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Sea cucumbers: the sustainability of emergent and historical resources

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Sea cucumbers: the sustainability of emergent and historical resources

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Editorial: Sea cucumbers: the sustainability of emergent and historical resources

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Editorial on the Research Topic
Sea cucumbers: the sustainability of emergent and historical resources

Sea cucumbers (Holothuroidea) are a class of echinoderms found worldwide from the intertidal to the deep sea, from the tropics to the poles, and on both hard- and soft-bottom substrates. They have a variety of important ecological roles including nutrient cycling, redistribution and oxygenation of surface sediments, enhancement of benthic habitat through excretion of inorganic nitrogen and phosphorus, and amelioration of local ocean acidification impacts on coral reefs. They are fished worldwide as human food and for their various nutraceutical and pharmacological properties. Unfortunately, the fisheries for many species have not been professionally managed, leading to overfishing and population declines across many regions of the globe. Aquaculture of a few species has been developed in order to bolster wild populations and for commercial sale to meet increasing market demand. That has led to increasing research on fisheries and aquaculture of previously unfished or underutilized sea cucumber species. The present Research Topic brings together a number of papers that address the utilization of various holothuroid resources, with goals of conservation and stock management of wild populations and increased production of cultured species.

Understanding the fundamental principles of sea cucumber biology and their ecological processes is crucial to deal with important holothuroid resources. With that in mind, Liu et al. reviewed papers on the fundamental principles, functions, and characteristics associated with various molecular markers employed across a number of both temperate and tropical sea cucumber species. Those markers serve pivotal roles in terms of genetic sex identification, germplasm resource evaluation, population structure assessment, as well as marker-assisted breeding in sea cucumber farming. Prata and Christoffersen assessed the knowledge on Brazilian sea cucumbers over the last 20 years, revealing asymmetries in the conducted studies, either geographical or biased towards specific subjects (*i.e.* taxonomy and molecular analysis) or species (*i.e. Holothuria* (*Halodeima*) *grisea*). Despite the progress, the authors concluded that much more information is required—especially on topics such as morphological variations, genetic distances between populations, population densities, and reproductive biology of a number of species—all of which are essential topics in ensuring the conservation of all harvested holothuroids.

Using genetic parentage analyses and statistical modelling and working in Papua New Guinea, Waldie et al. studied the population connectivity of sandfish (*Holothuria scabra*), based on larval dispersion. Their study demonstrates the relevance of local indigenous plans to protect spawning biomass of the species by establishing a network of locally-managed marine areas.

On a different ecological topic, Azevedo e Silva et al. determined the growth parameters of three commercial sea cucumber species—*Holothuria mammata*, *Holothuria forskali*, and *Holothuria arguinensis*—from the northeastern Atlantic Ocean, revealing species and seasonal effects. Knowledge on various biological aspects of these marine resources is valuable for both protecting wild populations and enabling or increasing aquaculture production.

Okada et al. examined gene expression in regenerating tissues of *Eupentacta quinquesemita* after evisceration to pinpoint the genes involved in the formation of the eviscerated organs, such as the gut tube and the digestive tract and even the central nervous system. The research is crucial in understanding the molecular mechanisms behind regeneration of eviscerated tissues in this and other holothuroid species.

Fished or cultured sea cucumbers are primarily destined for human consumption, hence, pollutant, including microplastics (MP), bioaccumulation is a pressing concern. Menéndez et al. examined MP pollution in genetically-connected populations of *Holothuria forskali* in the south Bay of Biscay (France/Spain) and found that MP load was higher in sea cucumber tissues than in nearby water or sediment, indicating bioaccumulation within the holothuroid. Moreover, the MP concentration was found to be linked with various MP sources like rivers, fishing ports, and aquaculture farms. The work is essential in understanding pollutant load in various holothuroids used, directly or indirectly, for human food in order to allow the development of suitable mitigation measures if needed.

Aquaculture is another key topic in the management of sea cucumber resources, creating an alternative source of supply to fisheries. In a most relevant review, Ciriminna et al. explored the status of aquaculture of sea cucumbers in the northeastern Atlantic Ocean and Mediterranean Sea. The paper highlights that most of the research has been conducted in Italy, Portugal, and Turkey, with *Holothuria tubulosa* and *Holothuria arguinensis* being the predominant species studied. The article is important as it outlines the most common farming practices used, while identifying gaps and future directions for research on European/Mediterranean sea cucumber aquaculture.

However, sea cucumber aquaculture faces several challenges. As sea cucumber farming becomes more important as a food production system, research on holothuroid diseases will undoubtedly take on more urgency and consequence. Yu et al. applied metagenomic analyses to examine microbial factors associated with intestinal atrophy in larvae of *Apostichopus japonicus*—a disease that has been reported at a number of Japanese sea cucumber farms, hampering production. Several different pathogenic bacteria were found in the diseased larvae of this species, with *Tenacibaculum* sp. suggested as being the most problematic. The use of such a molecular approach for examining the potential linkages between microbes and holothuroid diseases will be invaluable in the identification of the pathogenesis of such diseases and will be important to establishing commercially/biologically-sustainable production systems.

The aquaculture industry aims to produce high quality products. In this sense, and because body color influences several biological functions in sea cucumbers, Liu et al. aimed to understand the molecular mechanisms regulating body colour and examined gene expression in green and purple forms of *Apostichopus japonicus* exposed to different culture conditions. The research revealed that genes in the body walls of specific individuals with particular traits were affected by their environment, a result and technique that can aid selective breeding for production of high-quality individuals.

Landes et al. examined live-storage of *Parastichopus tremulus*, studying the effects of temperature on oxygen consumption rate and condition of individuals in long-term storage. Their results showed that lower temperatures reduced oxygen consumption and increased condition. Studies such as this have implications for live seafood storage and transportation, as well as farming, impact assessment, and management of wild stocks.

The studies presented here provide valuable insights into the biology and ecology of various holothuroid species, serving as basic knowledge for developing evidence-based conservation and management strategies, ensuring the sustainability of populations in the wild. Other findings contribute to the advancement of sea cucumber aquaculture, which is developing into a promising sector within the framework of the advancing blue economy, offering potential benefits for sector sustainability and economic development. While research on holothuroids has been on the rise in the last quarter century, there is a common theme throughout the papers in this Research Topic—there are many species and research areas that remain rife for further exploration!

Author contributions

PMF: Writing – review & editing. AP: Writing – review & editing. AR: Writing – review & editing. CMP: Writing – review & editing, Writing – original draft.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Inferring potential causative microbial factors of intestinal atrophic disease in the sea cucumber *Apostichopus japonicus*

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The overexploitation of the wild populations of *Apostichopus japonicus* has caused a significant decline in their numbers, prompting the establishment of a sea cucumber aquaculture industry that has become a major player in East Asia. Recent research has focused on identifying the various factors that may affect sea cucumber aquaculture, including water quality, diet, and the microbiome associated with their hosts. Notably, studies on host-associated microbiomes have emerged as a prominent area of research, particularly in the context of investigating the relationship of the microbiome with diseases that have caused significant economic losses. Intestinal atrophy of *A. japonicus* larvae has been reported at a number of sea cucumber farms in Japan, but there have been no factor screenings, and there is currently no efficient mitigation. In this study, we applied metagenomic analyses for the first time to an intestinal atrophic case of the sea cucumber *A. japonicus* larvae, which occurred in an aquaculture facility in Hokkaido, Japan, elucidating potential causative microbial factors and comparing the diseased microbiome against the healthy pan-microbiome. Through our metagenome comparisons, we have found significant increases in *Oceanicoccus*, *Ruegeria*, *Nioella*, *Labrenzia*, *Tenacibaculum*, and *Muricauda* in the diseased larvae. Among those, *Tenacibaculum* was estimated to potentially be the most pathogenic bacterium in the development of intestinal atrophic disease due to previous outbreaks of sea urchin diseases in the same facility. Our metagenomic approach demonstrates the further need of microbial screening of such causative agents and identification of the pathogenesis of such diseases, which could contribute to establishing more sustainable aquaculture in the sea cucumber industry.

