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The green menace: non-native green seaweed detected in Western Antarctica using molecular tools

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Abstract

Biological invasions threaten marine ecosystems worldwide, including Antarctica. The Western Antarctic Peninsula (WAP) is especially vulnerable because it concentrates research and tourism traffic, a recurrent source of non-native species (NNS) via shipping, and is one of the fastest-warming regions on Earth, gradually easing thermal barriers that have constrained marine NNS establishment. Rising temperatures may also enhance natural rafting on floating seaweeds and plastics, promoting passive transport of NNS from sub-Antarctic regions into the WAP. Green seaweeds (Chlorophyta) are prolific opportunistic invaders, yet in the WAP the lack of molecular baselines has prevented reliable discrimination of native from non-native lineages, weakening detection and management capacity. Here we combined specimen DNA barcoding (nuclear ITS and chloroplast *tuf*A markers; N = 128) from the WAP and adjacent sub-Antarctic regions with environmental DNA (eDNA) metabarcoding (*tuf*A) of seawater, vessel hulls, rafting seaweeds and plastic debris collected in the WAP (N = 88) to document green algal diversity and screen for incoming non-native Chlorophyta, with a particular focus on Ulva. Barcoding of adult specimens recovered a single, geographically restricted lineage, provisionally named *Ulva* sp. "Antarctica", with no close match to sub-Antarctic or temperate sequences, suggesting a possible endemic taxon and contrasting with earlier morphology-based records of cosmopolitan *Ulva*. Metabarcoding additionally detected several cosmopolitan, amphipolar and Northern Hemisphere lineages associated with multiple vectors, tentatively positioning taxa along the invasion continuum, from transient propagule arrival to possible naturalisation. *Ulva* sp. "Antarctica" was also detected on a sub-Antarctic vessel hull, suggesting possible bidirectional transport across the Antarctic Circumpolar Current. Although sampling was opportunistic and limited, these results provide a molecular baseline supporting early detection of non-native green seaweeds in a rapidly changing Southern Ocean.

Introduction

Invasive non-native species cause severe ecological and economic impacts on coastal and marine ecosystems, where high habitat connectivity facilitates dispersal and enables rapid range expansion (Anton et al. 2021; Cuthbert et al. 2021; Pyšek et al. 2020). Both anthropogenic activities—such as shipping and aquaculture—and natural passive dispersal (e.g., rafting on floating substrates like kelp rafts and plastic debris) charter these species over geographical barriers allowing them to colonize new habitats, sometimes across vast oceanic distances (Carlton 1996; Pyšek et al. 2020). However, successful biological invasions involve a sequence of steps beyond mere dispersal. Non-native species (NNS) must navigate ecological filters and establish during a favourable "environmental window." This window occurs when local abiotic factors (like temperature and nutrients) and biotic conditions (herbivory) allow for survival, often following an environmental disturbance (Carlton 1996). The competitive advantage that NNS may gain under such conditions can be decisive for their successful establishment in novel environments.

Seaweeds constitute over 14% of aquatic NNS introductions, with more than 230 species reported worldwide in recent decades (Bailey et al. 2020; William and Smith 2007). NNS seaweeds can significantly modify habitat complexity and biogeochemical processes, leading to shifts in native community composition and biodiversity loss (Anton et al. 2021; Pyšek et al. 2020). Economic consequences

particularly affect fisheries, aquaculture operations, and coastal tourism, which rely on ecosystem services that invasive seaweeds can disrupt (Anton et al. 2021; Barbier 2017; Pereira 2024; Schling et al. 2026). Some taxonomic groups are naturally prone to introduction and invasion, such as the green seaweeds (Chlorophyta), owing to intrinsic biological traits such as rapid growth, phenotypic plasticity, and versatile dispersal strategies (Williams and Smith 2007).

Several species within prominent families—including Caulerpaceae, Codiaceae, and Ulvophyceae—have been extensively documented as invasive worldwide (e.g., *Caulerpa taxifolia*, Arnaud-Haond et al. 2017; *Codium fragile*, Watanabe et al. 2009; *Ulva prolifera*, Wang and Wu 2021). Human-mediated vectors, namely shipping and aquaculture activities, have allowed for their global spread through ballast water discharge and hull fouling (Clarke et al. 2017; Cuthbert et al. 2021).

Although international regulations implement ballast water treatments such as UV radiation to reduce the risk of transport and introduction (Davidson et al. 2017), resilient and diverse fouling communities can persist on vessel hulls, sustaining long-distance dispersal abilities in algal taxa (Lacoursière-Roussel et al. 2012). Filamentous *Ulva* spp. in particular represents around 50% of marine NNS dispersed by hull fouling (William and Smith 2007). Additionally, passive dispersal vectors like kelp rafts and floating plastic debris significantly extend the range and likelihood of NNS colonization, as viable green seaweed propagules and adults can survive prolonged oceanic transit (Barnes 2002; Thiel and Gutow 2005; Saunders 2014).

The Antarctic region represents a major research gap in invasion ecology, with a limited number of studies, due to the inherent difficulties of working in an isolated and extreme environment. While establishing a direct correlation between NNS invasiveness and ecological impact is challenging (Ricciardi and Cohen 2007), the early detection of newly introduced marine NNS is essential for management and conservation, especially in this remote and vulnerable environment. The Antarctic's brief spring–summer, marked by longer days and higher temperatures, offers a much shorter seasonal window for annual species' growth and reproduction than temperate or tropical regions; during this time seaweeds need to complete their life cycles (Gómez et al. 2009, 2019). Endemic Antarctic seaweed species exhibit adaptations to temperatures consistently below 5°C and to pronounced light seasonality, traits that have historically constrained the establishment of marine NNS (de Broyer et al. 2014; Hughes et al. 2020). However, rapid climate warming in the Western Antarctic Peninsula (WAP) is reshaping benthic ecosystems, and arrival and installation of NNS from cold-temperate species from the sub-Antarctic region is expected (Pellizzari et al. 2020, Mitchell et al. 2020, Nasir et al. 2024; McCarthy et al. 2019).

The WAP is experiencing more frequent and intense storm events associated with global warming enhance wave-driven Stokes drift, which is increasingly enabling rafting seaweeds and their associated fouling communities to cross biogeographic barriers such as the Antarctic Circumpolar Current (ACC) (Fraser et al. 2018). Concurrently, anthropogenic activity has intensified. Ship traffic for research and tourism in the WAP during the spring and summer season has increased markedly in recent years (McCarthy et al. 2022). As a result, hull-fouling organisms arriving on vessels as well as rafting kelps and their associated fouling communities have potentially greater opportunities to exploit suitable environmental windows during the Antarctic summer. This increase in propagule pressure, combined with the relaxation of environmental barriers, could substantially enhance the risk and rate of NNS seaweed establishment. Indeed, several

marine NNS introductions have been reported in the WAP, one of the most vulnerable regions for biological invasions in Antarctica (Hughes et al. 2020; McCarthy et al. 2022), including introduced mussels and crustaceans occasionally detected during summer surveys. However, these taxa consistently fail to survive winter conditions, underscoring that environmental filtering mechanisms remain a persistent barrier to successful establishment (Cárdenas et al. 2020; Gómez et al. 2020; Navarro et al. 2024; López-Farrán et al. 2021). For seaweeds, the filamentous green alga *Ulva intestinalis* was the first non-native species recorded on Half Moon Island in the South Shetland Islands in the 1990s (Clayton et al. 1997), thought to be facilitated by biofouling on ships visiting the region. To date, approximately 32 Chlorophyta species have been reported from the WAP and sub-Antarctic islands, mostly based on morphological identifications, although molecular data are available for some taxa (Gómez et al. 2020). Of these, only four species are considered endemic to Antarctic waters: *Chaetomorpha irregularis*, *Lambia antarctica*, *Protomonostroma rosulatum*, and *Ulothrix australis* (Gómez et al. 2020). The remaining 28 species exhibit a cosmopolitan or amphipolar distribution, highlighting their broad ecological ranges. Notably, none of the *Ulva* species reported from Antarctic waters has been classified as endemic. The only filamentous *Ulva* taxon reported in Southern Ocean floras based on morphological character, *Ulva hookeriana* Kützing 1849 (syn. *Enteromorpha bulbosa*), was originally described from the Falkland (Malvinas) Islands. This species has also been recorded on several subantarctic islands (e.g. Macquarie, Gough, Kerguelen) as well as temperate coasts of South America and Africa, suggesting that its distribution is not restricted to Antarctica. Taxonomic resolution in *Ulva*, however, remains challenging when based solely on morphological traits, due to pronounced phenotypic plasticity and widespread cryptic speciation (Hayden et al. 2003; Kraft et al. 2010; Leliaert et al. 2009; Tran et al. 2022). Consequently, recent introductions of non-indigenous *Ulva* taxa may have gone undetected, given the scarcity of historical records and the current lack of comprehensive molecular baselines for Antarctic material. Likewise, truly endemic *Ulva* species, if present, may have been overlooked or misidentified due to reliance on morphological characters alone.

Molecular markers, such as the chloroplast *tuf*A gene or the nuclear intergenic ribosomal region (ITS), have improved global *Ulva* taxonomy by facilitating species-level delimitation (Famà et al. 2002; Leskinen et al. 2004; Saunders & Kucera, 2010). The *tuf*A gene particularly offers high resolution for discriminating among Ulvales and closely related taxa, outperforming other markers in sensitivity and species-level delimitation (Weinberger et al. 2021;2026). Recent taxonomic reassessments conducted on remote and temperate coastlines further underscore the vital role of these markers in clarifying green macroalgae species diversity and distribution (Gabrielson et al. 2024; Hughey et al. 2024; Maggs et al. 2024; Tran et al. 2022). Along the WAP, DNA barcoding using the chloroplast markers *tuf*A and *rbc*L has revealed the presence of one *Ulva* spp. and several other green algal taxa, including *Monostroma*, *Urospora*, *Blidingia*, *Protomonostroma*, *Capsosiphon*, *Ulvella*, and *Prasiola*, among others (Dubrasquet et al. 2021). Among these taxa, several exhibit amphipolar distributions (e.g., *Prasiola crispa*, Garrido-Bennavent et al., 2018; *Ulvella islandica*, Dubrasquet et al. 2021). The current lack of comprehensive molecular baselines for Antarctic Ulvales constrains the ability to distinguish native from non-native lineages and conduct accurate invasion assessments.

