

Long-Term Investigation of Marine-Derived *Aspergillus* Diversity in the Republic of Korea

Jun Won Lee , Wonjun Lee , Rekhani Hansika Perera  and Young Woon Lim 

School of Biological Sciences and Institute of Microbiology, Seoul National University, Seoul, Korea

ABSTRACT

Aspergillus species play a crucial role in terrestrial environments as degraders and are well known for producing various secondary metabolites. Recently, *Aspergillus* species have been discovered in marine environments, exhibiting adaptability to high salinity and producing diverse secondary metabolites with valuable properties. However, limited research has focused on their marine diversity, leading to inaccurate species identification. The current study addresses this gap by investigating diverse marine habitats in the Republic of Korea, including sediment, seawater, seaweed, and marine animals. From three coasts of the Korean Peninsula, 472 *Aspergillus* strains were isolated from the various marine habitats. A total of 41 species were accurately identified using multigenetic markers: internal transcribed spacer, calmodulin, and β -tubulin. The findings underscore the importance of accurate identification and provide a basis for elucidating the functional role of marine-derived *Aspergillus* species in marine ecosystems.

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1. Introduction


Aspergillus is a genus of diverse fungal species that commonly grow on the surface of various substrates in terrestrial environments. The genus *Aspergillus* plays an important role in ecosystems as degraders of a wide range of natural organic substrates. *Aspergillus* species possess asexual spore-forming structure, aspergillum and many teleomorph genera have been designated to *Aspergillus* anamorph such as *Chaetosartorya*, *Emericella*, *Eurotium*, and *Neosartorya* [1]. Even though they were recognized as distinct sexual genera, various studies have shown that they all belong to monophyletic *Aspergillus* [2,3]. The genus comprises six subgenera, 27 sections, and 446 species [4]. *Aspergillus* contributes significantly to the industry by producing organic acids, antibiotics, and degrading polysaccharides such as cellulose [5–9]. *Aspergillus* species have also been known as endophytes of plants [10]. Some species are mycotoxin producers, food spoilers, and human pathogens [11].

Recently, *Aspergillus* species have also been reported from marine environments. Marine-derived *Aspergillus* have been primarily isolated from intertidal zones, deep-sea sediment, sponges, and algae [12–15]. There are 47 *Aspergillus* species reported from marine habitats [16]. Marine-derived *Aspergillus* can grow in marine environments with more than

30% salinity [17,18]. They produce a large number of secondary metabolites with antimicrobial, cytotoxic, insecticidal, neuroprotective, and antioxidant effects [19,20]. *Aspergillus* species contribute significantly to the degradation of marine waste as they are efficient at cellulose degradation [21]. Although research on marine-derived *Aspergillus* have been reported, few studies have been undertaken on the diversity of marine *Aspergillus*. Studies have primarily focused on discovering bioactive compounds or testing enzyme activity. Therefore, the identification of many *Aspergillus* species has been reported as inaccurate because the identification was dependent on morphological traits and/or the internal transcribed spacer (ITS) sequence. For the identification of *Aspergillus*, growth rate, the color of the colony, and size of conidial heads and conidia are crucial characteristics [22]. However, both the morphological features and ITS are insufficient for species level identification of *Aspergillus* [22].

Marine-derived *Aspergillus* species were isolated from mudflats and sea sand of the eastern and southern coasts [14] and decaying spot of macroalgae (*Agarum clathratum*) in the Republic of Korea [23]. Since then, we have isolated many strains of *Aspergillus* from various marine environments. In this study, we investigated the diversity of marine-

CONTACT Young Woon Lim  ywlim@snu.ac.kr

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derived *Aspergillus* species in the Republic of Korea as a part of a project organized by the Marine Fungal Resource Bank (MFRB). We used multigenetic markers, ITS, calmodulin (*CaM*), and the β -tubulin (*BenA*) for *Aspergillus* identification using a standardized method [22].