KEYWORDS

***Apostichopus japonicus*, host-microbe interaction, larval development, disease susceptibility, metagenomics**

Introduction

The sea cucumber *Apostichopus japonicus* (Selenka, 1867), belonging to Echinodermata: Holothuroidea, is one of the most important and valuable aquaculture resources in Asia, and it has been consumed as both a delicacy and a traditional medicine for over 100 years (Chen and Chang, 2015). Due to the growing commercial value of sea cucumber, wild populations of *A. japonicus* were overfished in the 20th century, leading to declines in wild populations and an increase in the price of the product, and it has been listed as a threatened species in the IUCN due to this high demand and the lack of regulations, (Hamel and Mercier, 2013). To address the decline in wild populations, aquaculture of *A. japonicus* was first developed in the 1950s (Yang et al., 2015). Since the first successful artificial cultivation of *A. japonicus*, aquaculture bloomed and rapidly developed into a major industry in East Asia, with China being the largest producer of cultured *A. japonicus* (Han et al., 2016). A study used genetic markers to investigate the genetic diversity and structure of wild and cultured *A. japonicus* populations in China, indicating that wild populations exhibited higher genetic diversity than cultured populations, and there was a genetic gap in different geographic regions, highlighting the importance of preserving genetic diversity in both wild and cultured populations for sustainable management of the industry (Chen et al., 2008).

In recent decades, research efforts have been devoted to identifying factors that impact the aquaculture of the sea cucumber *A. japonicus*, such as investigating areas such as water quality, stock density, genetic factors, and diet (Li and Li, 2010; Gao et al., 2017; Feng et al., 2022). In recent years, the study of host-associated microbiomes has achieved a prominent position in research, with a growing body of literature highlighting its importance in elucidating the biology of host organisms and enabling a range of practical applications (Adair and Douglas, 2017). Expanding research into sea cucumber has accumulated knowledge on the microbiomes associated with the host organism, in particular, by providing valuable insights into host growth performance and disease prevention and treatment and encouraging the development of novel aquaculture applications in sea cucumber (Yamazaki et al., 2016; Chen et al., 2022a; Zhao et al., 2022). Our own previous research has also demonstrated a relationship between the gut microbiota of sea cucumber and growth performance and also during gut regeneration and larval development (Yamazaki et al., 2016; Yamazaki et al., 2020; Yu et al., 2022). These findings suggest the potential for interventions targeting the host-associated microbiome to help enhance the aquaculture of *A. japonicus* by promoting host health, development, and growth performance.

The rapid expansion of sea cucumber aquaculture has given rise to various diseases, such as rotting edges syndrome and skin ulceration disease, which have resulted in significant economic losses and hindered sustainable development of the industry (Wang et al., 2004; Yang et al., 2015). Skin ulceration syndrome (SUS) is one of the most severe diseases affecting adult sea cucumbers and has been reported in multiple sea cucumber species worldwide, primarily caused by *Vibrio splendidus, Vibrio alginolyticus, Vibrio cyclitrophicus*, and *Shewanella marisflavi* bacteria (Deng et al., 2009; Li and Li, 2010; Zhang et al., 2015). SUS causes skin ulcers that lead to a decline in sea cucumber health and eventual death (Zhang, 2006). In addition, sea cucumber larval development is affected by diseases such as rotting edges syndrome, caused by *Vibrio lentus* during the auricularia stage, and off-plate syndrome, caused by pathogenic *Vibrio* sp. during the doliolaria and pentactula settlement stages (Yu et al., 2010). The impact of these diseases has prompted extensive research to better understand their underlying causes and to develop effective prevention and control measures (Yasoda et al., 2006). For instance, recent studies have explored the use of probiotics, such as *Bacillus*, and dietary supplements, including β-glucan, to regulate the growth, immune response, intestinal microbial homeostasis, and pathogen resistance of *A. japonicus* (Zhao et al., 2012; Yang et al., 2015; Yang et al., 2017). Moreover, ecofriendly agents such as immunostimulants are being investigated to enhance immune response in *A. japonicus* and to aid resistance against bacterial diseases in aquaculture (Zhang et al., 2021). During the study of a healthy sea cucumber microbiome in its early life stages from fertilized eggs to benthic-settled juveniles under laboratory aquarium conditions in our laboratory, a disease characterized by an atrophic digestive system occurred, and it halted metamorphosis at the auricularia stage at a local sea cucumber farm on site. Outbreaks of such a disease, named as intestinal atrophic disease, have occurred sporadically in various facilities in Hokkaido, Japan. Despite these sporadic occurrences, we have been unable to find causative agents related to this disease in sea cucumber larvae. This knowledge gap places an obstacle to the sustainable seed production of sea cucumber in related aquaculture industries. Addressing these challenges requires further investigation of the mechanisms and pathogenesis of intestinal atrophic disease in sea cucumber larvae.

To our knowledge, this is the first study to assess the structure, function, and dynamics of the microbiome on intestinal atrophic diseased larvae using a metagenomic approach. We also used a unique metagenome data set of the pan-microbiome of healthy larvae as a baseline control to identify the microbial factors causing the structural and functional differences in larvae affected by intestinal atrophic disease. The broad assessment of the atrophic-associated microbiomes performed in this study identified significantly increased numbers of potential pathogenic microorganisms, such as *Tenacibaculum*, which is known to be a fish and shellfish pathogen. Furthermore, this study highlights the relative abundance of specific microbial metabolisms, including succinate dehydrogenase, cytochrome c oxidases, and pathogenic islands, in larvae with internal atrophic disease. Additionally, the influence of environmental rearing seawater on the intestinal microbiome was also evaluated. These findings offer novel insights into the disturbance of the microbiome and the potential involvement of pathogenic bacteria in the development of intestinal atrophic disease during the larval stage of sea cucumber, thereby advancing our understanding of host–microbe interactions and aiding in disease prevention in sea cucumber aquaculture.

Materials and methods

Sample collection of healthy and atrophic larvae

Due to the limitations in collecting healthy samples from the farm during the disease outbreaks, we decided to utilize a pan-microbiome from healthy larvae reared in our laboratory as a baseline control. For healthy samples, fertilized egg or gastrula samples of the sea cucumber *A. japonicus* were collected on three occasions in 2021. Fertilized eggs or gastrula were prepared at 18.7°C at a farm in Hokkaido Aquaculture Promotion Corporation Kumaishi Branch, Japan (42.12574, 139.99966), at 11:00 am on 30th June 2021 and 12th July 2021. Other fertilized egg samples were prepared at 18.7°C at a farm in Hokkaido Aquaculture Promotion Corporation Shiriuchi Branch, Japan (41.61463, 140.38335), at 11:00 am on 30th July 2021. Fertilized egg or gastrula samples were further reared in our laboratory breeding system for sample collection at different developmental stages, and the sub-sampling procedures were performed as described in a previous study (Yu et al., 2022). In brief, the final density of fertilized eggs in this study reached 7,500 eggs per liter in an 8-liter volume aquarium. The aquarium was prepared using a sterilized 8-liter glass bottle (Ishizuka Glass Co. Ltd., Aichi, Japan). Each bottle was filled with 7.5 liters of sterilized artificial seawater (Sea Life, MARINETECH, Japanese Seawater Co. Ltd., Japan). Additionally, 0.22 µm Sterivex filters were installed at the exit of the aeration system to prevent contamination. A commercially available diatom, *Chaetoceros gracilis* (Hakodate Fisheries Research, Japan), was fed to the sea cucumber larvae daily. Larvae samples with intestinal atrophic were collected directly from a farm in the Shiriuchi Branch, Japan (42.12574, 139.99966), on the day disease occurred on August 12th, and the larvae were starved for 3 days from August 15th, and feeding was restarted on August 16th 2021. As described above, the fertilized eggs collected from the Shiriuchi farm were subsequently cultured in our laboratory system to establish a healthy pan-microbiome profile for early-stage sea cucumber, *A. japonicus*. This approach was employed as a control due to the limited availability of healthy samples from the farm during the atrophic sample collection. We acknowledge the limitations in obtaining an adequate number of healthy controls and have addressed this concern by using the laboratory-raised larvae as a comparative reference (Figure S1).

Microbial DNA extraction and 16S rRNA gene sequencing

Microbial DNA extraction from sea cucumber was performed using the NucleoSpin Soil Kit (MACHEREY-NAGEL, Düren, Germany) according to the manufacturer's protocol. Microbial DNA extraction from seawater was performed using the NucleoSpin Tissue kit (MACHEREY-NAGEL) according to the modified manufacturer's protocol. In brief, seawater samples were heated at 55°C for 1 hour to add an active cell lysis process in the TE buffer (10 mM Tris-HCl, 1 mM EDTA) containing 20% SDS and proteinase K (20 mg mL^{-1}) instead of buffer T1. In the third step, for the lyse sample, 1 mL of buffer B3 was used instead of 200 µL.