Environmental DNA (eDNA) metabarcoding, coupled with metagenomic sequencing, has revolutionized biodiversity assessments by enabling sensitive, non-invasive detection of both established and transient species—including incoming marine NNS—across wide spatial scales in Antarctic coastal waters (Cowart et

al. 2018; Fonseca et al. 2023; Nester et al. 2024). This approach enables detection of species that may fail to establish viable populations but still pose invasion risks, permitting early intervention (Cristescu 2014). While eDNA applications around the WAP have predominantly focused on marine invertebrates and fish—groups with greater biodiversity and more extensive molecular reference databases—the monitoring of marine flora, including macroalgae, remains comparatively underdeveloped (Jeunen et al. 2024; Suter et al. 2025; Wang et al. 2025). Challenges such as limited reference databases for algal taxa, complex life cycles, and the presence of cryptic species complicate the application of eDNA for marine flora (Gold et al. 2022). Nonetheless, recent progress in DNA barcoding targeting chloroplast regions (*rbc*L, *tuf*A, UPA) and nuclear ribosomal DNA (ITS) is rapidly improving macroalgal detection and diversity estimation capabilities (Bombin et al. 2024; Ortega et al. 2020).

The goal of this study was to assess *Ulva* diversity along the WAP and in subantarctic regions using combined traditional DNA barcoding and environmental DNA (eDNA) metabarcoding. Our first objective was to characterize *Ulva* spp. diversity using nuclear ITS and chloroplast *tuf*A markers by comparing Antarctic and subantarctic established populations and generating new genetic data for the region. We further used eDNA metabarcoding (targeting *tuf*A) on (1) opportunistic water samples from locations near human settlements and frequently visited sites, and (2) swab samples collected from floating substrates such as vessel hulls, seaweed rafts, and plastic debris, to track the presence of non-indigenous *Ulva* spp. propagules on these vectors of transport. This integrated approach provides means to detect NNS signals across vectors and locations, contributing to the development of early-warning strategies for invasion monitoring in Antarctic coastal ecosystems.

Materials & Methods

1) Sampling

● Adult specimen sampling for DNA-barcoding approach

Adult *Ulva* spp. specimens were collected from the Western Antarctic Peninsula (WAP) and the sub-Antarctic region to establish a reference library using two genetic markers (ITS and *tuf*A). This reference database served as the basis for species delimitation analyses but also for taxonomic assignment in the metabarcoding approach (see below).

Sampling in the WAP was conducted during two summer seasons (2022/2023 and 2023/2024) aboard the MS Fridtjof Nansen operated by HX Expeditions (Table 1, Supplementary Table S1) in seven locations spanning three key areas: Deception, Rey Jorge and Half Moon Islands in the South Shetland Islands; Danco Island & Orne Harbour in the North Gerlache Strait, and Pléneau Island in the South Gerlache Strait. Tubular morphotypes were preferentially targeted to detect *Ulva intestinalis*, the first non-native species with a filamentous thallus reported in the WAP in the late 1990s (Clayton et al. 1997). This focus also minimized confusion with blade-forming taxa commonly associated with *Monostroma* spp. At each sampling location, up to ten individuals morphologically identified under a microscope as *Ulva* spp. were collected and preserved in silica gel (approximately 10 mg fresh tissue each). Additional opportunistic samples (n = 19)

were also collected and preserved following the same protocol in the South Shetland Islands during the LX Expedición Científica Antarctica (ECA60 2023/2024) operated by the Chilean Antarctic Institute (INACH). Sampling locations included Almirantazgo Bay, Deception Island, Livingston Island (in Punta Hanna & Punta Byers), Rey Jorge Island (Elefantera, Bahia Fildes and China Base) and the Rizopatron Chilean Base (Table 1, Supplementary Table S1). From these samples collected during the ECA60 2023/2024, two were recovered from the biofouling community attached to the hull of the research vessel *Betanzo*: one while the ship was in Antarctic waters (B1M5), and another after its return, while docked in Punta Arenas (B1M8) (Table 1, Supplementary Table S1).

In the sub-Antarctic region, sampling occurred during summer 2022 in the Magallanes region with six sampling sites around Punta Arenas (from San Gregorio to Cape Horn, 52°S to 55°S) and the cool maritime and temperate coasts of Chile (Niebla, Valdivia 41°S and Renaca, Valparaiso, 32°S), following the same protocol as in the Antarctic (Table 1, Supplementary Table S1). Additionally, samples were collected, preserved as dried material and sent out from the cool maritime regions around New Zealand (Chatham Islands, N-Z, 43°S), Australia (Tasmania, Hobart 42°S) and the French Austral Islands (Kerguelen and Crozet Islands, in Port-aux-Français, Baie des Marins et Baie du Sphinx, 49°S). In total, 46 adult specimens were collected from the WAP and 184 from the sub-Antarctic region (Table 1, Supplementary Table S1). Voucher specimens from New Zealand are deposited in the Herbarium of the Museum of New Zealand Te Papa Tongarewa (WELT), whereas specimens from Chile and Tasmania are housed in the Herbarium of the Universidad Austral de Chile (VALD) (Table 1; Supplementary Table S1).

● Water sample sampling for eDNA approach

Sampling in the WAP was conducted during two summer seasons (2022/2023 and 2023/2024) aboard the MS Fridtjof Nansen, operated by HX Expeditions, facilitating access to multiple sites. Surface water samples were collected opportunistically in the WAP whenever weather permitted. Between 7 and 10 L of surface water were collected using two 5 L plastic bottles, each pre-disinfected by rinsing with 10% bleach for 5 minutes and subsequently rinsed with distilled water. Water collection was realized within an angle of 90° from the ship's bow toward the starboard side, in the direction of the thrusters of the ship when anchored. Samples were collected within 100m of the vessel to ensure proximity to locations where tourists disembark or where human activity is documented, including scientific research stations. GPS coordinates were registered and samples were vacuum filtered on a 47mm diameter nylon fiber 0.45µm mesh size (Sartorius Celulose Nitrate Filters) within the 2 hours following water collection. If the filter collapsed due to high density of suspension material, another filter was used and noted accordingly to keep track of duplicates. Additionally, negative samples were taken for each sampling effort by filtering 3L of tapwater. Filters were preserved in 99% ethanol (N = 56) or dry (N = 18) in disinfected individual 2 mL tubes at − 20°C while on board and then stored at − 80°C in the laboratory until DNA extraction (Supplementary Table S1). Dry-preserved filters correspond to additional opportunistic samples collected by Zambra López-Farrán during the ECA60 (INACH, 2023/2024), namely in Punta Hanna and Byers in the Livingston Island (n = 6), Punta Spring close to Danco Island (n = 3), and Almirantazgo Bay (n = 3) and Deception Island (n = 6) in the South

Shetland Islands. In total, 74 filters from 14 sampling sites were collected around the WAP (Table 1, Supplementary Table S1).

● Swab sampling for eDNA approach

Swab samples were recollected opportunistically using individual sterile sponges (Whirl-Pak® Cellulose Speci-Sponge Bags) whenever floating structures such as plastic debris and rafting seaweeds were found during eDNA water sampling or stranded ashore when collecting *Ulva* adult samples in the WAP. Swabs were also taken from the vessel MS Fridtjof Nansen hull when the ship was anchored in the harbour in Ushuaia coming back from Antarctic voyage. Finally, opportunistic swabs were taken from the surface of floating or stranded seaweeds where a green biofilm was visually detected on three different occasions around the O'Higgins Chilean Antarctic Base during ECA60 (INACH, 2023/2024).

Each time, three swabs were taken from each object (plastic- rafting seaweed or vessel hull), kept at -20°C while on-board and then stored at − 80°C in the laboratory until DNA extraction. Negative samples were taken for each sampling site by leaving the sponges out of their sterile bag for one minute. In total, 48 swabs were collected around the WAP and 3 in the Magallanes region (i.e., vessel hull) (Table 1 and Table S1 Supplementary Materials).

Table 1
Sampling sites for DNA barcoding approach and eDNA survey within and outside the WAP.

Sampling Region	Sampling Site	Latitude	Longitude	Adult Sample	Water Samples	Swab Samples
ANTARCTICA	SOUTH SHETLAND ISLANDS	-62,084	-58,391	31	38	19
	O'HIGGINS BASE	-63.32	-57.90	0	0	24
	NORTH GERLACHE STRAIT	-64.62	-62.54	4	29	4
	SOUTH GERLACHE STRAIT	-65.07	-64.03	11	7	1
SUBANTARTICA	MAGALLANES REGION, CHILE	-53.32	-70.106	70	0	3
	FALKLAND ISLANDS	-51.84	-61.08	4	0	0
	ISLAS CROZET AND KERGUELEN	-49.35	70.22	72	0	0
COOL TEMPERATE MARITIME REGIONS	HOBART, TASMANIA	-42.95	147.35	11	0	0
	ISLAS CHATHAM, NEW ZEALAND	-43.82	-176.64	9	0	0
	NIEBLA, VALDIVIA, CHILE	-39.874	-73.40	7	0	0
TEMPERATE MEDITERRANEAN CLIMATE	RENACA, VALPARAISO	-32.95	-71.55	10	0	0
TOTAL				**230**	**74**	**51**

DNA Extraction and PCR protocols

● DNA-Barcoding Approach

Raw DNA was extracted from adult *Ulva* samples using a modified CTAB extraction protocol (Doyle 1991). Briefly, approximately 10 mg of dried material was grounded with BioSpec Mini-Beadbeater x 24 at 1200 rpm for 1 minute, a 2% CTAB buffer lysis solution including 1uL Proteinase K (20 mg/mL) was added, and the sample was incubated at 56°C for 2 hours. DNA was then separated from the protein phase using chloroform:isoamylalcohol 24:1 solution and washed three times with ethanol. DNA pellets were dried

overnight under a sterile hood and then resuspended in sterile water. To check DNA quality, 2uL of each sample was loaded onto a 2% agarose gel at 110V for one hour in TAE (Tris-Acetate-EDTA) buffer. DNA quantity was determined using a Nanodrop photometer (Thermo Scientific Nanodrop Nd-1000). Samples with high DNA quality and quantity were then subjected to PCR for the two genes of interest using primers already published: ITS (forward: TCTTTGAAACCGTATCGTGA; reverse: GCTTATTGATATGCTTAAGTTCAGCGGGT, Saunders & Kucera 2010) and *tuf*A (forward *TufA*gf4: TGAAACAGAAMAWAWCGTCATTATGC; reverse: CCTTCNCGAATMGCRAAWCGC; Famà et al. 2002). All PCR reactions were performed in a Perkin Elmer Gene Amp PCR system 9700 thermal cycler (Applied Biosystems). For both genes, PCR amplification followed the published protocols with some modifications. The PCR protocols used to amplify the ITS gene considered an annealing temperature of 45°C, while for *tuf*A the annealing temperature was 56°C. Gel-agarose purification was performed to eliminate PCR product with non-specific amplification using an E.Z.N.A.® Gel Extraction Kit (V-spin), and sequencing was performed on the Austral-OMICS platform at the Universidad Austral de Chile using an ABI PRISM®310 Genetic Analyzer (Applied Biosystems).