2. Materials and methods

2.1. Sample collection and isolation

The marine-derived samples were collected around marine environments of the Republic of Korea during 2006–2022 (Table S1). Mudflat and sea sand samples were collected at 2–3 cm depth after removing the topsoil to avoid surface contamination. The deep-sea sediment samples were collected using a Smith-Mcintyre grab sampler. 5 g of each sediment was diluted tenfold or hundredfold with sterilized seawater (SSW). Each dilution with 100 μ L was spread on dichloran rose bengal chloramphenicol agar (DRBC, Difco, Becton Dickinson, Sparks, MD, USA), glucose yeast extract agar (GYA; 1 g L⁻¹ glucose, 0.1 g L⁻¹ yeast extract, 0.5 g L⁻¹ peptone, and 15 g L⁻¹ agar, Oxoid, Hampshire, UK) media, or Sabouraud dextrose agar (SNA, Difco, Becton Dickinson, Sparks, MD, USA). Seawater was sampled from the surface or bottom with sterilized bottles or using Niskin bottles equipped in the conductivity, temperature, and depth (CTD) rosettes and filtered through a sterile polycarbonate track-etched membrane filter ($d = 47$ mm, $\phi = 0.2$ μ m, GVS Filter Technology, Sanford, USA) by using a vacuum pump as done in a previous study [24]. The filters were placed and cultured on DRBC agar for 7–28 days. The crab, sandfish egg, seaweed, shellfish, sponge, and starfish were collected and transported in sterile bags on ice. To remove surface debris and soil, each sample was washed with SSW. Using sterilized scissors, samples were cut into 5 \times 5 mm pieces. Each sample was transferred to DRBC, GYA, or SNA. Plates were incubated at 25 °C for 7–14 days. Each culture was transferred to a new PDA plate to obtain pure cultures. A map of where the samples were collected was conducted using R version 4.1.3 [25]. Identified strains were visualized on the map by using the R ggmap package with Adobe Photoshop 2022 (Adobe Systems, USA) software [26].

2.2. DNA extraction, PCR amplification, and sequencing

AccuPrep Genomic DNA extraction kit (Bioneer Co., Daejeon, Korea) was used to extract genomic DNA from the fungal mycelia, following the manufacturer's protocol. The procedure was slightly modified using CTAB buffer instead of a TL buffer.

PCR amplifications of ITS, *BenA*, and *CaM* markers were done using primer pairs of ITS1F/ITS4 [27], Bt2a/Bt2b [28], and CF1/CF4 or CMD5/CMD6 [29,30], respectively. PCR was conducted on a C1000 thermal cycler (Bio-Rad, Hercules, California, USA) with the AccuPower[®] PCR PreMix (Bioneer Co., Daejeon, Korea) in a final volume of 20 μ L, containing 10 pmol of each primer and 10 ng of DNA. PCR amplification was done following the conditions mentioned in [22]. The PCR products were purified using the Expin[™] PCR SV Kit (GeneAll Biotechnology, Seoul, Korea) or an ExoSAP-IT Express PCR Product Cleanup (Thermo Fisher Scientific, Waltham, Massachusetts, USA) following the manufacturer's guidelines. Both forward and reverse directions were sequenced. Sequencing was done at Macrogen (Seoul, Korea) using an ABI PRISM 3700 Genetic Analyzer (Life Technologies, Carlsbad, California, USA). Forward and reverse sequences were assembled using the *De novo assemble* function in the Geneious Prime software ver. 2022.0.2. (Biomatters Ltd., San Diego, California, USA) [31]. Sequences were deposited in GenBank. GenBank accession numbers including reference sequences are provided in Table S2.