The hypervariable V1-V2 region of the 16S rRNA gene was amplified by PCR with barcoded 27Fmod and 338R primers with Illumina adaptor sequences (Yamazaki et al., 2019; Yu et al., 2022). PCR amplicons were purified using AMPure XP magnetic purification beads (Beckman Coulter, Brea, CA, USA), and quantified using the Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies Japan). Equal amounts of each PCR amplicon were mixed and then sequenced using MiSeq Reagent Kit v3 (600-cycles) with the MiSeq Illumina platform. Based on sample-specific barcodes, obtained reads were assigned to each sample.

Meta16S analysis and plot construction

The paired-end sequence data with quality scores were analyzed using Quantitative Insights Into Microbial Ecology 2 (QIIME 2, version 2022.2) (Bolyen et al., 2019). Quality controls and merging paired-end sequences were performed using DADA2 (Callahan et al., 2016). Reads with 100% similarity constituted an amplicon sequence variance (ASV). ASVs were assigned to taxonomy using the naïve Bayes classifier and Greengenes database. Using subsampled reads, unweighted UniFrac distances as beta-diversity were calculated and visualized in PCoA plots (Lozupone et al., 2011). Significant differences of unweighted UniFrac distance were tested by permutational multivariate analysis of variance (PERMANOVA) (FDR-corrected $p<0.05$). The phylogenic tree was generated by FastTree (Price et al., 2010). The Z-score was calculated using the genefilter package in R (Gentleman et al., 2023). Pheatmap, ggplot, and the DESeq2 package in R (Version 4.0.5) were used for heatmap construction and statistical analysis (McMurdie and Holmes, 2013; Love et al., 2014). Venn diagrams of shared ASV between sea cucumber and seawater were generated by Venny 2.1 (Oliveros, 2007-2015). Re-analysis of individual parameters using linear-constrained ordination redundancy analysis (RDA) was performed by vegan-2.6-4 (Oksanen et al., 2015). Spearman correlations were used for correlation network construction by R package Hmisc and psych (Revelle, 2017; Harrell and Dupont, 2019). LDA Effect Size (LEfSe) was used for assessing the significantly different taxa and microbial functions (Segata et al., 2011). EZR package was used for the *t*-test, *F*-test, and Shapiro–Wilk test to compare the size of digestive systems between diseased and healthy individuals (Kim, 2020).

Metagenomic sequencing and functional profile

A total of 20 templates with ≥ 300 ng DNA material extracted from sea cucumber larvae were used for paired-end shotgun metagenomic sequencing on the HiSeq platform. For analysis of sea cucumber larval metagenome samples, the metagenomics RAST server (MG-RAST) was used for eukaryotic taxonomic classification and microbial functional annotation (Meyer et al., 2008). The low-quality regions were trimmed by using SolexaQA, followed by dereplication using the k-mer approach (Cox et al., 2010). Duplicate Read Inferred Sequencing Error Estimation (DRISEE) was used to analyze the sets of Artificial Duplicate Reads (ADRs) (Gomez-Alvarez et al., 2009; Keegan et al., 2012). Reads with 97% identity were clustered, and the longest sequences were picked as the cluster representatives. The cluster representatives were further assigned to taxonomy using a BLAT similarity search, which integrates SILVA, Greengenes, and RDP (Cole et al., 2003; DeSantis et al., 2006; Pruesse et al., 2007). After the abundance profile was generated, the SEED subsystem was used for the functional profiles (Overbeek et al., 2005).

Results

Descriptions of signs of intestinal atrophic disease

Microscopic observation of sea cucumber with internal atrophic indicated that the digestive system, including the intestines and stomach, were clearly atrophic (Figures 1A, B). The size of the intestine and stomach in diseased larvae was significantly smaller than in healthy larvae, showing a digestive system with a width of 57.1-107.1 μm and length of 128.5-199.9 μm. Compared to the healthy larvae, the width and length of intestine and stomach was significantly narrower and shorter (*t*-test, $p < 0.01$, Table S1). No parasitic eukaryotes-like cells were observed in those atrophic samples. In healthy larvae, it was very easy to define the intestine and stomach under light microscopy (Figure 1C), but in atrophic larvae, the digestive system was atrophied, and it was very difficult to distinguish the different digestive organs, such as the intestines and stomach. An additional sign of diseased larvae was the halt in metamorphosis when the disease occurred.

The significantly different bacterial diversity and composition in larvae with intestinal atrophic disease compared to healthy pan-microbiome

In order to understand whether the microbial community varied in larvae with intestinal atrophic disease and which taxa showed increases in atrophic individuals, we compared the atrophic Meta16S data against the healthy pan-Meta16S data set. A total of 165,675 and 256,140 Meta16S sequence reads were obtained to create a "healthy pan-Meta16S data set" from 17 sea cucumber and 14 seawater samples collected from healthy larvae, respectively. A total of 32,759 and 41,608 Meta16S sequence reads were obtained from 3 sea cucumber and 3 seawater samples collected from larvae with intestinal atrophic disease, respectively. Alpha and beta diversity of larval microbiota were assessed and compared (Figure 2). Alpha diversity based on Chao1 and the Shannon index indicated a relatively higher alpha diversity in atrophic larvae, but beta diversity based on the Unweighted UniFrac distance showed a significantly decreased beta diversity in atrophic larvae (PERMANOVA, $p<0.05$), indicating a more complex but conservative microbiota in larvae with intestinal atrophic disease (Figures 2A, B). The PCoA plot based on the Unweighted UniFrac distance also illustrated a clustering microbiota in atrophic larvae, which separated the healthy larvae at same developmental stage as the atrophic samples, indicating a distinct microbiota in atrophic larvae (Figure 2C).

Since the significantly different microbiota in atrophic larvae was revealed, we wondered how microbial compositions were different in the atrophic larvae. A total of 1,915 ASVs from sea cucumber and seawater samples were obtained and further assigned to 210 families and 457 genera. *Flavobacteriaceae* (23.0 ± 5.1%) and *Rhodobacteraceae* (22.8 ± 8.3%) were the most dominant bacteria families in the microbiota of atrophic larvae, followed by *Spongiibacteraceae* (7.5 ± 4.0%), *Desulfuromonadia* PB19 (5.3 ± 2.6%), *Stappiaceae* (4.7 ± 4.0%), *Alphaproteobacteria* NRL2 (3.5 ± 1.7%), *Halieaceae* (3.4 ± 0.9%), *Crocinitomicaceae* (2.8 ± 2.2%), *Hyphomonadaceae* (2.5 ± 0.9%), and *Bdellovibrionaceae* (1.7 ± 1.7%), consisting of over 77% microbiota in atrophic larvae (Figure S2). Comparing the microbial composition between atrophic and healthy larvae, there were several bacterial families that were significantly different in the atrophic microbiome found by LEfSe analysis (Figure S3). *Flavobacteriaceae* was significantly enriched in atrophic larvae but relatively rare in healthy larvae (5.6 ± 6.3%). Other families dominant in atrophic larvae, including *Spongiibacteraceae*, *Desulfuromonadia* PB19, *Stappiaceae*, *Alphaproteobacteria* NRL2, *Halieaceae*, and *Crocinitomicaceae*, were barely or not detected in healthy larvae (Figures S3A, B, Table S2). On the other hand, *Alteromonadaceae*,

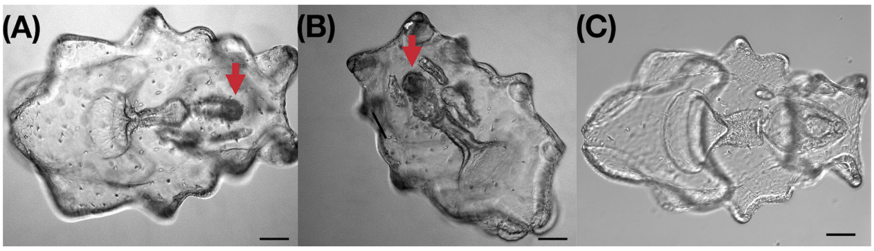

FIGURE 1
Light microscopy image of healthy larvae and larvae with intestinal atrophic disease of *Apostichopus japonicus*. **(A, B)** Larvae with intestinal atrophic disease. Size of digestive system (stomach and intestine) is obviously smaller, width is between 50-70μm, length is between 150-170μm; **(C)** Healthy larvae. Width of digestive system is between 170-200μm, length is between 250 and 270μm; bar:100μm.