● eDNA Barcoding Approach

DNA was extracted from dry and ethanol-preserved filters and from swab samples using a modified CTAB protocol (Doyle 1991). For dry filters, CTAB buffer was added directly to the collection tubes, which were then centrifuged (10 min, 10,000 rpm) to concentrate the material prior to extraction. Ethanol-preserved filters were transferred to sterile 2 mL tubes, ethanol was removed, and samples were centrifuged (10 min, 10,000 rpm) before adding 800 µL CTAB buffer for starting extraction. For swab samples, sponges were immersed in CTAB buffer and manually massaged for 2 min; the resulting liquid was then transferred to 50 mL tubes and centrifuged (10 min, 10,000 rpm). In all cases, the supernatant was discarded and the DNA was extracted from the remaining pellet.

The chloroplast gene *tuf*A was used to generate the genetic library for Illumina sequencing. A three-step PCR amplification procedure was carried out using the AmpliTaq Gold Polymerase (Fast PCR Mix 2X). The first PCR used the Fama et al 2002 *tuf*A primers and included a denaturation at 94°C for 1 min, an annealing at 55°C for 30 s, followed by an extension at 72°C for 1 min, the program included 37 cycles. The second PCR used a pair of internal primers specifically designed for *Ulva* spp. and used to improve the amplification process due to the high rate of failure during the first PCR (Weinberger et al. 2026). The PCR was run for 25 cycles, starting with a denaturation phase at 94°C for 1 min, followed by an annealing phase at 59.2°C for 30 s and ending with an extension phase at 72°C for 1 min. The third and final PCR integrated the primer sequence containing the llumina adaptor + index + PCR adaptor (Weinberger et al. 2021). The third PCR was run for 10 cycles and parameters were the same as the ones described just above. Illumina sequencing was performed at the Max Planck Institute of Evolutionary Biology at Plön/Germany in December 2024.

2) Species Delimitation based on DNA Barcoding

Newly obtained DNA sequences (ITS and *tuf*A) were cleaned and edited using Chromas v.2.33 (McCarthy 1997) and aligned using MEGA v.5 (Tamura et al. 2011) and the online version of MAFFT v7 (Katoh et al.

2019). For ITS sequences, due to the presence of gaps causing difficulties during alignment, the data were subdivided into three main subgroups according to their dissimilarity levels. For a preliminary assessment of species diversity in our data set, unrooted UPGMA trees were constructed based on pairwise sequence dissimilarities using MEGA v.5 (Tamura et al. 2011) for each sequence alignments (one *tuf*A and 3 ITS alignments). Monophyletic groups were visually identified by selecting clusters of sequences that consistently grouped together. One or a few sequences were selected from each of these a priori genetic groups and BLAST searches (Altschul et al., 1990) were conducted to identify closest matches in the GenBank repository; GenBank sequences with ≥ 99% similarity and 100% query coverage were downloaded from GenBank. Additional *tuf*A (n = 46) and ITS (n = 35) GenBank sequences were used to complement our dataset for giving robustness to further species delimitation analysis; these sequences are provided in Supplementary Table S2.

Following this preliminary step, Maximum Likelihood phylogenetic trees (ML trees) were inferred from our data sets complemented by the sequences from Genbank using IQ-TREE (Trifinopoulos et al. 2016; http://iqtree.cibiv.univie.ac.at/). Best-fit nucleotide substitution models were selected using IQ-TREE's statistical criteria; TIM + F + R3 was used for the three ITS sequence subgroups and *tuf*A. Node support was evaluated by 10,000 ultrafast bootstrap replicates. Resulting phylogenies were visualized with the Interactive Tree Of Life (iTOL) online tool (Letunic & Bork 2024; https://itol.embl.de/upload.cgi).

Species delimitation employed three complementary DNA-based methods: Assemble Species by Automatic Partitioning (ASAP; Puillandre et al. 2020), Generalized Mixed Yule Coalescent (GMYC; Luo et al. 2018), and Poisson Tree Processes (PTP; Zhang et al. 2013). ASAP, a distance-based method, was applied independently to each of the three ITS subgroups and the *tuf*A dataset using online interface (https://bioinfo.mnhn.fr/abi/public/asap). Due to alignment gaps in the ITS dataset, GMYC and PTP analyses were performed exclusively on *tuf*A data. For these, the ML tree from IQ-TREE served as input for Bayesian inferences. Ultrametric trees required for GMYC were generated using BEAST v1.10.4 (Suchard et al. 2018) under a GTR + I+G substitution model and a relaxed clock log normal molecular clock model with coalescent constant population size prior. Markov Chain Monte Carlo ran for 50 million generations, sampling every 1,000 generations, with convergence of parameters confirmed via Tracer v1.7 (Rambaut et al. 2018). Maximum clade credibility trees were summarized with TreeAnnotator v1.10.1 (Drummond & Rambaut 2007), after discarding 25% burn-in. Multiple-threshold GMYC analyses (Fujisawa & Barraclough 2013) were performed using the 'Splits' R package using the "gmyc" function (Ezard et al. 2009). PTP analyses were conducted through the Exelixis Lab species delimitation web server (https://species.h-its.org; Zhang et al. 2013) using an unrooted *tuf*A ML tree as input, with setting 500,000 MCMC generations, thinning value of 100, a burn-in of 10%. This approach integrates both distance-based and tree-based delimitation methods to robustly identify different species.

A group of sequences was considered a putative species when supported by most delimitation methods (i.e., at least three out of six), including ML tree-based monophyly (for *tufA* and ITS), ASAP partitions (*tufA* and ITS), and GMYC and bPTP species delimitations inferred from *tufA*. All assigned sequences (*tuf*A and ITS) were deposited in Genbank, see Access Number in Supplementary Table S1.

3) Bioinformatics & Diversity Analysis for eDNA metabarcoding

Demultiplexed raw sequencing reads in FASTQ format were processed using the qiime2 software package (Bolyen et al. 2019). Adapter sequences were removed using the cutadapt procedure, thereafter low-quality bases were trimmed, paired-end reads were merged and chimeric sequences were excluded using the DADA2 pipeline (Callahan et al. 2016) as implemented in qiime2. The resulting amplicon sequence variants (ASVs) were taxonomically annotated using a custom-curated local reference database compiled from green seaweed *tuf*A sequences retrieved from GenBank, complemented by newly generated *tuf*A sequences from Antarctic and subantarctic Ulva samples (n = 4) from the present work (see above), via the classify-consensus-vsearch procedure implemented in qiime2, considering only matching reference sequences that exhibited 100% of query cover and at least 98% of sequence identity.

Summary tables produced by the DADA2 pipeline were imported into R v4.5.1 (R Core Team 2024) as phyloseq class objects using the “phyloseq” package (McMurdie & Holmes 2013). Potential contaminant ASVs identified in negative control samples were removed using the “decontam” package (Davis et al. 2018) with the prevalence method at a threshold of 0.5 (Saenz-Agudelo et al. 2020). Additionally, ASVs represented by fewer than two reads were excluded to minimize inclusion of spurious sequences. All raw sequencing data generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) under accession number XXXX (pending submission), along with complete metadata to ensure reproducibility and enable future reanalysis.

Following automated ASV generation with DADA2, manual taxonomic curation was performed to retain only high-quality sequences (> 98% of identity) and to restrict analyses to Ulvophyceae, resulting in a final dataset of 300 high-quality ASVs. ASV sequences were aligned and trimmed to 430 bp together with a custom reference library of green seaweed *tuf*A sequences (n = 71 from GenBank; n = 33 newly generated in the barcoding component of this study; Supplementary Table S3). A maximum-likelihood phylogenetic tree was inferred using IQ-TREE multicore v1.6 (Trifinopoulos et al. 2016) with default parameters and 10,000 ultrafast bootstrap replicates, and the resulting tree was visualized using iTOL v6 (Letunic & Bork 2024). ASVs clustering with GenBank sequences and forming monophyletic clades (≥ 98% bootstrap) were then checked for taxonomic consistency and named. Using ASVs representing putative species-level taxa facilitate diversity comparisons and reduce intra-specific noise for downstream community analyses.

Exploratory diversity analyses were conducted within the “phyloseq” package (R v4.5.1). Two factors were included to examine variation in diversity: Geographic Zone and Sample Type. Five geographic zones were defined: South Gerlache Strait (65–66°S), North Gerlache Strait (64–65°S), O’Higgins (62°S, site located along the continental part of the WAP), South Shetland Islands (61–62°S), and the Subantarctic region (60 to 54°S). Sample Type comprised two categories: filters from sea-surface water and swabs from floating substrata. Alpha diversity was assessed based on observed species richness and the Shannon and Simpson indices across Geographic Zone and Sample Type factors. Statistical differences between sample types were evaluated using Wilcoxon rank-sum tests, while variation among geographic zones was tested with the Kruskal–Wallis non-parametric test. Analyses were performed using raw diversity values. For beta diversity analysis, ASV read counts were normalized by transforming to relative abundances prior to calculation of pairwise Bray–Curtis dissimilarities among samples. Unconstrained ordination techniques, including principal coordinates analysis (PCoA) and non-metric multidimensional scaling (NMDS), were applied to

visualize patterns of community similarity in low-dimensional space. To test whether Geographic Zone, Sample Type, or their interaction explained significant variation in the community structure, permutational multivariate analysis of variance (PERMANOVA) was performed on the transformed data. Finally, indicator species analysis was conducted using the “indicspecies” R package to identify ASVs significantly associated with specific sample types, geographic zones, or combinations thereof, providing further insight into taxa driving observed community differentiation.

Results

1. **DNA- Barcoding**

Genetic material was successfully extracted and amplified from 98 individuals, resulting in 35 *tuf*A and 95 ITS sequences. Short sequences (less than 400 bp; n = 2 for *tuf*A and n = 4 for ITS) were excluded from the species delimitation analyses. Taxonomic assignment for these shorter sequences was carried out using Blast searches (see below for more details). The final *tuf*A dataset used for species delimitation analyses included 33 newly generated sequences and 46 sequences already published in GenBank of 764 bp in length. For the ITS, 91 newly obtained sequences to which 35 already published in GenBank were added (688 bp alignment), were retained for subsequent species delimitation analyses. Sampling locations, specimen IDs, and GenBank accession numbers for *tuf*A and ITS sequences from the present work are listed in Supplementary Table S1; sequences downloaded from GenBank are listed in Supplementary Table S2.

1.1) Phylogenetic Reconstruction and Species Delimitation

Phylogenetic analyses based on *tufA* and ITS sequences from 93 individuals (30 with both markers), consistently revealed that our samples belong to two main lineages corresponding to the genera *Ulva* and *Ulvaria* (Fig. 1). In the *tufA*-based Maximum Likelihood tree, the *Ulvaria* lineage was strongly supported (bootstrap > 90%; Fig. 1), whereas the *Ulva* lineage showed lower support (bootstrap < 90%). Within the *Ulva* lineage, a clade corresponding to “true *Ulva*” (i.e., *U. australis*, *U. fenestrata*, *U. intestinalis*, and *U. compressa*) showed very strong support (bootstrap = 99%), while a second group was less resolved (bootstrap = 93%). This second group includes sequences belonging to less resolved clades such as *Ulva stenophylloides*, *U. clathratioides/torta*, *U. californica*, *U. flexuosa/aragoensis*, *U. stenophylla*, *Ulva* sp. “Antarctica” and *U. smaragdina*. For ITS, *Ulvaria* and *Ulva* sequences could not be aligned together and were analysed separately. Within *Ulva*, two ITS datasets were analysed independently due to alignment gaps, these correspond to the two main clades recovered using *tufA* (Fig. 1).