2.3. Phylogenetic analysis

Reference sequences were downloaded from GenBank based on NCBI blast search and following recent publications. Sequences were aligned using MAFFT v. 7 online server (<http://mafft.cbrc.jp/alignment/server/index.html>) [32] and improved manually using the Geneious Prime (Biomatters Inc., USA) software. *Aspergillus* isolates were assigned to sections using the maximum likelihood (ML) tree generated based on ITS sequences. The phylogenetic analysis for entire species was conducted with combined sequences of ITS, *BenA*, and *CaM*. ML phylogenetic analyses were conducted using RAxML v. 8.2 [33] with 1000 rapid bootstraps. The final tree was selected among suboptimal trees from each run by comparing likelihood scores with the GTRGAMMA nucleotide substitution model.

3. Results

Based on the ITS sequence data, 472 *Aspergillus* strains were recognized from various marine habitats along three seashores of the Republic of Korea (Figure 1). They were further identified as 41 different *Aspergillus* species in 15 sections using phylogenetic analysis of combined ITS, *BenA*, and *CaM* markers. All *Aspergillus* strains were visualized with ML tree with 91 reference type strains (Figure 2). The final alignments comprised 632 bases for ITS,

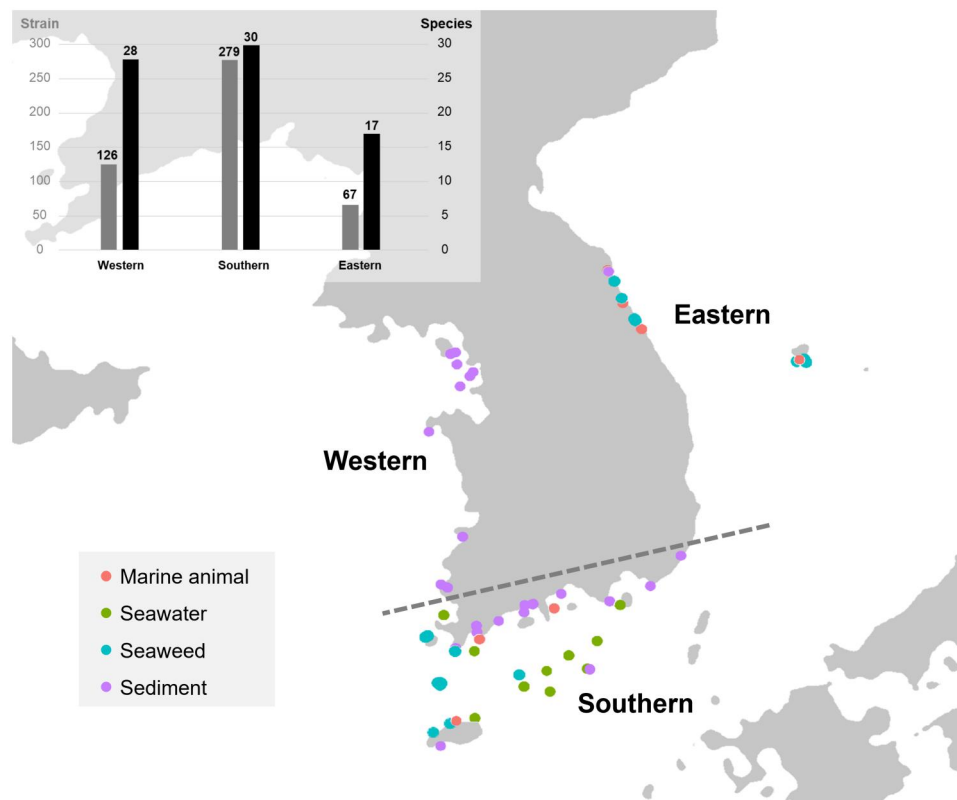


Figure 1. Map showing the location of sampling sites and bar graph with the number of *Aspergillus* strains and species isolated from each seaside. The colored dots indicate the habitats: marine animal (red), seawater (green), seaweed (blue), sediment (purple).

524 for *BenA*, and 779 for *CaM*. The *Aspergillus* and *Nidulantes* sections are the most dominant sections in the marine habitats in the Republic of Korea, where each of six species were found (Figure 2, Table 1).