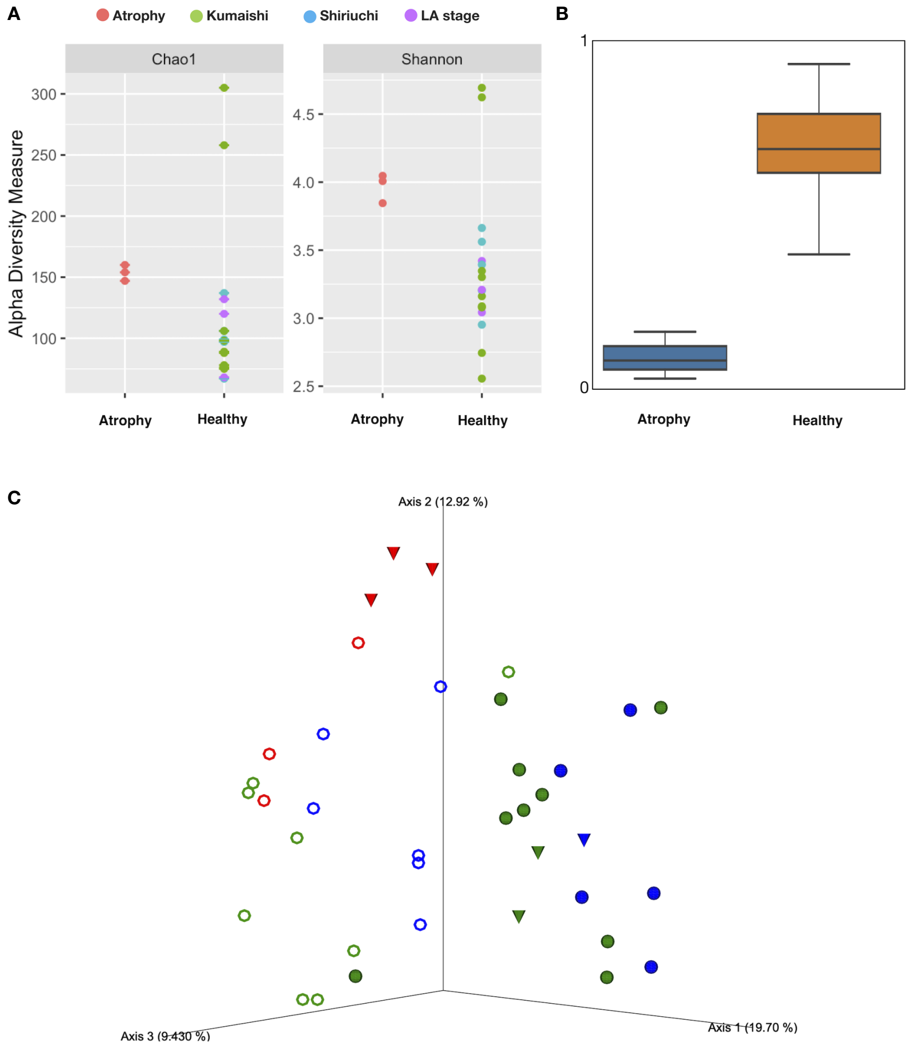

FIGURE 2
Alpha and beta diversity among sea cucumber samples. **(A)** Alpha diversity based on Chao1 and Shannon index. Red: atrophic larvae samples; green: healthy larvae collected from Kumaishi; blue: healthy larvae collected from Shiriuchi; purple: healthy larvae at late auricularia stage. **(B)** Boxplot based on unweighted UniFrac distance (p<0.05, q<0.05). Scale represents similarity within samples. **(C)** PCoA plot based on unweighted UniFrac distances obtained by comparison of microbiotas of sea cucumber larvae at different health status. Red: atrophic larvae and their rearing seawater; blue: healthy larvae collected from Shiriuchi; green: healthy larvae collected from Kumaishi; closed circle: healthy larvae; open circle: larvae's rearing seawater; triangle: larvae at late auricularia stage.

Methylophagaceae, and *Lachnospiraceae* were significantly abundant in healthy samples but dramatically rare in atrophic larvae, with a relative abundance of 0.7 ± 0.6% and below the undetectable limit (BUL), respectively. Since a significantly different microbial structure and composition was observed in atrophic larvae, we assumed these significantly varied bacteria in atrophic larvae were related to intestinal atrophic disease.

In order to further understand how bacteria were different in atrophic larval microbiota and whether there were pathogenetic bacteria resulting in intestinal atrophic disease, we also assessed the significantly different bacteria at the genera level (Figure 3; Figure S4). In atrophic larvae, *Oceanicoccus* (7.1± 3.6%) and *Ruegeria* (5.6 ± 3.0%) were the most dominant genera, followed by *Nioella* (4.4 ± 3.0%), *Labrenzia* (4.2 ± 3.9%), *Tenacibaculum* (3.6 ± 3.1%), and *Muricauda* (3.2 ± 1.3%). These dominant bacteria significantly increased in atrophic larvae but were barely or not detected at all in healthy larvae (Figure 3). The abundances of *Nioella*, *Labrenzia*, *Tenacibaculum*, and *Muricauda* in healthy sea cucumber were 0.6 ± 1.0%, 0.0% ± 0.1%, 0.2 ± 0.4%, and 0.3 ± 0.4%, respectively. *Sulfitobacter*, which includes probiotic candidate strains, belonging to *Rhodobacteraceae* was significantly dominant in healthy samples (3.5 ± 7.2%) but not detected in atrophic larvae (Figure 3; Table S3). *Neptunibacter* and *Alteromonas* were also significantly abundant in healthy larvae compared to atrophic larvae, indicating these bacteria may be responsible for different functions associated with host biology in healthy larvae.

Further linear-constrained ordination redundancy analysis (RDA) with forward selection revealed that health status significantly impacts on microbial community composition (ANOVA, p<0.05) (Figure 4). Four families, namely, *Alteromonadaceae*, *Methylophagaceae*, *Myxococcaceae*, and *Lachnospiraceae*, showed positive correlations with healthy larval

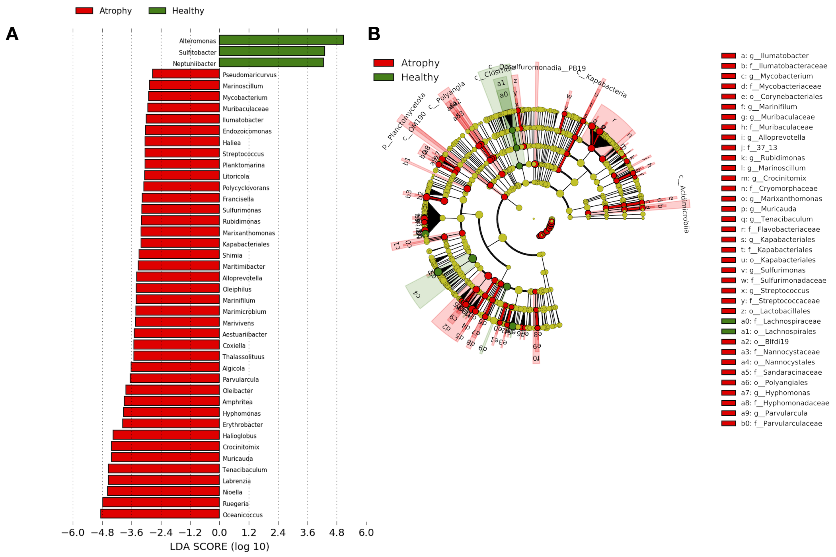

FIGURE 3
The linear discriminant analysis effect size (LEfSe) analysis of microbial abundance among sea cucumber larvae samples with different health status. **(A)** Only genera-level taxa with significant difference in atrophic and healthy larvae were detected by LEfSe analysis with an LDA threshold score of 3.5 and a significance of **0.05.** Red: atrophic larvae; green: healthy larvae. **(B)** The cladogram of detected prokaryotic taxa for atrophic and healthy larval microbial community. Red: atrophic larvae; green: healthy larvae.

microbiota, whereas *Flavobacteriaceae*, *Spongiibacteraceae*, *Cellvibrionaceae*, *Stappiaceae*, *Crocinitomicaceae*, *Halieaceae*, *Alphaproteobacteria* NRL2, and *Desulfuromonadia* PB19 were positively correlated with atrophic larval microbiota (Figure 4A). At the genera level, our analysis revealed a positive correlation between healthy microbiota and the presence of *Alteromonas*, *Sulfitobacter*, and *Neptunibacter*. Conversely, we observed a positive correlation between atrophic microbiota and the presence of *Oceannicoccus, Tenacibaculum, Labranzia, Ruegeria, Nioella*, and *Muricauda* (Figure 4B). These findings highlight the potential association between specific bacterial groups and the health status of sea cucumber larvae, providing valuable insights into the microbial dynamics and potential contributors to the development of intestinal atrophic disease.