Results of species delimitation analyses (ASAP, bPTP, GMYC) are summarized in Table 2 and Fig. 1 (see Supplementary Table S4 for details). For *tuf*A, ASAP recovered 15 lineages and 6 singletons, bPTP 19 lineages and 5 singletons, and multi-threshold mGMYC 9 lineages and 2 singletons. ASAP and bPTP results were largely congruent, although bPTP tended to over-split some lineages (e.g., *U. compressa*; Fig. 1). bPTP mainly supported highly divergent lineages (e.g., *Ulvaria* sp. “Kerguelen”, *U. intestinalis*, *U. fenestrata*, *U. stenophylla*, *U. prolifera*, *Ulva* sp. “Antarctica”). Most ASAP and bPTP groups corresponded to well-supported monophyletic clades. In contrast, mGMYC was not significant (p = 0.63) and lumped distinct, well-supported lineages (e.g., *U. australis*, *U. compressa*, *U. intestinalis*, *U. fenestrata*; Fig. 1). For ITS, ASAP identified 14

clusters and 4 singletons. Sequences generated in this study clustered into 10 genetic species (1 *Ulvaria*, 9 *Ulva*), supported by concordant evidence from *tuf*A and ITS, multiple delimitation methods, and well-supported ML clades (Fig. 1; Supplementary Table S4). The identified species are: *Ulvaria* sp. “Kerguelen”, *Ulva fenestrata*, *U. intestinalis*, *U. compressa*, *U. clathratioides/torta*, *U.* cf *stenophylloides*, *U. californica/flexuosa/aragoensis*, *U. stenophylla*, *Ulva* sp. “Antarctica”, and *Ulva smaragdina*. The rationale for species delimitation and nomenclature is provided below.

All specimens from the Kerguelen and Crozet Islands (N = 27) clustered into a single, strongly supported genetic lineage based on both markers (supported by all species delimitation methods except mGMYC–*tuf*A), here named *Ulvaria* sp. “Kerguelen”. No closely matching sequences were found in public databases; sequences showed pairwise divergences of ~ 7% (*tuf*A) and ~ 15% (ITS) relative to *Ulvaria splendens*.

All Antarctic specimens (N = 13) formed a cohesive and fully congruent genetic group across both markers and all species delimitation methods, here named *Ulva* sp. “Antarctica”. This group included previously published ITS and *tuf*A sequences from King George Island (Khan et al. 2017; Dubrasquet et al. 2021) and was closely related to *Ulva prolifera* (pairwise distance ~ 1.8% for both markers).

Sample ASV354 from New Zealand clustered unambiguously with sequences identified as *U. stenophylloides*, *U. ohnoi*, *U. fasciata*, *U. uncialis*, and *U. lacinulata* for both genes, forming a supported group within this broader unresolved assemblage (*tuf*A bootstrap: 98%; ITS: 82%). Given its high sequence similarity to Chilean *U. stenophylloides* (Mutizabal-Arros et al. 2024) and only two of six delimitation criteria met, ASV354 is here named *U.* cf. *stenophylloides*.

For both genes, all species delimitation methods (except mGMYC–*tuf*A) assigned various Chilean specimens (Magallanes and southern Chile) to *U. fenestrata* (N = 8) and *U. intestinalis* (N = 12). For *U. fenestrata*, only one *tuf*A sequence from Magallanes (HD20) was obtained and matched a Falkland Islands sample (Mrowicki & Brodie 2023). Most *Ulva intestinalis* samples clustered with Tasmanian specimens for both markers.

Tasmanian samples TAS5 and TAS7, together with Magallanes samples HD25 and HD65, clustered with reference sequences of *Ulva linza* from China, Korea, Australia, Canada and California (Fig. 1). As a recent taxonomic revision has renamed *U. linza* as *U. smaragdina* (Hughey et al. 2026), the updated nomenclature has been used here. Given the well-documented taxonomic uncertainty of *Ulva linza* species complex (Steinhagen et al. 2019), all samples were conservatively named *Ulva smaragdina* (previously *Ulva linza*) pending further taxonomic advances.

For *Ulva compressa*, species delimitation was marker-dependent. *tuf*A recovered a single lineage, whereas ITS resolved two lineages diverging by ~ 7%. One lineage included samples from Central Chile and Magallanes plus a Tasmanian reference sequence; the second comprised samples from Central Chile, Magallanes, Tasmania, and all Falkland Islands specimens. No close GenBank matches were obtained for the latter; *tuf*A sequencing failed for these specimens. Despite ITS divergence suggesting possible cryptic species, all samples were conservatively retained as *U. compressa* pending further data.

ASAP-ITS recovered a single group corresponding to the *U. clathratioides* / *U. torta* lineage, consistent with a weakly supported ITS clade but a strongly supported *tuf*A clade (bootstrap 68% and 100%, respectively). Although bPTP oversplit this lineage in *tuf*A, this pattern was not supported by ASAP or mGMYC. Given their known genetic proximity and occasionally conspecific or cryptogenic treatment (Kraft et al. 2010; Kirkendale et al. 2013), samples CENT03, CENT04, CENT06, and CENT07 were named "*U. clathratioides*/*torta*".

For both markers, all delimitation methods recovered a single unresolved *U. californica*/*U. flexuosa*/*U. aragoensis* complex. None of the marker retrieved this lineage as monophyletic. ITS shows weak support for a lineage including *U. californica*, *U. flexuosa*, *U. aragoensis* and *U. tanneri* (bootstrap 73%), whereas for *tuf*A the group was polyphyletic. For *tuf*A, HD35 grouped with *U. flexuosa*/*aragoensis* (bootstrap 92%) and HD38 and HD40 grouped with *U. californica* (bootstrap 96%). As the species names are frequently treated as synonyms (Guiry & Guiry 2025), and given inconsistent boundaries and low support, these samples were conservatively assigned to an unresolved *U. californica*/*flexuosa*/*aragoensis* species complex.

Short sequences were assigned using BLAST on the data set considering all full-length sequences used for species delimitation analyses. For *tuf*A (n = 2), HD52 (Magallanes) matched *U. intestinalis* (99.46% similarity to a Finnish sequence), and PL01 (Antarctic Peninsula) matched *Ulva* sp. "Antarctica" (100% similarity). For ITS (n = 4), LLG9120 (Kerguelen) was assigned to *Ulvaria* sp. "Kerguelen"; CENT05 (Central Chile) to *U. clathratioides*/*torta*; and hull-fouling samples B1M5 and B1M8 to *Ulva* sp. "Antarctica" and *U. intestinalis*, respectively.

Overall, Antarctic (n = 13) and Kerguelen–Crozet (n = 27) samples formed two genetically distinct and apparently endemic lineages. All remaining samples—from Magallanes (n = 32), South and Central Chile (n = 11), the Falkland Islands (n = 3), Tasmania (n = 4), and New Zealand (n = 5)—were assigned to cosmopolitan or regionally widespread taxa (*U. compressa*, *Ulva smaragdina* (Hughey et al 2026), U. *intestinalis*, *U. fenestrata*, *U. clathratioides*/*torta*, *U. californica*/*flexuosa*/*aragoensis*, *U. stenophylla*, *U.* cf *stenophylloides*). Across their ranges, these lineages show broad but partially unresolved Southern Hemisphere distributions, indicating that several temperate *Ulva* taxa occur widely across southern coastal systems.

Table 2
Comparison of delimitation methods results for each marker, and Results of the Generalized Mixed Yule-Coalescent (GMYC) analyses under the multiple threshold model.

Species Delimitation Method		**TUFA**			**ITS**		
		ASAP	**mGYMC**	**bPTP**	**ASAP**	**mGYMC**	**bPTP**
#lineages		15	9	19	16	-	-
#singleton		6	2	5	4	-	-
tufA mGMYC results	**NCLUSTER**	**NGMYC**	**LNULL**	**LGMYC**	**Λ**	**P**	**Threshold**
	8 (4–11)	9 (5–22)	228.3862	231.1655	5.55875	0.0620773n.s.	–0.04553609

2) eDNA- Metabarcoding

1. Metabarcoding Dataset Summary

The initial sequencing library comprised a total of 96 samples, including 55 filter samples (out of 74 initially collected), 33 swab samples (out of 51 initially collected) and eight negative controls (five swabs and three filters). Out of those 96 initial samples, 51 yielded results (Supplementary Table S5). All negative controls (N = 2) were first used to identify putative contaminant ASVs, which were then removed from every sample in the dataset. After this quality-filtering step, the two negative controls that yielded reads and one low-read environmental sample (< 10 reads, HD101) were excluded from downstream analyses. This filtering resulted in a refined dataset of 48 environmental samples (Supplementary Table S5), comprising 9,435,201 reads clustered into 613 ASVs, with an average of 196,566.7 (± 354,327.6) reads per sample and a mean read length of 430 base pairs.

2. ASV Clustering and Taxonomic Assignment

Of the initial 613 ASVs, all sequences not assigned to Ulvophyceae were removed. This filtering was performed by manually trimming and aligning ASV sequences against our curated *tuf*A reference database (Supplementary Table S3). Among the sequences filtered out, two belonged to *Florenciella parvula* (Heterokonta), while the remainder represented diatoms. Following phylogenetic reconstruction, 211 ASVs corresponding to 34.42% of the initial dataset, remained and were clustered phylogenetically under species-level taxa (see Materials and methods section for more details). This clustering analysis identified 16 distinct genetic taxa, four of which corresponded to species previously recognized through DNA barcoding: *Ulva* sp. “Antarctica”, *Ulva intestinalis*, *Ulva smaragdina*, and *U. californica/flexuosa/aragoensis*. For clarity, this last group will hereafter be referred to as *Ulva flexuosa* throughout the results and figures. The twelve additional taxa identified belonging to Ulvophyceae included: *Blidingia marginata*, *Capsosiphon* sp., *Monostroma hariotii*, *Monostroma sp.2 grevillei*, *Protomonostroma undulatum*, *Ulvella islandica*, *Urospora sp.1 penicilliformis* and *Urospora sp.*, *Umbraulva dangeardii*, *Ulva* sp. HD1, *Ulva* sp. HD2 and *Ulva maeotica/shanxiensis*/sp. 3 (see Fig. 2). *Ulva* sp. HD1 and sp. HD2 were each represented by a unique ASV. The sequence of *Ulva* sp. HD1 matched 100% with three GenBank sequences: OR734320 (*Ulva* sp. 1 SAM1166 from Italy; Armeli Minicante et al. 2025), OL421114 (*Ulva* sp. 2 SSt-2022 from Germany; van der Loos et al. 2023), and JN029338 (*Ulva* sp. 10GWS from Tasmania; Kirkendale et al. 2013). *Ulva* sp. HD2 exhibited 100% identity with three GenBank sequences: OP267647 (Ulva sp. DK_018 from Denmark; Steinhagen et al. 2023), MT859878 (*Ulva paradoxa* from USA; Melton & Lopez-Bautista 2021), and MZ582192 (*Ulva flexuosa* from Vietnam; Tran et al. 2022) (see Supplementary Figure S1). *Ulva maeotica/shanxiensis*/sp. 3 designation was based on 100% identity with sequences from the Black Sea, Baltic Sea, North Sea and China (Steinhagen et al. 2023; van der Loos et al. 2021; Le Chen et al. 2015) (see Supplementary Figure S1).