Aspergillus species were reported from a variety of substrates. From marine sediment, the most diverse *Aspergillus* species were discovered from mudflats (37 species) followed by sea sand (14 species) and deep-sea sediment (2 species). Eight species were isolated from seawater at the surface and/or at bottom depth. Eighteen species were isolated from the seaweed and 12 species from marine animals including crabs, sandfish eggs, shellfish, sponges, and starfish (Table 1). The most diverse species were discovered from the southern seaside with 30 species, followed by 28 from the western and 17 species from the eastern seaside (Figure 1). Of the 41 species, 12 species were isolated from all three seashores (Table 1). Detailed information for each strain is provided in Supplementary Table 1.

4. Discussion

Although marine-derived *Aspergillus* species have been discovered in various habitats, no research has been conducted on its overall marine diversity. In this study, we isolated 472 *Aspergillus* strains from diverse marine habitats, including sediments,

seawater, seaweed, and marine animals around the seashores of the Republic of Korea. Overall, 41 species (in 15 sections) were identified based on the analyses of ITS, *CaM*, and *BenA* genetic markers (Table 1). We used the latest version of the name of *Aspergillus* species [4,34,35–37]. Thirty one *Aspergillus* species were first discovered in new to marine environments compared with the previous list [16]. Twelve species have been recorded for the first time from the marine environments in South Korea. Even though our research was limited to the marine environment of the Republic of Korea, we discovered a number of *Aspergillus* species from marine sites.

Based on our collection, the section *Aspergillus* (6 species) and *Nidulantes* (6) are the most diverse sections followed by *Fumigati* (5), *Nigri* (5), and *Circumdati* (3) in marine habitats. However, at the subgenus level, all subgenera contained marine-derived species. This suggests that there is no specific marine-adapted clade, and that the majority of the *Aspergillus* living on terrestrial environments can survive in marine environments with salt tolerance and osmotolerant mechanisms [38–40]. Dominantly isolated species such as *A. clavatus*, *A. creber*, *A. fumigatus*, *A. niger*, *A. sydowii*, and *A. terreus* are also commonly found in indoor or terrestrial environments [41,42]. To date, salt-barriers have been known to play a role in dividing communities between species. However, fungi are strong