Since the bacterial structure and composition were significantly different in atrophic larvae from those of healthy larvae, and no parasitic eukaryotes-like cells were observed under a microscope, we also performed meta16S comparison to determine whether the eukaryote community also varied and was associated with intestinal atrophic disease using mitochondrial reads. Although alpha diversity based on the Shannon index was not significantly different between atrophic and healthy larvae (Figure S5), Onchocercidae and Perkinsidae were significantly increased in atrophic larvae after LEfSe analysis, with relative abundances of 1.1± 0.1% and 0.2± 0.2%, respectively (Figure S5).

Impact of environmental rearing seawater on microbiome of atrophic larvae

In order to understand the rearing seawater impact on the microbial differences in atrophic larvae and its relationship with intestinal atrophic disease, the microbial structure and composition of rearing seawater were also assessed. The microbial diversity of alpha diversity based on the Shannon index and beta diversity based on the Unweighted UniFrac distance showed significant differences between atrophic larvae and their rearing seawater (p<0.05) (Figures 5A, B). A total of 118 ASVs (33.5%) were shared within atrophic larvae and the rearing seawater, indicating the seawater also influenced the atrophic microbiome moderately (Figure 5C). *Rhodobacteraceae*, *Flavobacteriaceae*, *Alteromonadaceae*, *Pseudohongiellaceae*, OM182 Clade (*Gammaproteobacteria*), *Microbacteriaceae*, *Legionellaceae*, *Hyphomonadaceae*, *Stappiaceae*, and *Saccharospirillaceae* were the dominant families in the rearing seawater of atrophic larvae, comprising over 86% of microbiota in the rearing seawater (Table S2). The significantly abundant bacteria in atrophic larvae *Spongiibacteraceae* (7.5± 4.0%), *Desulfuromonadia* PB19 (5.3 ± 2.5%), and *Halieaceae* (3.4 ± 0.9%) were only enriched in atrophic larvae but significantly decreased in their rearing seawater, with relative abundances of 0.4 ± 0.2%, 0.1 ± 0.1%, and 0.1 ± 0.2%, respectively (Figure S3C, Table S2). Other dominant bacteria in atrophic larvae including *Flavobacteriaceae*, *Stappiaceae*, *Alphaproteobacteria* NRL2, and *Crocinitomicaceae* that might relate with intestinal atrophic disease were also abundant in their rearing seawater (Figure S3C, Table S2). Furthermore, a heatmap based on significantly different genera between atrophic and healthy larvae was generated to better understand how bacteria varied in atrophic larvae, their rearing seawater, and healthy larvae (Figure 6). At the genus level, *Planktomarina*, the NS3a marine group (*Flavobacteriaceae*), *Pseudohongiella, Nioella, Aestuariibacter, Jejudonia, Pontimonad, Marivita, Alteromonas*, and *Labrenzia* were the most dominant bacterial taxa in the rearing seawater of atrophic larvae, consisting of over 60% of the microbiota in rearing seawater (Table S3).

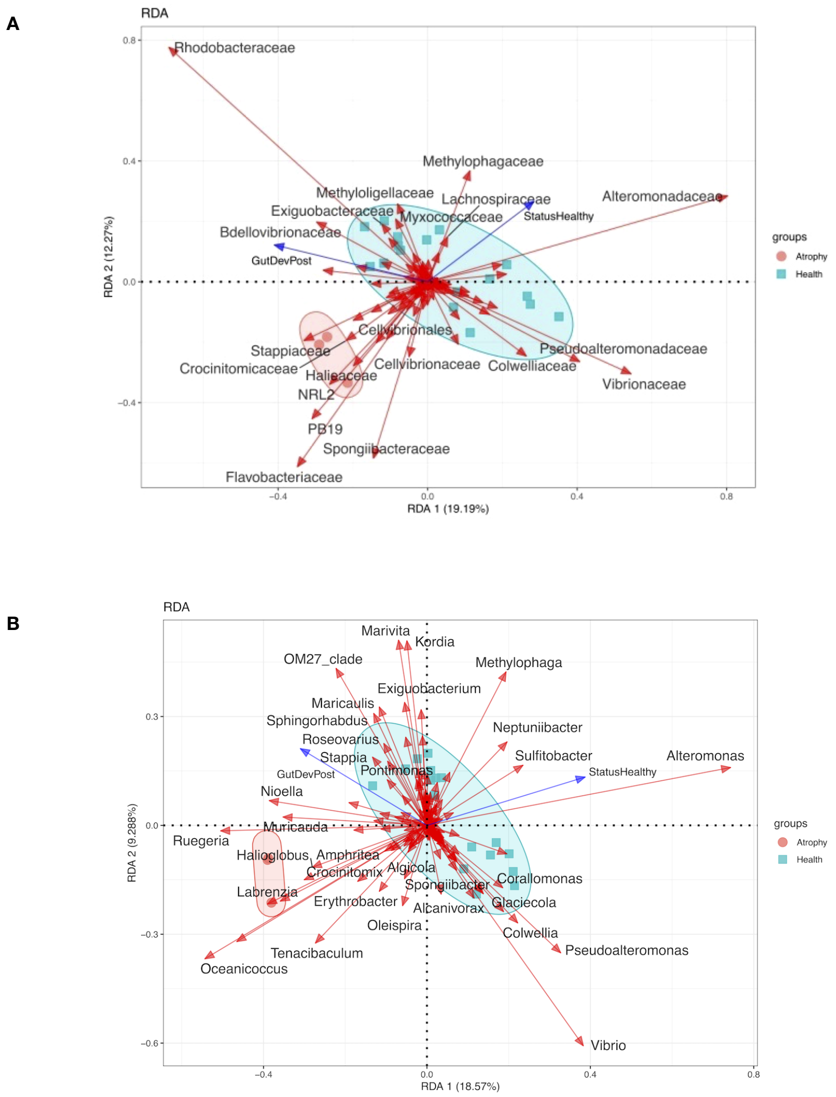

FIGURE 4
Redundancy analysis (RDA) of the relationship between the health status and the relative abundance of bacterial group of atrophy and healthy larvae samples. Circles represent atrophy samples, cubes represent healthy samples. The abbreviation of samples is shown in Table S1. Arrows indicated the direction and magnitude of variables (p<0.05). **(A)** RDA of the relationship between the health status and the relative abundance of bacterial group at family level. **(B)** RDA f the relationship between the health status and the relative abundance of bacterial group at genera level.

Among these dominant bacteria in the rearing seawater of atrophic larvae, *Nioella* and *Labrenzia* were also significantly abundant in atrophic larvae and might be affected by their rearing seawater. Whereas *Oceanicoccus*, *Ruegeria*, *Crocinitomix*, and *Muricauda* were significantly increased in atrophic larvae compared to healthy larvae, they were significantly reduced in their rearing seawater. The same tendency could also be observed in *Halioglobus* and *Amphritea*. Moreover, while *Tenacibaculum* was dominant in atrophic samples and potentially associated with intestinal atrophic disease, it was only abundant in seawater collected on the first day when disease occurred and not detected in later seawater samples.

In addition, we compared the microbiota between atrophic larvae rearing seawater and healthy larvae rearing seawater to further investigate whether seawater impacted the alteration of the larval microbiome and induced potential pathogenetic bacteria. Although alpha diversity based on the Shannon index was not significantly different between diseased larval seawater and healthy larval seawater, beta diversity based on the unweighted UniFrac distance was significantly different between environmental seawater in atrophic and healthy larvae (Figure 5D). At the family level, *Alphaproteobacteria* NRL2, *Micrococcaceae*, *Parvularculaceae*, *Porticoccaceae*, and *Thalassobaculaceae* significantly increased in atrophic larval rearing seawater, and *Alphaproteobacteria* NRL2 was significantly enriched in atrophic larvae, which could be induced by their rearing seawater (Table S2, Figure S6A). At the genus level, *Planktomarina*, the NS3a marine group (*Flavobacteriaceae*), *Pseudohongiella, Nioella, Aestuariibacter, Jejudonia, Pontimonad, Marivita, Alteromonas*, and *Labrenzia* were the most dominant bacterial taxa in the rearing seawater of atrophic larvae, consisting of over 60% of the microbiota (Table S3). Among these bacteria, *Nioella*, *Oleibacter*, *Muricauda*, and *Algicola* were found to