3. Read Distribution and Sample-Level Variation

● Abundance and Prevalence

Figure 2 displays a heatmap illustrating read abundance per sample following OTU reassignment to species-level taxa. *Ulva maeotica/shanxiensis/sp.*3 showed the highest prevalence (40/48 samples), followed by *Capsosiphon* sp. (15 samples) and *Ulva* sp. “Antarctica” (7 samples) (Fig. 2). Moderate prevalence (> 5 samples) included *Monostroma hariotii*, *Blidingia marginata*, *U. intestinalis*, *U. smaragdina*, and *U. flexuosa*. Rare taxa (≤ 2 samples) comprised *Urospora* sp.1 *penicilliformis* and *Urospora* sp., *Protomonostroma undulatum*, *Umbraulva dangeardii*, *Ulva* sp. HD1, *Ulva* sp.HD2 and *Monostroma* sp. 2 *grevillei* (Fig. 2).

● Filters VS swabs

Filters yielding data (N = 34) were collected in a wide latitudinal range from approximately 65°S at the southern end of Lemaire Channel to 62°S in the South Shetland Islands, including Half Moon Island and Deception Island, in waters close to well-visited sites by tourism and research vessels (Fig. 3A). Swab samples (N = 14) yielding data were collected in Pléneau Island (65°S), the South Shetland Islands, the vicinities of the O’Higgins Chilean Base and the harbour of Ushuaia, from a variety of substrates, including floating and stranded native seaweeds (n = 11) such as *Cystosphaera jacquinotii*, *Palmaria decipiens* and *Desmarestia menziesii*, as well as from floating nylon rope (n = 1) and vessel hull surfaces (n = 2) (Fig. 3A, 3D).

Filter samples yielded the highest read counts, occasionally exceeding one million reads, but showed low taxonomic diversity (< 3 species per sample; Fig. 3B), whereas swabs exhibited significantly higher richness (mean > 5 taxa per sample) despite lower read abundance (< 500,000 reads) (Fig. 3B). *Ulva maeotica/shanxiensis*/sp. 3 dominated most filter samples (> 7.6 million reads) and occurred only at low relative abundance in swabs, followed by *Capsosiphon* sp. (~ 190,000 reads). Rare taxa were detected in a small number of filter samples from Deception Island (e.g., *Protomonostroma undulatum*, *Urospora* spp.) while *Ulva* sp. HD1 and *Ulva* sp. HD2 were detected in swabs from stranded and floating native seaweeds (Fig. 3D). The rare taxon *Umbraulva dangeardii* was detected only once in the dataset (HD94), at very high read abundance (~ 390,000 reads) on a native stranded seaweed sampled near O’Higgins Chilean Base.

Swabs from stranded seaweeds around O’Higgins exhibited the highest richness (up to eight taxa; 11 of 16 taxa overall), whereas swabs from nylon rope and vessel hulls showed lower richness (2–4 taxa) and commonly shared taxa such as *Capsosiphon* sp. and *Ulva* sp. “Antarctica”. *Ulva* sp. “Antarctica” contributed over > 300.000 reads and was found ubiquitous on all types of substrates except nylon rope. Swabs from floating *Cystosphaera jacquinotii* showed intermediate diversity (4–6 taxa). The southernmost *C. jacquinotii* individual (HD81) hosted four species: *Monostroma hariotii*, *Monostroma* sp. 2 grevillei, *Capsosiphon* sp. and *Blidingia marginata*. At O’Higgins, three swabs from a single *C. jacquinotii* (HD85, HD86 and HD87) all contained *Ulva* sp. “Antarctica”, *U. maeotica/shanxiensis*/sp. 3, *U. smaragdina* and *U. intestinalis*, while only one of the three (HD86) additionally harboured *Ulva* sp. HD1 and *Blidingia marginata*. The two vessel hull samples collected in Ushuaia from MS *Fridtjof Nansen* upon its return from Antarctica (HD76 and HD79) yielded *Capsosiphon* sp., *Ulva maeotica/shanxiensis/sp. 3*, and *Ulva* sp. “Antarctica”.

Shannon and Simpson diversity indices were significantly higher in swabs than in filters (Wilcoxon test, $p < 0.002$; Fig. 3C; Supplementary Tables S6.1 and S6.2), while among latitude groups only observed richness

differed significantly (Kruskal–Wallis test, $p < 0.05$) with O'Higgins showing the highest richness, and no significant variation in Shannon or Simpson indices (Fig. 3C).

A) Sampling locations for Antarctic and sub-Antarctic filter and swab samples, grouped by region; B) Sequencing read distribution by sample type, showing only the most prevalent taxa; C) Boxplots of alpha diversity indices (Observed richness, Shannon, Simpson); there were no significant differences in Shannon or Simpson indices among latitudinal groups based on Kruskal–Wallis tests (see Table S6.1, Supplementary Material);

D) Bubble plot of raw read abundance per taxon and sample, grouped by sample type (filters vs. swabs) and ordered by decreasing latitude from HD81 at 65°S (Pléneau Island) to HD76 at 56°S (Ushuaia). Swab samples substrates are indicated by colours: blue for drifting nylon rope; dark green for drifting native seaweed; light green for stranded native seaweed; dark red for vessel hull. For the complete list of samples metadata see Table S5, Supplementary Materials.

4. Beta Diversity and Community Structure

PERMANOVA analyses indicated that both latitude and sample type had significant independent effects on species composition, as well as a significant interaction between these factors (Supplementary Table S6.3). This demonstrates that community structure varies not only across geographic locations but also according to the type of sample collected, with these factors jointly shaping assemblage composition. Consistent with these findings, Principal Coordinates Analysis (PCoA) explained 80% of the variance across two axes and revealed three distinct clusters of samples (Fig. 5). Clusters 1 and 3 included a mixture of samples from multiple locations and sampling methods (filters and swabs), whereas Cluster 2 was exclusively composed of swab samples from O'Higgins, representing a distinct cohesive community. The two vessel-hull samples from Ushuaia (HD76 and HD79), taken from MS *Fridtjof Nansen* after its Antarctic cruise, clustered together with environmental samples from O'Higgins and the Gerlache Strait within Clusters 1 and 3. Indicator species analysis (IndVal) further clarified these patterns by identifying the taxa that most strongly characterized each cluster. Cluster 2 (O'Higgins swabs on native macroalgae) was dominated by cosmopolitan species such as *U. intestinalis* and *U. smaragdina*, each contributing approximately half of the cluster's composition and together accounting for nearly the entire assemblage, whereas Cluster 1 was almost entirely associated with *Capsosiphon* sp., detected in both filters and swabs over a broad latitudinal range, and Cluster 3 was primarily characterized by *M. hariotii* and *M.* sp. 2 *grevillei* (Fig. 4).

Discussion

By combining specimen DNA barcoding with eDNA metabarcoding, this study substantially advances the resolution and scope of green macroalgal diversity documented across the Western Antarctic Peninsula (WAP) and adjacent sub-Antarctic regions. The two approaches proved complementary as specimen barcoding primarily captured established, locally abundant taxa whereas eDNA metabarcoding revealed a far broader and more dynamic assemblage that included both native and probable non-native lineages transported via multiple vectors. Together, these results allow some of these taxa to be positioned along distinct stages of the invasion continuum (Carlton 1996; Richardson et al. 2000; Richardson et al. 2010),

from episodic propagule arrival to persistence and putative incipient naturalization, and provide a molecular baseline against which future changes in green algal diversity can be assessed.

Seven *Ulva* (*U. compressa*, *U. smaragdina*, *U. intestinalis*, *U. fenestrata*, *U. californica/flexuosa/aragoensis*, *U. stenophylla, U.*cf. *stenophylloides*) and one *Ulvaria* (*Ulvaria* sp. “Kerguelen”) species were identified from the subAntartic region, known for its high macroalgal diversity, with for example up to 234 species reported in the Magallanes region (Ramírez 2010). While Chlorophyta accounts for only 12% of the reported species in that region (Mansilla et al. 2013), our results showed a greater diversity than the two previously species currently described for the fjords where our samples were taken (i.e *U. smaragdina* and *U. prolifera*; Mansilla et al. 2013). This pattern likely reflects the susceptibility of the Magallanes region to species immigration, given the convergence of intense maritime traffic and major oceanographic features that facilitate long-distance propagule transport. Specifically, the southern tip of South America, including Punta Arenas (Chile) and Ushuaia (Argentina), constitutes one of the world's most active shipping hubs, identified as “Antarctica gateway” (McCarthy et al. 2022), generating elevated propagule pressure from NNS transported as hull-fouling organisms (McCarthy et al. 2019). The Antarctic Circumpolar Current (ACC) has similarly been implicated in passive dispersal of propagules along subantarctic coasts of the Southern Ocean (Janosik et al. 2011). It remains challenging to determine whether the observed presence of the cosmopolitan species *U. compressa*, *U. smaragdina*, *U. intestinalis*, *U. fenestrata*, *U. californica/flexuosa/aragoensis* and *U. stenophylla* reflect ancient cosmopolitanism or recent, possibly cryptic, human-mediated dispersal.

Historical morphology-based floristic inventories emphasized strong similarities between Magallanes, the Falkland Islands, and the remote Kerguelen and Crozet archipelagos, reporting more than 90 shared species interpreted as evidence of ACC-mediated taxon redistribution (Féral et al. 2021). However, our molecular data tell a different story: while Magallanes and the Falkland Islands do share taxa such *Ulva compressa* or *U. fenestrata*, the Kerguelen and Crozet archipelagos shared no species with either region. This discrepancy suggests that morphology-based inventories may have overestimated floristic connectivity across the Atlantic and Indian Ocean sectors of the Southern Ocean, likely due to misidentification of cryptic or morphologically convergent taxa (Féral et al. 2021; Leliaert et al. 2014). The discovery of *Ulvaria* sp. "Kerguelen", an undescribed taxon recovered exclusively from our samples, further supports this interpretation. The comparatively low shipping traffic reaching these archipelagos likely compounds this isolation, reducing propagule pressure and fostering endemism.