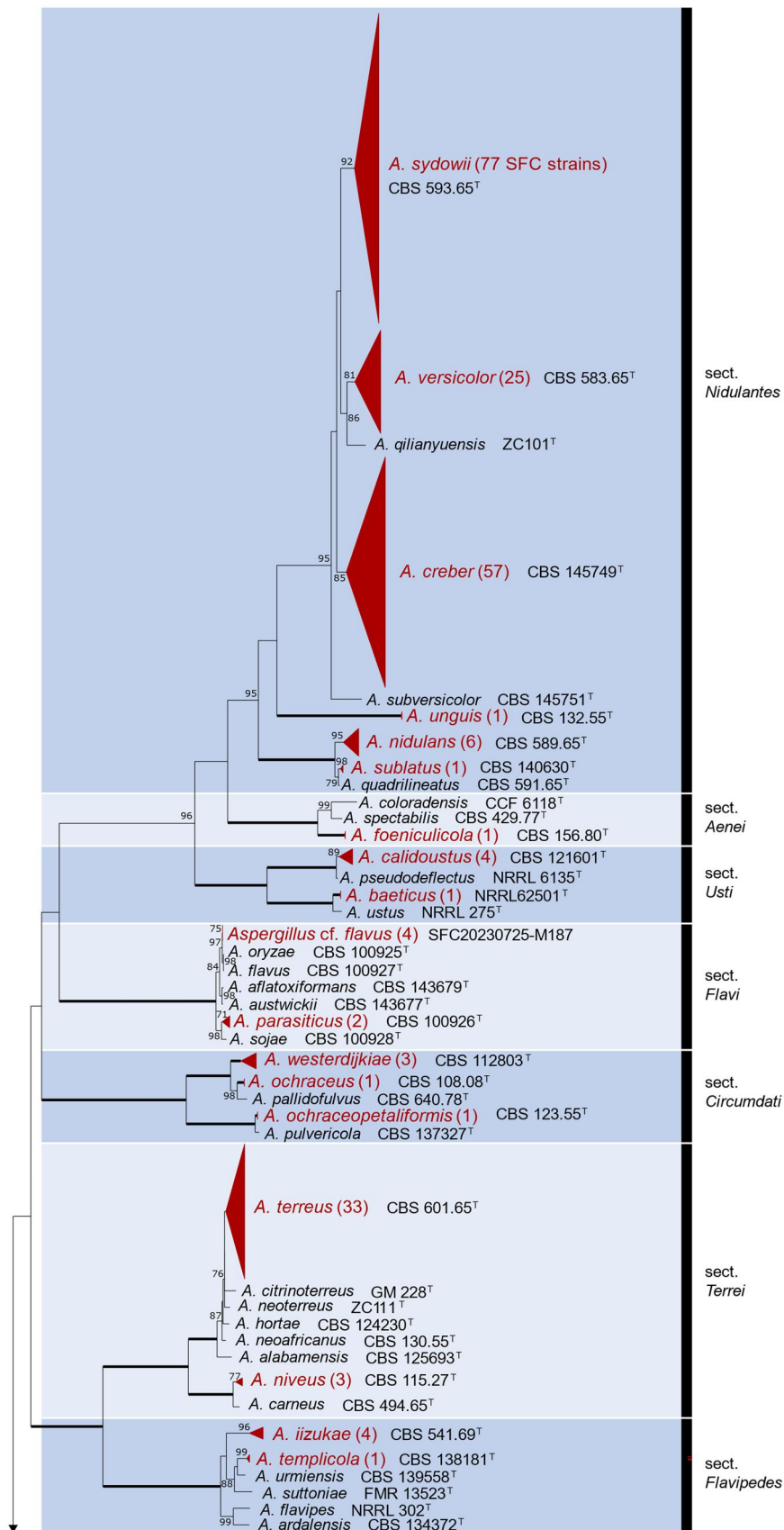


Figure 2. Maximum likelihood phylogenetic tree of *Aspergillus* species based on the combined data set of ITS, *BenA*, and *CaM* sequences. Bootstrap values >70 are presented at the nodes. Thick lines on branches indicate 100 bootstrap support. The scale bar represents the number of nucleotide substitutions per site. "T" indicates the ex-type strains. *Aspergillus* species reported in this study are collapsed at a species level, and the number of SFC strains is indicated in parentheses.

colonizers on both terrestrial and marine environments, and transitions between the environments occur frequently [43]. Further research is needed to

elucidate the unique characteristics and ecological role of marine-derived *Aspergillus* in the marine environment.