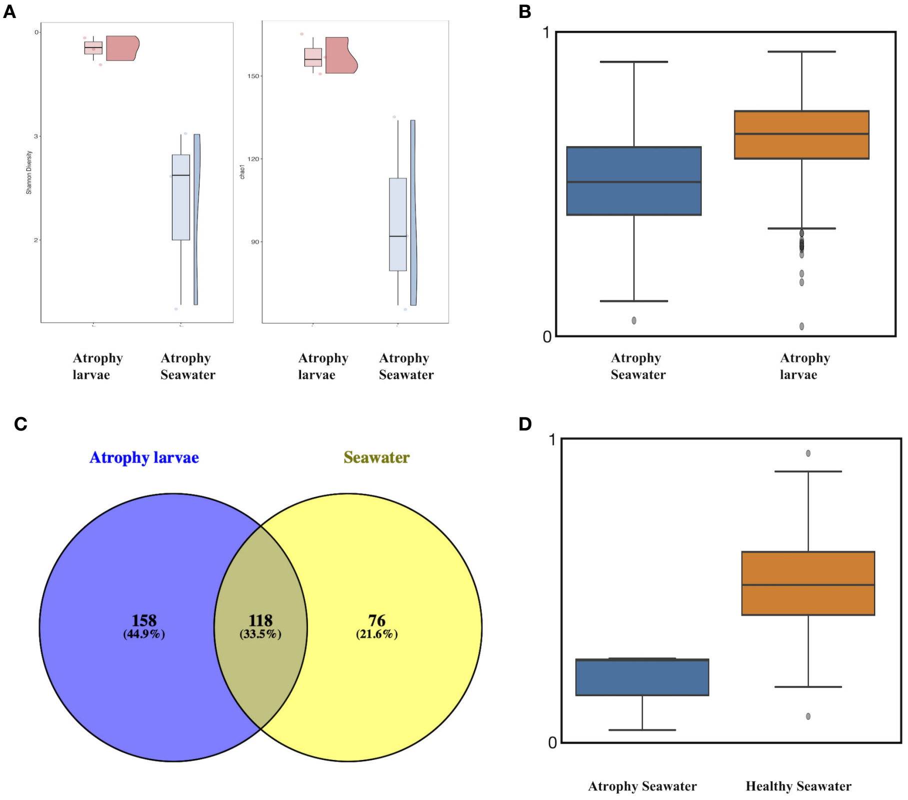

FIGURE 5
Alpha and beta diversity among atrophic sea cucumber and seawater samples. **(A)** Alpha diversity based on Chao1 and Shannon index. Red: atrophic larvae samples; blue: atrophic larvae rearing seawater. **(B)** Boxplot based on unweighted UniFrac distance of atrophic larvae and their rearing seawater (p<0.05, q<0.05). Scale represents similarity within samples. **(C)** Venn diagram depicting unique and shared OTUs among atrophic larvae and their rearing seawater. **(D)** Boxplot based on unweighted UniFrac distance of atrophic larvae's rearing seawater and healthy larvae's rearing seawater (p<0.05, q<0.05). Scale represents similarity within samples.

significantly increase in the seawater used for rearing atrophic larvae but decrease in the seawater used for rearing healthy larvae. These bacteria were also found to be significantly abundant in atrophic larvae, suggesting a potential influence of the rearing seawater on their presence and abundance in atrophic larval microbiome (Figure 6; Figure S6B, Table S3). In addition, the proportion of *Tenacibaculum* (BUL) in healthy larval rearing seawater was very small compared to atrophic larval rearing seawater (1.1% ± 1.6%), indicating *Tenacibaculum* was likely to be introduced in the rearing seawater of atrophic larvae. These results suggested rearing seawater impacted on atrophic larval microbiota in a moderate way, causing the differences in microbiome of atrophic larvae.

Microbial function analysis of healthy and atrophic larvae

In order to further understand how microbial functions varied in larvae with intestinal atrophic disease, microbial function profile was also performed by MG-RAST, and it revealed the significantly different microbial functions in atrophic larvae. In general, the most abundant microbial functions were Serine-glyoxylate cycle, methionine biosynthesis, ribosome LSU bacterial, cobalt–zinc–cadmium resistance, purine biosynthesis, Ton and Tol transport systems, respiratory complex I, phosphate metabolism, and RNA polymerase bacterial and DNA replication in atrophic samples (Figure 7A). Heatmaps based on LEfSe analysis revealed the significantly different functions in atrophic larvae (Figure 7B). Compared to healthy larvae, there were more microbial functions enriched in atrophic larvae related to vitamin B (Thiamin) biosynthesis, metabolism of aromatic compounds (phenylalkanoic acid degradation), carbohydrates metabolism (glycolysis and gluconeogenesis), amino acid metabolism (lysine biosynthesis), and protein metabolism (metallocenter biosynthesis). In addition, microbial functions related to respiration including biogenesis of c-type cytochromes, succinate dehydrogenase, and ATP synthase increased in atrophic larvae. These functions may contribute to bacterial pathogens regulating their metabolism for growth in atrophic larvae, enabling them to colonize larval microbiota and induce pathogenic damage. In addition, the function of pathogenicity islands, detected in large numbers of bacterial pathogens, was also enhanced in atrophic larvae, probably in relation to intestinal atrophic disease.

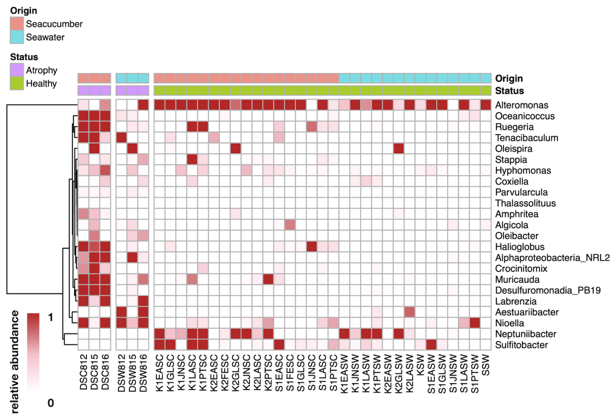

FIGURE 6
Dynamics of significantly different genera between atrophic larvae, atrophic larvae's rearing seawater, healthy larvae, and healthy larvae's rearing seawater. Scale represents relative abundance. DSC812-816 represent atrophic larvae samples; DSW812-816 represent atrophic larvae's rearing seawater; K*SC and S*SC represent healthy larvae; K*SW and S*SW represent healthy larvae's rearing seawater. Legend bar shows the sample types and health status. Origin shows the original samples collected from sea cucumber or seawater, red represents sea cucumber, blue represents seawater, Status shows the healthy status of samples, purple represents atrophic samples, and green represents healthy samples.

Correlation network analysis revealed a more complex microbiome in atrophic larvae

To further understand the species–species association in the microbiome of atrophic larvae, a correlation network of larval microbiota based on Spearman correlation was generated (Figure S7). In healthy larval microbiome, *Spongiibacter* and *Nitrosomonas* were the most important bacteria linked to seven other genera, followed by *Reinekea* and *Enrythrobacter* related to six other genera (Figure S7A). Compared to healthy larval microbiota, there were more microbial connections observed in atrophic larvae (Figure S7B). *Nioella* and *Ruegeria* were in the center of the correlations, related to most other bacteria in atrophic larvae. *Algicola* was negatively correlated with *Nioella* and *Oceanicaulis*, and *Aestuariibacter* was negatively correlated with unclassified *Pseudomonadaceae*. The dominant taxon in healthy larval *Alteromonas* was correlated with abundant taxa in atrophic larvae *Stappia*. The potential pathogen *Tenacibaculum* was correlated with *Amphritea* and unclassified *Cryomorphaceae*, which was rare in healthy larvae. The microbial correlations in atrophic larvae revealed a more complex microbial correlation in atrophic larval microbiota, which is probably related to intestinal atrophic disease.

Discussion

Disease in sea cucumber *A. japonicus* has been exclusively studied in recent decades to reduce the mortality of sea cucumber and contribute to the development of its aquaculture, including skin ulceration disease, viscera ejection syndrome, and acute peristome edema disease (Wang et al., 2007; Deng et al., 2009). However, most studies only concentrated on the disease of adult sea cucumber, and there has been a lack of knowledge of the disease that occurs during larval ontogenesis in *A. japonicus*, which has impacted seed production and resulted in enormous economic losses (Chen et al., 2022b). This study represents the first report of intestinal atrophic disease occurring at the auricularia stage of sea cucumber larval ontogenesis, highlighting it as a novel warning sign during larvae production in sea cucumber aquaculture. These findings have important implications for the industry and emphasize the need for further research and preventive measures to ensure the sustainable development of sea cucumber aquaculture. We revealed the significantly varied microbiome and their correlations in atrophic larvae, in which significantly abundant *Tenacibaculum*, *Oceanicoccus*, and *Labrenzia* were detected. In addition, it was demonstrated that significantly different microbial functions in atrophic larvae related to adaptations in the metabolism contributed to the colonization of bacterial pathogens in the host. Environmental rearing seawater impacted on host microbiome in atrophic larvae moderately, probably associated with the introduction of potential pathogens, highlighting the importance of standardized aquaculture management.

