang, H.; Gittenberger, A.; Smith, K.F.; Holsinger, K.; Lin, S.; Whitlatch, R.B. Determining the native region of the putatively invasive ascidian *Didemnum vexillum* Kott, 2002. *J. Exp. Mar. Biol. Ecol.* **2012**, *422–423*, 64–71. [CrossRef]
12. Nydam, M.L.; Giesbrecht, K.B.; Stephenson, E.E. Origin and dispersal history of two colonial ascidian clades in the *Botryllus schlosseri* species complex. *PLoS ONE* **2017**, *12*, e0169944. [CrossRef]
13. Shenkar, N.; Gittenberger, A.; Lambert, G.; Rius, M.; Moreira da Rocha, R.; Swalla, B.J.; Turon, X. Ascidiacea World Database. Accessed through: World Register of Marine Species. 2021. Available online: http://www.marinespecies.org/aphia.php?p=taxdetails&id=103529 (accessed on 26 April 2021).
14. Cohen, A.N.; Harris, L.H.; Bingham, B.L.; Carlton, J.T.; Chapman, J.W.; Lambert, C.C.; Lambert, G.; Ljubenkov, C.; Murray, S.N.; Rao, L.C.; et al. Rapid assessment survey for exotic organisms in southern California bays and harbors, and abundance in port and non-port areas. *Biol. Invasions* **2005**, *7*, 995–1002. [CrossRef]
15. Simkanin, C.; Fofonoff, P.W.; Larson, K.; Lambert, G.; Dijkstra, J.A.; Ruiz, G.M. Spatial and temporal dynamics of ascidian invasions in the continental United States and Alaska. *Mar. Biol.* **2016**, *163*, 1–16.
16. Pyo, J.; Shin, S. A new record of invasive alien colonial tunicate *Clavelina lepadiformis* (Ascidiacea: Aplousobranchia: Clavelinidae) in Korea. *ASED* **2011**, *27*, 197–200. [CrossRef]
17. Pyo, J.; Lee, T.; Shin, S. Two newly recorded invasive alien ascidians (Chordata, Tunicata, Ascidiacea) based on morphological and molecular phylogenetic analysis in Korea. *Zootaxa* **2012**, *3368*, 211–228. [CrossRef]
18. Tokioka, T. *Pacific Tunicata of the United States National Museum*; United States National Museum Bulletin 251; Smithsonian Press: Washington, DC, USA, 1967; Volume 251, pp. 1–247.
19. Rho, B.J. The ascidians (Tunicata) from Chindo island, Korea. *Korean J. Syst. Zool.* **1995**, *11*, 125–145.
20. Folmer, O.; Black, M.; Hoeh, W.; Lutz, R.; Vrijenhoek, R. DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. *Mol. Mar. Biol. Biotechnol.* **1994**, *3*, 294–299. [PubMed]
21. Brunetti, R.; Manni, L.; Mastrototaro, F.; Gissi, C.; Gasparini, F. Fixation, description and DNA barcode of a neotype for *Botryllus schlosseri* (Pallas, 1766) (Tunicata, Ascidiacea). *Zootaxa* **2017**, *4353*, 29–50. [CrossRef]
22. Darriba, D.; Taboada, G.; Doallo, R.; Posada, D. jModelTest 2: More models, new heuristics and parallel computing. *Nat. Methods* **2012**, *9*, 772. [CrossRef]
23. Guindon, S.; Dufayard, J.F.; Lefort, V.; Anisimova, M.; Hordijk, W.; Gascuel, O. New Algorithms and Methods to Estimate Maximum-Likelihood Phylogenies: Assessing the Performance of PhyML 3.0. *Syst. Biol.* **2010**, *59*, 307–321. [CrossRef]
24. Huelsenbeck, J.P.; Ronquist, F. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **2001**, *17*, 754–755. [CrossRef]
25. Kimura, M. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* **1980**, *16*, 111–120. [CrossRef] [PubMed]
26. Kumar, S.; Stecher, G.; Tamura, K. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* **2016**, *33*, 1870–1874. [CrossRef]