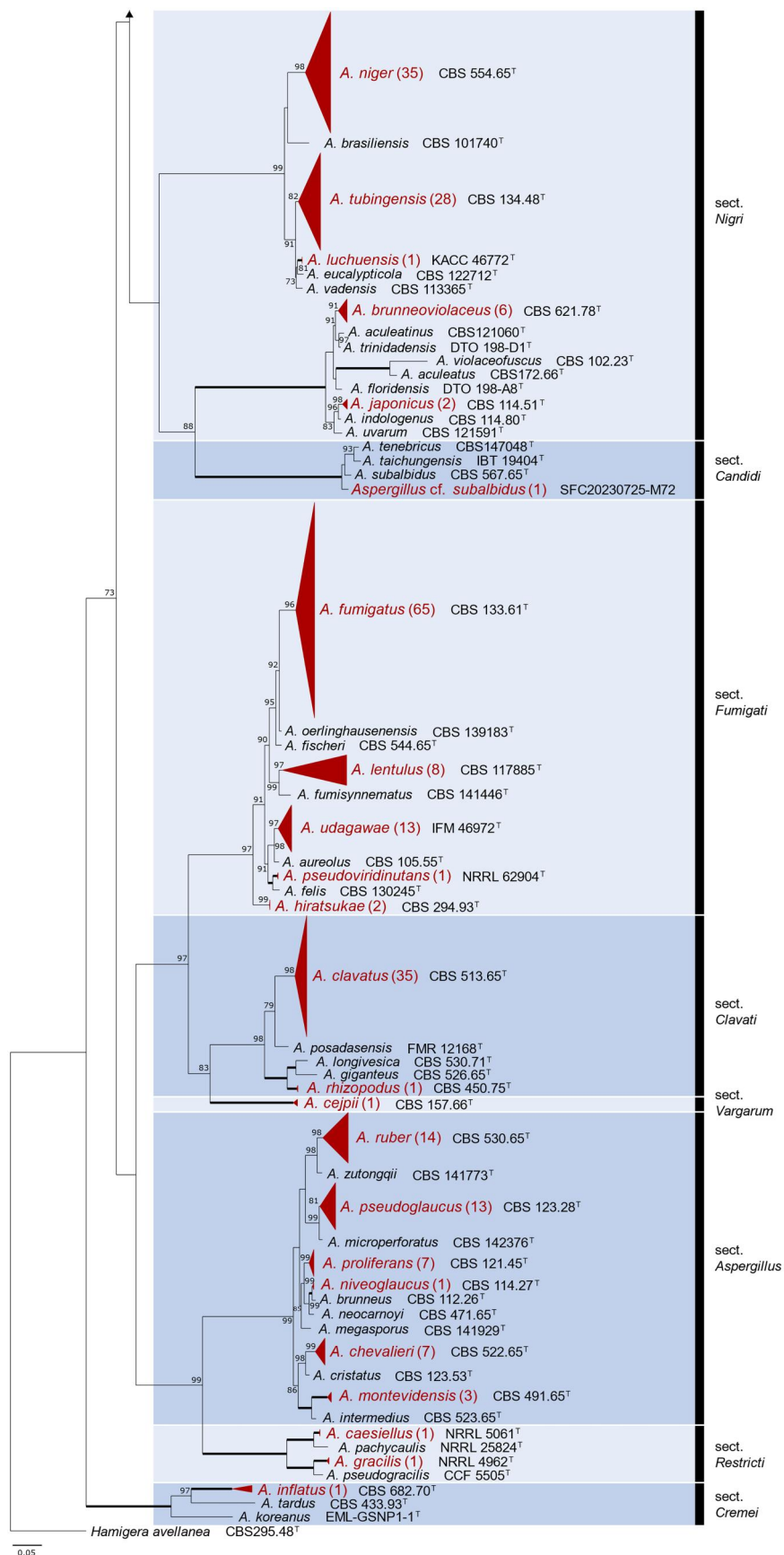


Figure 2. Continued

The three seasides of the Republic of Korea have different characteristics and diversities because of differences in ocean currents and the depth of the

surrounding oceans [44,45]. In some organisms, such as planktonic animals or protists, each seaside constitutes a significantly different community

Table 1. Discovered *Aspergillus* species with the habitat and seaside information.