Host-associated microbiomes in marine invertebrates play an important role in host health maintenance, immunity development, and disease resistance (O'Brien et al., 2019). The disturbance of microbial composition and function is directly related to outbreaks of diseases in marine organisms (Hou et al., 2018; Rubio-Portillo et al., 2021). Our study indicated the significantly different microbial structure and function in larvae with intestinal atrophic disease, indicating the microbial alteration in atrophic larvae is probably related to such diseases. In the sea cucumber *A. japonicus*, diseases caused by bacterial pathogens such as *Vibrio* spp. and *Pseudoalteromonas* spp. have been well studied to understand the symptoms, histopathology, and possible treatment for those diseases (Zhang et al., 2021). A study on skin ulceration disease revealed the significantly changed microbiota in diseased sea cucumber and indicated a potential pathogen, *Lactococcus garviaeae*, related to disease outbreaks (Zhang et al., 2018). *V. lentus* can cause rotting edges syndrome in sea cucumber larvae at the auricularia stage, which results in the autolysis of larvae and significantly increased mortality (Yang et al., 2015). *V. splendidus* and *V. alginolyticus* are also reported to be related to several diseases in *A. japonicus*, including skin ulceration disease and viscera ejection syndrome (Deng et al., 2009; Zhang et al., 2014). *Pseudoalteromonas* spp. cause skin ulceration disease in *A. japonicus*, indicating different bacterial pathogens that could be related to the same disease in sea cucumber (Liu et al., 2010). According to our study, neither *Vibrio* spp. nor *Pseudoalteromonas* spp. were enriched in atrophic larval microbiota, showing that these two common pathogens in *A. japonicus* may not be related to intestinal atrophic disease.

The significantly increased bacterial taxa in atrophic larvae, including *Muricauda, Oceanicoccus, Ruegeria, Tenacibaculum, Labrenzia*, and *Nioella*, have been identified as potential causative microbial factors related to disease. While *Muricauda* spp. have been reported as relatively abundant in the microbiome of diseased

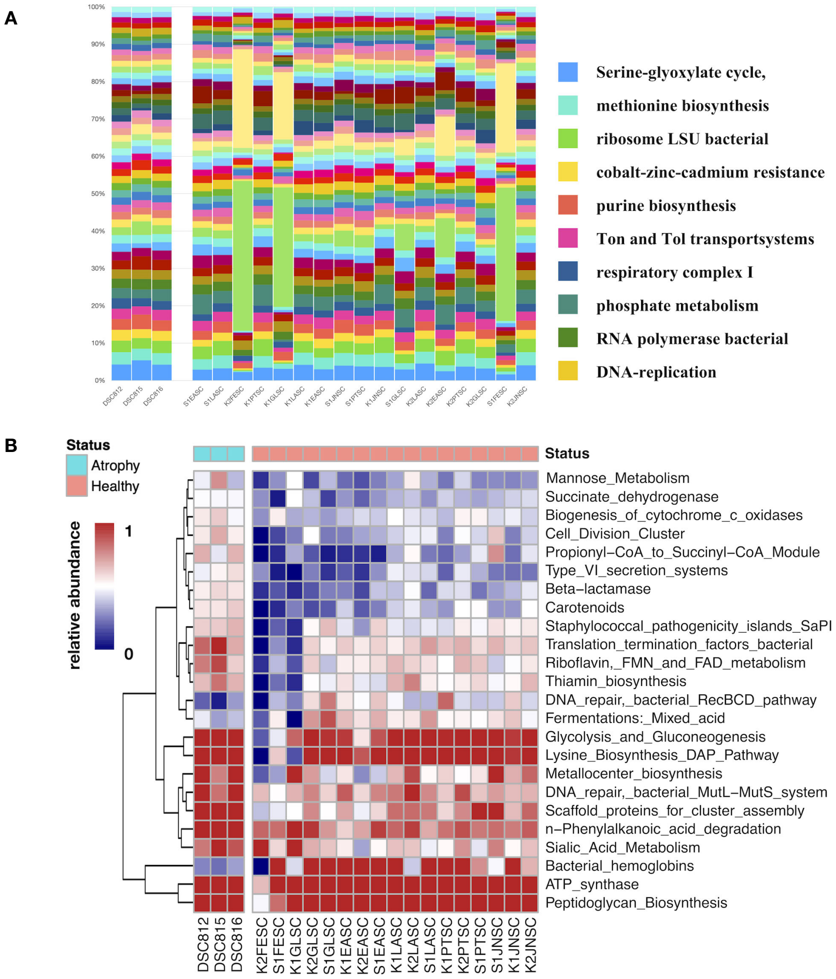

FIGURE 7
Microbial function analysis of atrophic and healthy larvae. **(A)** Distribution of microbial functions among atrophic and healthy larvae samples. Bars represent the relative percentage of each microbial functions. DSC812-816 represent diseased sea cucumber larvae and others represent healthy sea cucumber larvae. **(B)** Dynamics of significantly different microbial functions between atrophic larvae and healthy larvae. Scale represents relative abundance. DSC812-816 represent atrophic larvae samples; K*SC and S*SC represent healthy larvae. Legend bar shows health status. Purple represents atrophic samples, and green represent healthy samples.

sea grape, it has not been directly implicated as a pathogenic bacterium causing disease outbreaks (Kopprio et al., 2021). Bacteria in the genus *Ruegeria* are associated with coral diseases such as *Montipora* white syndrome (MWS), but they can also act as commensal bacteria in healthy corals (Casey et al., 2015). The genus *Nioella* has been detected in shrimp larvae with low survival rates and in the gut microbiome of shrimp with white feces syndrome caused by *Vibrio* spp. (Lu et al., 2020; Callac et al., 2023). However, apart from *Tenacibaculum*, there have been no studies reporting these bacteria as pathogenic bacteria associated with diseases or syndromes in marine invertebrates. The increased abundance of these bacteria may be associated with disturbances in the host microbiome and can serve as indicators of diseased status. On the other hand, significantly decreased *Sulfitobactor*, *Alteromonas*, and *Neptunibacter* in atrophic larvae are common core microbes in the pan-microbiome of healthy larvae. The significantly increased bacteria in healthy larval microbiome such as *Alteromonas* spp. and *Sulfitoacter* spp. have been used as probiotics in marine mollusks and fish against pathogens, which could probably be applied to investigating probiotics to modulate the host microbiome and prevent intestinal atrophic disease (Emilia Noor and Eguchi, 2012; Chi et al., 2014).

Although there have been no studies reporting significantly increased bacteria *Tenacibaculum* in atrophic larvae and related diseases in sea cucumber, *Tenacibaculum* was shown to have been able to cause spotting disease in sea urchin in the Shiriuch farm in 2000 (Masuda et al., 2004). Sea urchins that were exposed to *Tenacibaculum* F2 exhibited 100% mortality within 24 hours of cultivation and displayed detachment of spines and the development of dark violet spotting lesions (Tajima et al., 1997). Considering the high occurrence of intestinal atrophic disease and the cessation of metamorphosis in sea cucumber larvae cultivated in the same farm at Shiriuchi, we hypothesize that *Tenacibaculum* spp. present a strong candidate as the potential pathogenic bacterium responsible for causing atrophic disease in sea cucumbers. However, we did not isolate any *Tenacibaculum* strains from the atrophic larvae in this study, so further investigations are needed to confirm their role and elucidate the underlying mechanisms of pathogenesis. *Tenacibaculum* spp. are also reported to be a fish pathogenetic bacterium causing "tenacibaculosis" in different fish species, resulting in tissue loss, exposure of jaw bones, and frayed caudal fins (Spilsberg et al., 2022). In one study, Atlantic salmon and rainbow trout were exposed to *Tenacibaculum dicentrarchi*, resulting in the development of tail and peduncle lesions. The mortality rates significantly increased up to 65% and 93% within one hour, respectively (Avendaño-Herrera et al., 2006). The genomic study on *Tenacibaculum* revealed virulence-associated genes, including putative virulence, antimicrobial resistance genes, pathogenicity islands, toxins, and hemolysins, being present in most *Tenacibaculum* species (Nowlan et al., 2023). A metagenomic functional profile revealed that pathogenicity islands were also enriched in atrophic larvae, probably related to the high abundance of *Tenacibaculum*. Although *Tenacibaculum* spp. show their virulence factors and potential pathogenicity in marine organisms, the isolation and *in situ* detection of *Tenacibaculum* spp. in intestinal atrophic disease during sea cucumber larval ontogenesis also need to be further examined.