27. Nydam, M.L.; Lemmon, A.R.; Cherry, J.R.; Kortyna, M.L.; Clancy, D.L.; Hernandez, C.; Sarah Cohen, C. Phylogenomic and morphological relationships among the botryllid ascidians (Subphylum Tunicata, Class Ascidiacea, Family Styelidae). *Sci. Rep.* **2021**, *11*, 8351. [CrossRef]
28. Hebert, P.D.N.; Cywinska, A.; Ball, S.L.; Jeremy, R. Biological identifications through DNA barcodes. *Proc. Biol. Sci.* **2003**, *270*, 313–321. [CrossRef] [PubMed]
29. Dias, P.J.; Lukehurst, S.S.; Simpson, T.; Rocha, R.M.; Tovar-Hernández, M.A.; Wellington, C.; McDonald, J.; Snow, M.; Kennington, J. Multiple introductions and regional spread shape the distribution of the cryptic ascidian *Didemnum perlucidum* in Australia: An important baseline for management under climate change. *Aquat. Invasions* **2021**, *16*, 297–313. [CrossRef]
30. Salonna, M.; Gasparini, F.; Huchon, D.; Montesanto, F.; Haddas-Sasson, M.; Ekins, M.; McNamara, M.; Mastrototaro, F.; Gissi, C. An elongated COI fragment to discriminate botryllid species and as an improved ascidian DNA barcode. *Sci. Rep.* **2021**, *11*, 4078. [CrossRef]
31. Tokioka, T. Contributions to Japanese ascidian fauna XX. The outline of Japanese ascidian fauna as compared with that of the Pacific coasts of North America. *Publ. Seto Mar. Biol. Lab.* **1963**, *11*, 131–156. [CrossRef]
32. Nishikawa, T. The ascidians of the Japan Sea II. *Publ. Seto Mar. Biol. Lab.* **1991**, *35*, 25–170. [CrossRef]
33. Rho, B.J. Taxonomic study on the prochordates from Korea 1 (Ascidians). *Korean Cult. Res. Inst. Trans.* **1966**, *8*, 209–216.
34. Rho, B.J. A study on the classification and the distribution of the Korean ascidians. *J. Korean Res. Inst. Better Living* **1971**, *6*, 103–166.
35. Rho, B.J. On the classification and the distribution of the marine benthic animals in Korea (3. Ascidians). *J. Korean Res. Inst. Better Living* **1975**, *15*, 121–169.
36. Rho, B.J.; Huh, M.K. A systematic study on the ascidians in Korea. *J. Korean Res. Inst. Better Living* **1984**, *33*, 99–136.
37. Rho, B.J.; Lee, J.E. A systematic study on the ascidians from Cheju island, Korea. *Korean J. Syst. Zool.* **1989**, *5*, 59–76.
38. Rho, B.J.; Lee, J.E. A systematic study on the ascidians in Korea. *Korean J. Syst. Zool.* **1991**, *7*, 195–220.
39. Rho, B.J.; Choe, B.L.; Song, J.I. Biosystematic studies on the marine fouling invertebrates in Korea—A systematic study on the ascidians from Chundo island (Onsan Bay), Korea. *Korean J. Syst. Zool.* **1996**, *7*, 195–220.
40. Rho, B.J.; Park, K.S. Taxonomy of ascidians from Geojedo island in Korea. *Korean J. Syst. Zool.* **1998**, *14*, 173–192.
41. Carlton, J.T. Setting Ascidian Invasions on the Global Stage. In Proceedings of the International Invasive Sea Squirt Conference, 21–22 April 2005; Woods Hole Oceanographic Institution: Woods Hole, MA, USA, 2005. Available online: http://www.whoi.edu/page.do?pid=11421&tid=282&cid=16303 (accessed on 20 April 2007).
42. Carlton, J.T. Deep invasion ecology and the assembly of communities in historical time. In *Biological Invasions in Marine Ecosystems: Ecological, Management, and Geographic Perspectives*; Rilov, G., Crooks, J.A., Eds.; Springer: Berlin, Germany, 2009; pp. 13–56.