Section	Species	Habitat					Seaside		
		Sediment	Seawater	Seaweed	Marine animal	Western	Southern	Eastern	
<i>Aenei</i> <i>Aspergillus</i>	<i>A. foeniculicola</i>	Mudflat							
	<i>A. chevalieri</i>	Mudflat, Sea sand		Agarum clathratum	Sandfish egg				
	<i>A. montevidensis</i>	Mudflat, Sea sand		Sargassum sp.					
	<i>A. niveoglaucus</i>	Mudflat							
	<i>A. proliferans</i>	Mudflat, Sea sand		<i>Dictyopteris proliferata</i> , <i>Hypnea</i> sp., <i>Sargassum</i> sp., <i>Chondria crassicaulis</i> , <i>Eisenia bicyclis</i> , <i>Hypnea</i> sp., <i>Sargassum</i> sp.	Sponge				
	<i>A. pseudoglaucus</i>	Mudflat		<i>Dictyopteris proliferata</i> , <i>Eisenia bicyclis</i> , <i>Hypnea</i> sp., <i>Sargassum</i> sp.	Crab, sponge				
<i>Candidi</i> <i>Circumdati</i>	<i>A. ruber</i>	Mudflat							
	<i>A. subalbidus</i>	Mudflat							
	<i>A. ochraceopetaliformis</i>	Mudflat							
	<i>A. ochraceus</i>	Mudflat							
	<i>A. westerdijkiae</i>	Mudflat, Sea sand							
	<i>A. clavatus</i>	Mudflat, Sea sand							
	<i>A. rhizopodus</i>	Mudflat							
	<i>A. inflatus</i>	Mudflat		Seaweed		Sponge (<i>Hymeniacidon</i> sp.)			
	<i>Aspergillus</i> cf. <i>flavus</i>	Mudflat							
	<i>Flavipedes</i>	<i>A. parasiticus</i>	Mudflat	Surface seawater					
<i>A. tizukae</i>		Mudflat							
<i>A. templicola</i>		Mudflat							
<i>A. fumigatus</i>		Mudflat, Sea sand	Surface seawater	<i>Agarum clathratum</i> , <i>Chondria</i> sp., <i>Eisenia bicyclis</i> , <i>Hypnea</i> sp., <i>Sargassum fusiforme</i> , <i>Sargassum</i> sp., <i>Eisenia bicyclis</i>	Sandfish egg				
<i>Nidulantes</i>	<i>A. hirsutukae</i>	Mudflat							
	<i>A. lentulus</i>	Mudflat, Sea sand							
	<i>A. pseudoviridinutans</i>	Mudflat							
	<i>A. udagawae</i>	Mudflat, Sea sand							
	<i>A. creber</i>	Mudflat, Sea sand							
	<i>A. nidulans</i>	Mudflat	Bottom seawater,	<i>Chondria crassicaulis</i> , <i>Eisenia bicyclis</i> , <i>Hypnea</i> sp., <i>Sargassum</i> sp., <i>Sargassum thunbergii</i> , unknown	Sandfish egg,				
	<i>A. sublatius</i>	Mudflat	surface seawater	Unknown	Sponge, Starfish				
	<i>A. sydowii</i>	Deep-sea sediment, mudflat, sea sand	Surface seawater		Sandfish egg				
	<i>A. unguis</i>	Mudflat	Bottom seawater,	<i>Chondria</i> sp., <i>Codium fragile</i> , <i>Sargassum fusiforme</i> , <i>Sargassum</i> sp., unknown	Sponge				
	<i>A. versicolor</i>	Deep-sea sediment, mudflat	Surface seawater	<i>Chondria</i> sp., <i>Dictyopteris proliferata</i> , <i>Sargassum</i> sp.	Sponge				
<i>Nigri</i>	<i>A. brunneoviolaceus</i>	Mudflat	Surface seawater	<i>Sargassum fusiforme</i> , unknown					
	<i>A. japonicus</i>	Mudflat		Unknown					
	<i>A. luchuensis</i>	Mudflat, Sea sand		<i>Agarum clathratum</i> , <i>Codium fragile</i> , <i>Laurencia</i> sp., unknown	Sandfish egg				
	<i>A. niger</i>	Mudflat, Sea sand		<i>Sargassum</i> sp., unknown	Sandfish egg				
	<i>A. tubingenis</i>	Mudflat, Sea sand	Surface seawater						
<i>Restricti</i>	<i>A. caesiellus</i>	Mudflat							
	<i>A. gracilis</i>	Mudflat							
<i>Terrei</i>	<i>A. niveus</i>	Mudflat, Sea sand							
	<i>A. terreus</i>	Mudflat, Sea sand							
	<i>A. baeticus</i>	Mudflat		<i>Agarum clathratum</i> , <i>Sargassum thunbergii</i>					
<i>Usti</i>	<i>A. baeticus</i>	Mudflat							
	<i>A. calidoustus</i>	Mudflat							
<i>Vagarum</i>	<i>A. calidoustus</i>	Mudflat							
	<i>A. ceipii</i>	Mudflat		<i>Agarum clathratum</i>					