Antarctica's long geographic and oceanographic isolation has been critical to the evolution of high levels of endemism characterizing many of its marine taxa (Barnes et al. 2006). Our molecular results reveal that this isolation continues to actively shape local *Ulva* diversity, as evidenced by the recovery of a single, coherent endemic lineage, *Ulva* sp. "Antarctica", detected consistently across 450 km of the WAP (Fig. 5). This lineage was further recovered across diverse substrata in our eDNA dataset — including stranded native seaweeds, vessel hulls, and waters near touristic sites — underscoring its ubiquity and ecological versatility within the WAP (Fig. 5). Its endemic status is further supported by the absence of any close molecular match to sequences from sub-Antarctic or temperate regions, and stands in sharp contrast with the only filamentous *Ulva* taxon previously reported from Antarctic waters based on morphological evidence alone, *Ulva*

hookeriana Kützing 1849 (syn. *Enteromorpha bulbosa*), whose broad reported distribution explicitly precludes designation as an Antarctic endemic.

No endemic Ulva species had previously been described from the WAP, suggesting that our specimens represent undescribed diversity warranting formal taxonomic characterization using an integrative approach. Our results contradict earlier reports of the cosmopolitan *U. intestinalis* in the WAP, which were based solely on morphological observations (Clayton et al. 1997; Macaya et al. 2020; Pellizzari et al. 2023). In particular, the population from Half Moon Island that Clayton et al. (1997) reported as *U. intestinalis* was resampled in this study, and all sequenced specimens from this locality fell within the *Ulva* sp. "Antarctica" lineage, rather than clustering with reference material of *U. intestinalis*. While our sample size is limited and additional collections are needed, these data suggest that at least some previous records may correspond to this endemic Antarctic lineage rather than to *U. intestinalis* sensu stricto. This is particularly relevant given that *U. intestinalis* has been flagged as a macroalga of potential concern (Macaya et al. 2020); whether this endemic taxon co-occurs along WAP shores with as-yet-undetected NNS such as *U. intestinalis* remains an open and pressing question that extensive molecular surveys should address.

The strong endemism uncovered here for *Ulva* reinforces Antarctica as a region where biogeographic isolation remains a powerful structuring force for marine flora. Yet this isolation is not absolute, and a distinct question arises when considering species with cold-water affinities distributed across both polar regions; the so-called amphipolar taxa (Dubrasquet et al. 2021). Non-native species are considered naturalized when they form self-sustaining populations persisting and reproducing without ongoing human assistance (Richardson et al. 2000; Pyšek et al. 2004; Richardson et al. 2010). Several amphipolar species of green algae have been repeatedly reported as established adult populations across the WAP over at least a decade through specimen-based DNA barcoding, including *Capsosiphon* sp., *Protomonostroma undulatum*, *Ulvella islandica*, and *Urospora* spp. (Mystikou et al. 2014; Pellizzari et al. 2017; Dubrasquet et al. 2021). These taxa were consistently detected by eDNA in water samples and across multiple substrata (i.e., vessel hulls, stranded *Durvillea antarctica*, and floating *Cystosphaera jacquinotii*) spanning from 65°S to 62°S, generally at higher read abundance than transient lineages (see below), suggesting a more permanent regional presence. For example, *Capsosiphon* sp. was the third most abundant species in our eDNA dataset, further supporting establishment rather than sporadic occurrence (Fig. 5). These four taxa may therefore represent the first candidate naturalized NNS in the WAP. However, an equally plausible alternative to naturalization must be considered; these amphipolar species could represent cryptic introductions predating systematic monitoring. Distinguishing a long-standing undetected native from a recent natural colonist or a human-mediated introduction is a key dilemma where baseline data remain scarce (Hughes & Convey 2012). Such cryptic introductions are well documented in terrestrial systems. For example, *Poa annua*, first recorded in maritime Antarctica in 1953 yet likely introduced decades earlier, has since expanded across the WAP, colonising native habitats (Chwedorzewska et al. 2015; Frenot et al. 2005). Taken together, the decade-long barcoding record (Mystikou et al. 2014; Pellizzari et al. 2017; Dubrasquet et al. 2021) and the consistent, spatially extensive eDNA detection across anthropogenic and natural substrates provide strong evidence that *Capsosiphon* sp., *P. undulatum*, *U. islandica*, and *Urospora* spp. have achieved persistence in the WAP consistent with naturalization. Whether this reflects historical colonisation or continued propagule input via shipping and rafting remains unresolved.

Beyond amphipolar taxa already present in the WAP, the high *Ulva* diversity documented in the Magallanes region, where multiple cosmopolitan species co-occur near heavily trafficked southbound shipping routes, may constitute a substantial source pool for future introductions to Antarctica. Despite this, only three of these cosmopolitan species (i.e., *U. smaragdina, U. intestinalis*, and *U. flexuosa*) left any detectable trace in the WAP, and even then, only as scarce eDNA signals restricted to a single location, the O'Higgins Chilean scientific base, on stranded native seaweed. Their detection at very low read abundances and in fewer than five occurrences suggests that, for now, propagule pressure from the Magallanes region remains low and has not yet translated into detectable Antarctic adult populations (Fig. 5). Yet propagule transport to the WAP is not limited to geographically proximate sources. A suite of Northern Hemisphere-associated lineages (including *Blidingia marginata*, *Monostroma* sp. 2 "grevillei", *Ulva maeotica*/*shanxiensis*/sp. 3, *Ulva* sp. HD1, *Ulva* sp. HD2, and *Umbraulva dangeardii*) was recovered from water samples, hull-swabs, and stranded seaweeds, pointing to inter-hemispheric transport, likely via shipping (McCarthy et al. 2022; Lewis et al. 2003, 2004). Collectively, regardless of the vector involved, these lineages likely occupy the earliest stage of the marine invasion continuum — propagules episodically reaching Antarctic waters as transient arrivals without established adult populations — reflecting low propagule pressure and negligible short-term establishment risk (Blackburn et al. 2011).

Among the transient lineages detected, one stands out for its exceptionally high prevalence and read abundance across all sampled substrates: *Ulva maeotica*/*shanxiensis*/sp. 3. Its ubiquity in our eDNA dataset, combined with its apparent absence from established Antarctic populations, places it at the arrival stage of the invasion continuum (Blackburn et al. 2011). *Ulva maeotica* and *U. shanxiensis* were originally described from geographically restricted regions of the Northern Hemisphere, the Azov–Black Sea basin and Chinese inland and coastal waters, respectively (Stepanyan 2019; Le Chen et al. 2015). Yet our data suggest that propagules of this lineage are reaching Antarctic waters, most plausibly via repeated shipping introductions given the inter-hemispheric scale implied (McCarthy et al. 2019, 2022; Bayley et al. 2024). Both species are capable of rapid colonisation of new habitats, particularly under low salinity, turbid, and low-light conditions analogous to those found in Antarctic fjords. Although our short sequence fragments prevent definitive taxonomic assignment, the broad ecological tolerance of both *U.maeotica* and *U.shanxiensis*, combined with the exceptionally high propagule pressure documented here, makes this lineage a priority target for monitoring under projected scenarios of environmental change in Antarctica.

Beyond shipping, once propagules reach Antarctic waters, eDNA evidence suggests they are not simply passive arrivals; native floating macrophytes may actively redistribute them throughout the WAP. Indeed, thalli of *C. jacquinotii* hosted diverse biofouling assemblages simultaneously transporting endemic, amphipolar, and Northern Hemisphere-affiliated taxa, suggesting that a continuous pool of transient propagules is being redistributed via natural rafting vectors. Such internal redistribution may progressively raise the probability of successful establishment in thermally suitable microhabitats by increasing both the frequency and geographic spread of secondary arrival events (Lockwood et al. 2009).

An unexpected finding is the detection of *Ulva* sp. "Antarctica" on vessel hulls in sub-Antarctic waters (Fig. 5), suggesting that Antarctic-adapted endemic taxa could themselves cross the ACC and potentially establish in sub-Antarctic environments, a form of "reverse invasion" mediated by shipping (Wallingford et al.

2020) with potentially far-reaching consequences for sub-Antarctic benthic communities. Targeted monitoring of vessel hulls operating between Antarctica and sub-Antarctic gateways is therefore essential, as the directionality of biological exchange in the Southern Ocean may be more complex than previously assumed (McCarthy et al. 2022; Murphy et al.2021).

Importantly, our results highlight that none of the taxa documented here can yet be considered invasive *sensu* Richardson et al. (2000), as there is no evidence of rapid secondary spread or ecosystem-level impact. Strong environmental and biotic filters appear to continue structuring Antarctic coastal assemblages, as observed for other potential marine invaders that fail to maintain self-sustained populations through winter (e.g., Chilean mussel; Navarro et al. 2024; Cárdenas et al. 2020). However, these conclusions must be tempered by limited adult sampling and by the absence of successfully sequenced material from Deception Island, a site with documented NNS establishment (Chown et al. 2012; Hughes et al. 2020), meaning some diversity was likely missed.

This integrated framework should therefore be viewed as a molecular update to Antarctic marine flora baselines rather than an exhaustive census. Future studies broadening sampling scope, genetic resolution, and monitoring standardization will be essential to refine species delimitation, resolve native versus non-native status, and enable early NNS detection. Overall, these findings underscore the interplay of natural and anthropogenic forces shaping green algal diversity at the Antarctic gateway, where repeated introduction events and high propagule pressure are progressively reshaping biogeographic boundaries across the Southern Ocean. The arrival of non-native green seaweeds in Antarctic waters is no longer debatable; what remains uncertain is the temporal and ecological scale at which their presence will translate into measurable ecological change.

Declarations

Authors contributions

Conceptualisation: HD, MLG, FW. Developing methods: HD, ZL, IdelaB, LV, LLG, WN, CH. Conducting the research: HD. Data analysis: HD, MLG, FW, SK. Data interpretation: HD, ZL, IdelaB, MLG, FW. Preparation figures & tables: HD, MLG, FW, WN, LLG, CH. Writing: HD, MLG, FW, WN, LLG, CH. Funding acquisition: HD, MLG, FW.

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Data availability

Data that support the findings of this study are available from the corresponding authors upon reasonable request.

We confirm that this manuscript is original, has not been published elsewhere, and is not under consideration by any other journal. All authors have read and approved the final version of the manuscript and are aware of its submission. The authors declare no competing interests.