[46,47]. Additionally, habitat-dependent fungal species show differences in coastal distribution because of the different environments formed by each seaside [48]. However, most *Aspergillus* species were found in two or all the three seashores, except for single strain isolated species. The excellent decomposition ability and tolerance to extreme environments may have enabled the isolation of *Aspergillus* in various habitats [49]. Therefore, the unique species found in each seaside is also likely to be found on other seashores through further research.

The mudflat habitat was the most diverse environment, where 37 *Aspergillus* species were discovered. The intertidal mudflat habitats feature a high level of microbiological diversity [50,51]. In comparison, 14 species were identified from sea sand. These results are comparable to the findings of [14], in which 13 species were reported from mudflat, and five species were reported from sea sand. In our study, *A. fumigatus* (36 strains), *A. clavatus* (34), *A. terreus* (27), *A. niger* (24), and *A. sydowii* (18) were the commonly found species from mudflats. Among them, *A. fumigatus*, *A. niger*, and *A. sydowii* have high cellulose-degrading capabilities [52]. Of all marine-derived fungi, *Aspergillus* species produce diverse secondary metabolites [19]. Some *Aspergillus* species have been studied extensively as opportunistic pathogens of marine organisms [53,54]. *Aspergillus* species are well known for producing marine-derived enzymes [55]. Using these enzymes, they are able to inhabit intertidal zones by interacting with various marine organisms. Commonly isolated *A. sydowii* and *A. versicolor* were also detected in seawater and deep-sea sediment. According to their tolerance for osmotic pressure or salinity level [12,18,56,57], they possibly withstand other extreme or malnutrition environments. In this study, 18 *Aspergillus* species were isolated from seaweeds. They may play the role of saprotrophs by degrading seaweeds using enzymes such as cellulase [58]. Some *Aspergillus* species are adapted to colonize seaweed as endosymbionts, which explains the continuous isolation of certain species from seaweed [59–61]. Furthermore, several species have shown strong protease activity using caseinase and gelatinase in previous studies [62]. These enzymes were examined in the *Aspergillus* species isolated from marine organisms such as crabs, sandfish egg masses, sponges, and shells [62]. By decomposing these organisms, *Aspergillus* can contribute markedly to the nutrient circulation in the marine environment.

In conclusion, this study has focused on marine habitats in South Korea and reports several unrecorded *Aspergillus* species in marine environments. The findings suggest that *Aspergillus* diversity is high in marine environments, and more species are

yet to be discovered. Although *Aspergillus* species have been reported from a variety of substrates, their role in marine environment is still unclear. To date, little research has been conducted to study the importance of *Aspergillus* in the marine environment and its taxonomy. The continuous discovery of *Aspergillus* species in marine environments will expand our understanding of their ecological importance.

Disclosure statement

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ORCID

Jun Won Lee  <http://orcid.org/0000-0003-1416-3428>
 Wonjun Lee  <http://orcid.org/0000-0002-7227-0777>
 Rekhani Hansika Perera  <http://orcid.org/0000-0002-1360-5362>
 Young Woon Lim  <http://orcid.org/0000-0003-2864-3449>

Data availability statement

All the data that support the findings of this study are available within the article.

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