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Figures

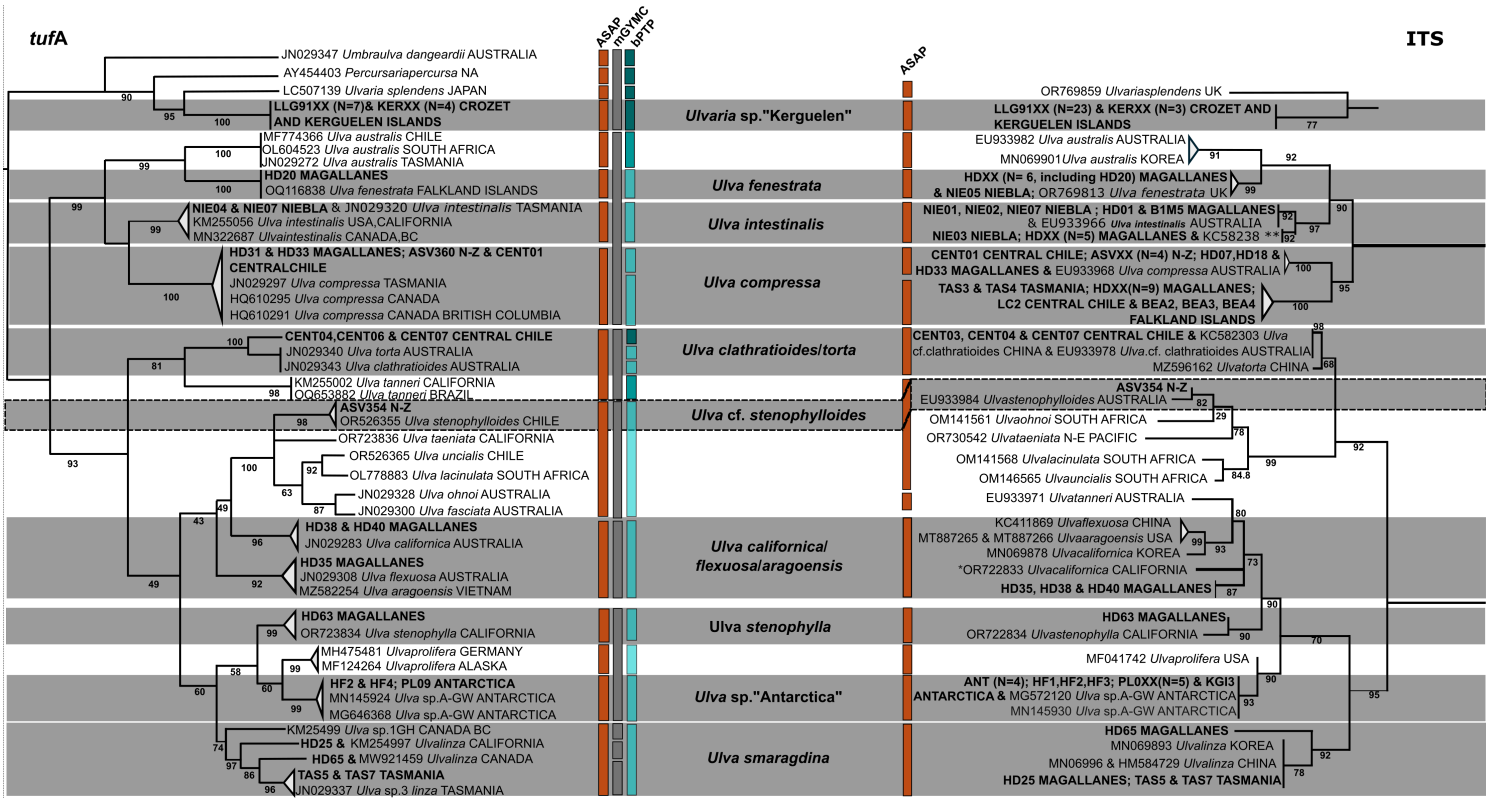


Figure 1

*Simplified phylogenetic reconstruction and species delimitation based on ITS and tufA datasets for Ulvaria and Ulva species identification. Maximum Likelihood (ML) trees were inferred separately for each marker using 10,000 bootstrap replicates. The tufA dataset includes 33 newly generated sequences and 46 sequences retrieved from GenBank, whereas the ITS dataset comprises 91 new sequences and 35 GenBank references. For clarity, only the closest matching reference sequences for each delimited genetic clade are displayed; the full list of sequences used is provided in Tables S1 and S2. Species delimitation support from the Bayesian Poisson Tree Processes (bPTP) analysis is represented by a colour gradient, with dark blue indicating strong support (posterior probability > 0.6), medium blue intermediate support (0.5–0.6), and light blue lower support (< 0.5). Branch bootstrap values above 70% are shown beneath the corresponding nodes, grey labels denote references sequences while taxa highlighted in bold correspond to the newly obtained sequences in this study. The final chosen name for each species based on delimitation methods are indicated at the centre of the figure. *: ITS sequence of the species type (USA); ** ITS sequence of Ulva cf.intestinalis USA.*

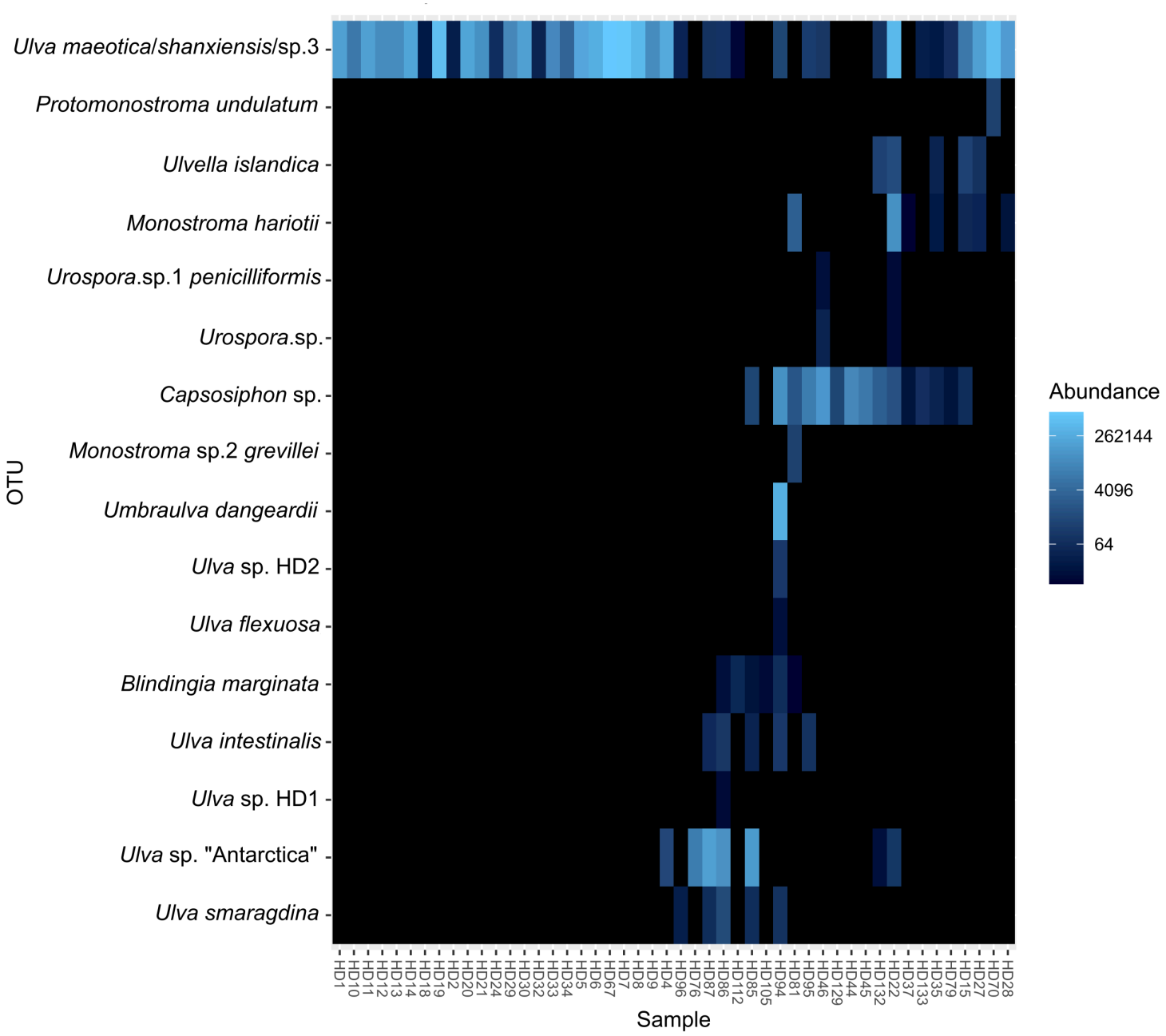


Figure 2

Heatmap of read abundance per sample after OTU reassignment at the species level. Sample metadata are provided in Supplementary Table S5.

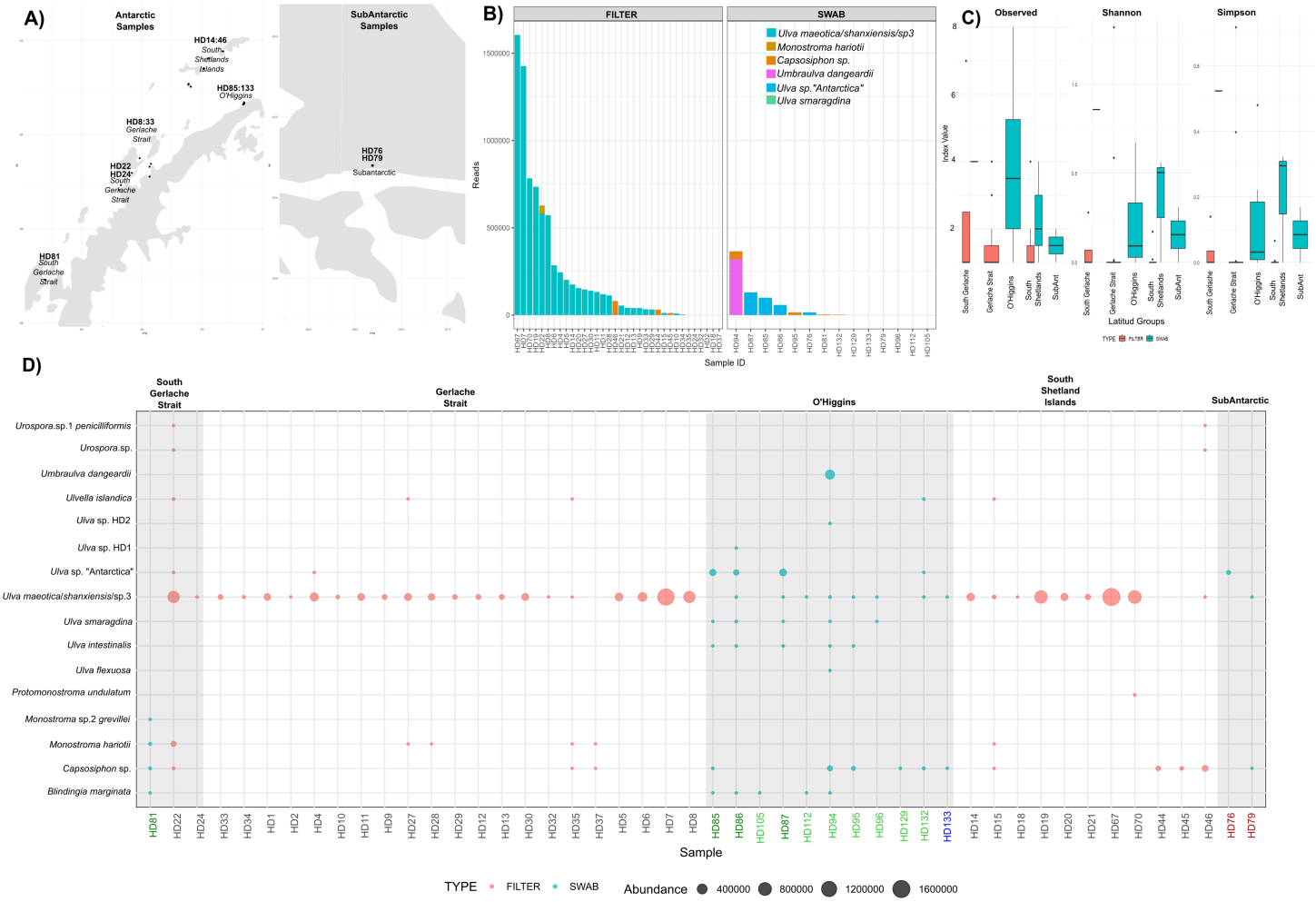


Figure 3

Distribution of sequencing read abundance and diversity across sample types and regions.
A) Sampling locations for Antarctic and sub-Antarctic filter and swab samples, grouped by region; B) Sequencing read distribution by sample type, showing only the most prevalent taxa; C) Boxplots of alpha diversity indices (Observed richness, Shannon, Simpson); there were no significant differences in Shannon or Simpson indices among latitudinal groups based on Kruskal–Wallis tests (see Table S6.1, Supplementary Material);
D) Bubble plot of raw read abundance per taxon and sample, grouped by sample type (filters vs. swabs) and ordered by decreasing latitude from HD81 at 65°S (Pléneau Island) to HD76 at 56°S (Ushuaia). Swab samples substrates are indicated by colours: blue for drifting nylon rope; dark green for drifting native seaweed; light green for stranded native seaweed; dark red for vessel hull. For the complete list of samples metadata see Table S5, Supplementary Materials.

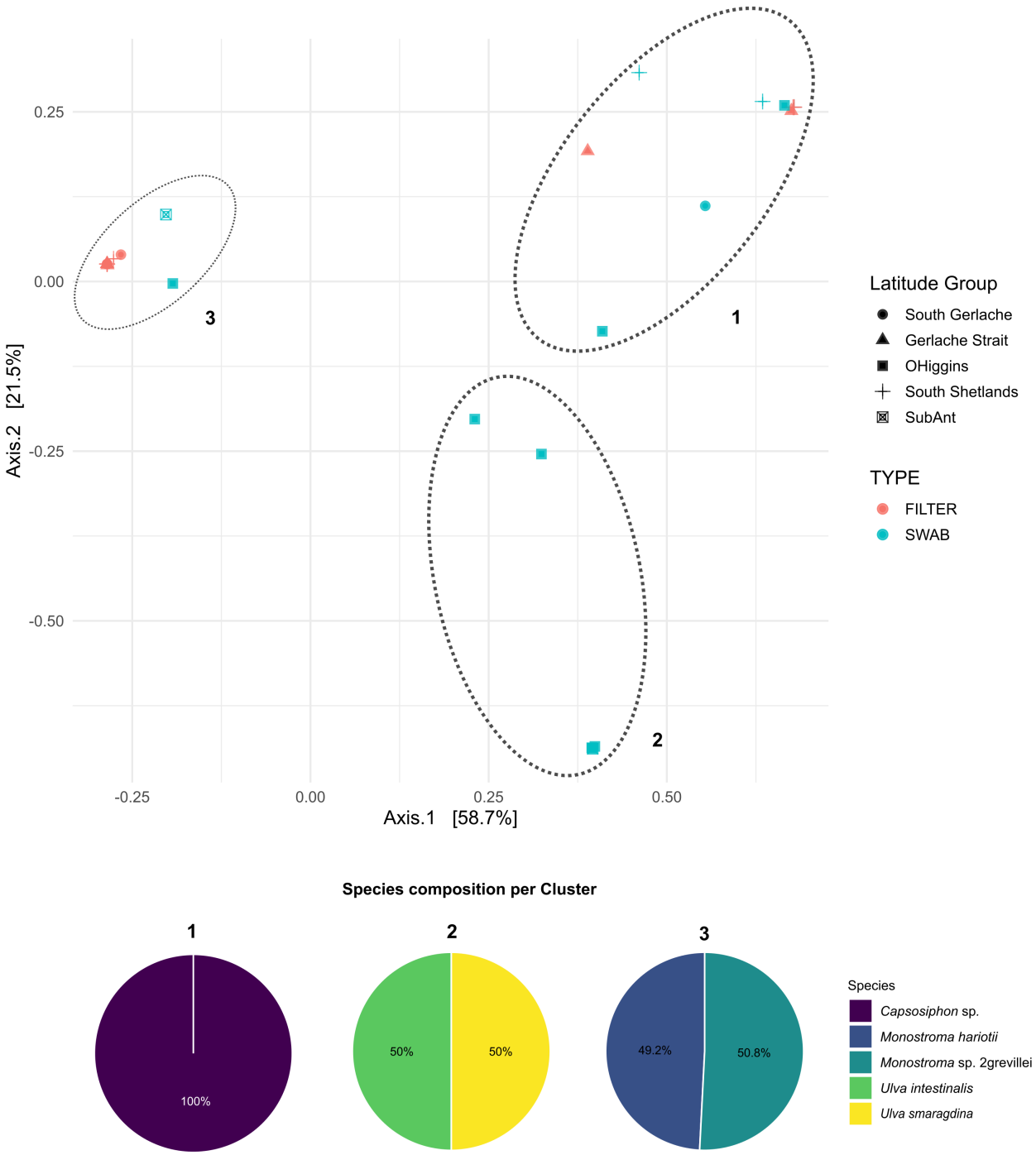


Figure 4

Principal Coordinates Analysis (PCoA) of transformed abundance data using Bray-Curtis distances, illustrating variation by sample type and latitude group. Clusters were determined by k-means analysis. Pie (camembert) plots depict the most significant species within each cluster, based on indicator species analysis (IndVal), with slice sizes proportional to species' relative contributions.

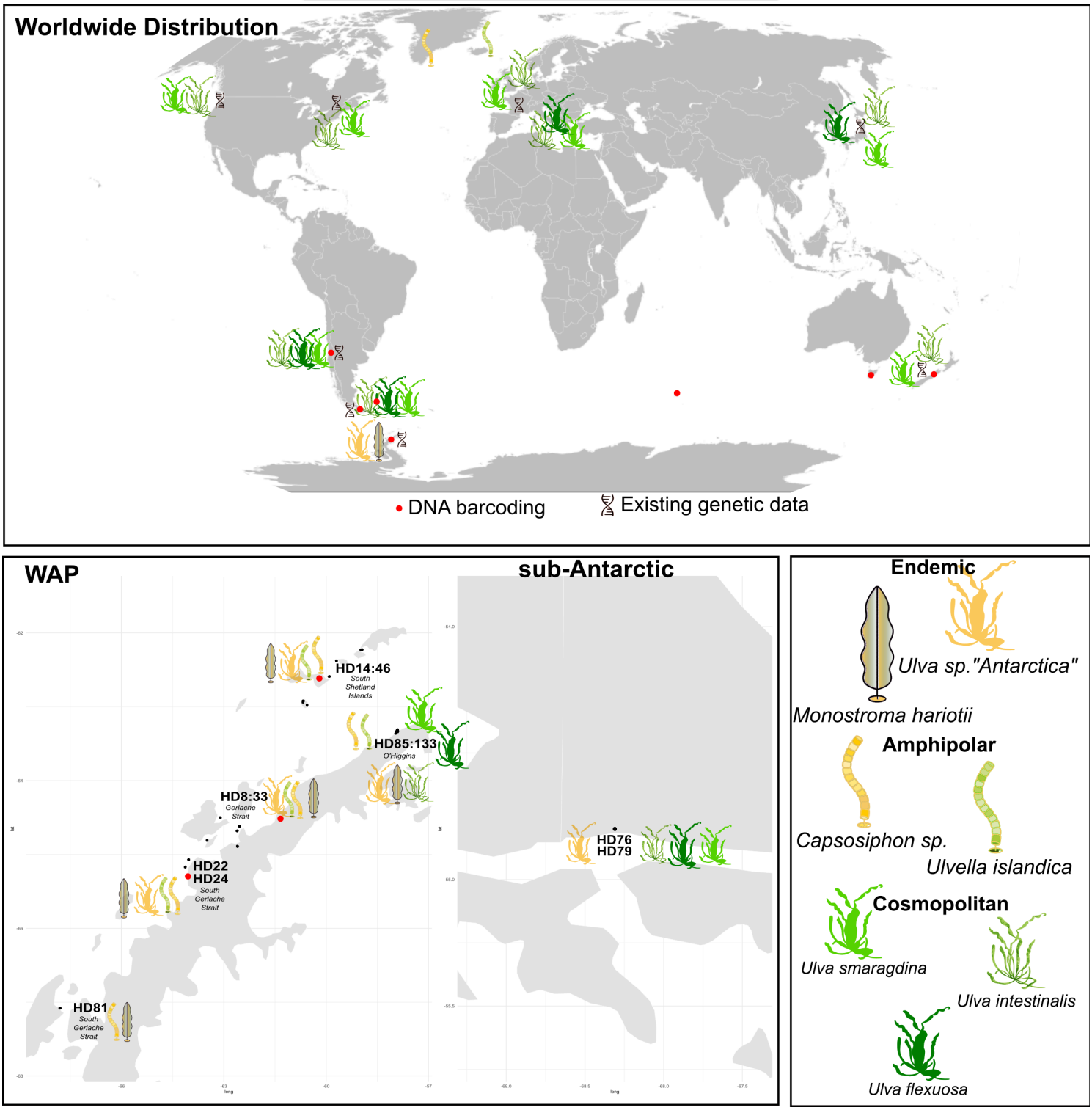


Figure 5

Geographic distribution of seven selected taxa, positioned along the invasion continuum, detected via combined DNA and eDNA surveys in the Western Antarctic Peninsula (WAP). The worldwide distribution map compiles global genetic data available in public repositories for adult populations sequenced by DNA barcoding, including this study. The regional map details species detected exclusively through eDNA within the WAP and sub-Antarctic zones. Red dots represent the specific sampling sites analyzed by DNA barcoding in this study, while DNA icons denote locations with available tufA sequences in public databases (e.g., GenBank).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigureS1OTUSdelimitation.png
- TableS1SupplMAtyMed.xlsx
- TableS2SupplMatyMed.xlsx
- TableS3SupplMatyMed.xlsx
- TableS4SupplMatyMed.xlsx
- TableS5SupplMatyMed.xlsx
- TableS6SupplMatyMed.docx