Our study also revealed parasitic disease-associated protozoans Perkinsidae belonging to Perkinsea, which is a microeukaryotic parasitic related to varieties of mollusk infection disease (Villalba et al., 2004). A study of sea cucumber with skin ulceration disease also indicated a significantly increased Perkinsidae detected in its eukaryotes community (Delroisse et al., 2020). The histology of *Perkinsus* species belonging to Perkinsidae exposed to clams indicated trophozoites (15 μm in diameter) of *Perkinsus*, which could be observed under microscopy at five days after infection (Waki et al., 2012). Since we did not observe any trophozoites nor protozoans in diseased larvae, the significantly increased Perkinsidae may not be the factor causing intestinal atrophic disease but is probably related to alteration of host physiology.

Microbial functions of succinate dehydrogenase, pathogenicity islands, and biosynthesis of cytochrome c oxidases enriched in atrophic larvae were related to bacterial pathogenicity. The metabolism of c-type cytochromes is essential for several pathogens as it enables respiration under both external and host environments. These cytochromes play a crucial role in the pathogen's virulence by contributing to various mechanisms (Wilson, 2015). Succinate dehydrogenase activity increased in sea cucumber *A. japonicus* during pathogen *V. splendidus* infection, involved in innate immunity through its participation in mitochondrial ROS generation (Sun et al., 2020). Such studies provide evidence that significantly increased succinate dehydrogenase in atrophic larvae might also be related to pathogen infection. In particular, studies of *Tenacibaculum maritimum* detected gene encoding with succinate dehydrogenase and cytochrome c oxidases in its genome, further explaining that *Tenacibaculum* is the potential pathogen, resulting in intestinal atrophic (Perez-Muñoz et al., 2017). The alteration of the microbial functions allowed the bacterial pathogens to adjust their metabolism for survival and growth in atrophic larvae and further cause pathogenetic infection.

In considering possible factors shaping the microbiome and inducing the bacterial pathogen, environmental factors including rearing water and diets are the most common source of contamination (Chen et al., 2022b). Free-living microbiota within aquatic ecosystems influence the microbiome of aquatic vertebrates such as aquatic mammals and fish, indicating that a shift of environment could lead to ecological disruptions that impact the microbiota and health of aquatic vertebrates (Sehnal et al., 2021). Our results revealed that 33.5% of ASVs were shared with the microbiome in atrophic larvae and their rearing seawater, including the significantly changed microbes and potential pathogen *Tenacibaculum*. In particular, on the first day we observed the occurrence of intestinal atrophic disease in sea cucumber larvae at the farm, the abundance of *Tenacibaculum* increased in both atrophic larvae and rearing seawater in the farm (Figure 6, Table S3). These results indicate that the rearing seawater impacted the atrophic larval microbiota, especially related to the natural presence of potential pathogens *Tenacibaculum.* Similar to our results, the microbiome of sea cucumber with Body Vesicular Syndrome (BVS) was significantly altered and shaped by rearing pond water, resulting in changes of the microbial metabolism and BVS occurrence (Zhao et al., 2022). Studies of environmental factors on skin ulceration syndrome (SUS) outbreaks in *A. japonicus* also demonstrated that the microbiome significantly changed in SUS-diseased sea cucumber under environmental stressors and pathogen *V. splendidus* exposure (Zhang et al., 2019). The environmental stresses, including high temperature, mechanical damage, nitrite-N, and ammonium, promoted pathogenetic infection by shaping the gut microbiome and increasing the possibility of pathogenic invasion (Zhang et al., 2019). Another study also emphasized the influence of high temperature on rearing water and sediments of *A. japonicus* and its microbiome, showing the microbiota was significantly changed with the increasing proportion of potential pathogens (Wang et al., 2021). When we considered the possible side factors accelerating intestinal atrophic disease during larval development, it was found that higher humidity in the breeding center during the summer season might cause the aerosol contamination in the rearing environmental seawater via the aeration system. Formation of dew was observed in the tubes of the aeration system, possibly affecting water quality and providing the possibility for bacteria to colonize the small tube or other components in the aeration system under humid conditions. We observed a significant increase in *Tenacibaculum* on the first day of disease occurrence and a high abundance of *Nioella* and *Labrenzia* in rearing seawater, indicating a contamination event that potentially altered the microbiome of the environmental seawater. This contamination could have induced the potential pathogens, leading to the disturbance of the larval microbiome and resulting in the development of intestinal atrophic disease in the sea cucumber larvae (Herpertz-Dahlmann et al., 2017). In addition, diet should be also considered as a possible reason causing the alteration of the microbiome in the rearing seawater and sea cucumber larvae. Unfortunately, since the diets fed for both healthy and atrophic larvae were originally collected from the same research center (Hakodate Fisheries Research, Japan) and stored under the same conditions (4°C), the microbial community of the diet was not investigated in this study. Our findings on the impact of environmental factors on the atrophic larval microbiome highlight the importance of aquaculture management, in particular, the breeding conditions including temperature, humidity, and salinity, which could be key factors influencing the seed production of sea cucumber. The disturbance of the microbiome in intestinal atrophic larvae also gives us new insights into microbial homeostasis contributing to host disease resistance and health maintenance. Further approaches to isolating potential pathogens and exposing them to sea cucumber larvae could help us better understand the mechanism of intestinal atrophic disease in sea cucumber larvae and contribute to strengthening its aquaculture industry.

Conclusion

In this study, we report for the first time the occurrence of intestinal atrophic disease during sea cucumber larval production in a Japanese farm and investigate the variation in the associated microbiome. Our analysis reveals a significant alteration in the composition of the microbiome in atrophic larvae compared to the healthy pan-microbiome, characterized by increased abundance of keystone bacteria, including *Oceanicoccus*, *Ruegeria*, *Nioella*, *Labrenzia*, and *Tenacibaculum*. Our findings suggest that the environmental rearing seawater was the factor impacting the atrophic larval microbiome moderately, emphasizing the need for standardized aquaculture management, particularly with regards to culture conditions. Additionally, this study infers that a potential pathogen, *Tenacibaculum*, is associated with intestinal disease, underscoring the need for further investigation into the role of potential pathogens in sea cucumber development. Such investigations provide new insights into host–microbe interactions and ultimately benefit the seed production and aquaculture of sea cucumber.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

Ethical review and approval were not required for the study on animals in accordance with the local legislation and institutional requirements.

Author contributions

TS and JY designed the study. JY, RY, SK, and YS performed the experiment. JY, TS, and SM obtained the data and performed the data analysis. JY and TS drafted the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2023.1225318/full#supplementary-material

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Effect of live-storage period and temperature on oxygen consumption rate in the cold-water sea cucumber *Parastichopus tremulus*

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Sea cucumbers are of great significance in marine ecosystems all over the world. The red sea cucumber *Parastichopus tremulus* is also considered commercially interesting but very little information is available on its ecology and physiology. This study aimed at investigating this North Atlantic species` oxygen demand in relation to live-storage period and temperature in an attempt to deliver some physiological puzzle pieces useful in a potential aquacultural context. Oxygen consumption rate (OCR) was measured in *P. tremulus* stored in a flow-through system for up to one year, and in recirculation systems at different temperatures (4, 8 and 12°C) for up to four weeks. Throughout the experimental period, biometric data were collected from the different treatment groups and a condition index (CI) calculated. The results showed that long-term storage affected OCR and CI, which increased and decreased with time, respectively. Storage at 4°C resulted in significantly lower OCR and higher CI compared to 8 and 12°C after four weeks, suggesting that 4°C may be best for maintaining body mass during short-term storage, whereas storage at 8 and 12°C may require supplementary feeding to avoid biomass loss. Data presented in this study may be useful for a range of applications, for example in a prospective aquaculture setting, for optimizing live seafood storage, but also for impact assessment and management of wild stocks.

KEYWORDS

sea cucumber, Holothuroidea, Parastichopus tremulus, oxygen consumption, live-storage, temperature, condition index

1 Introduction

Several sea cucumber species are commercially exploited due to their importance as a luxury seafood and application in traditional medicine (Purcell, 2014; Yang et al., 2015). Rising Asian demand and the associated depletion of wild stocks in tropical and temperate regions (Purcell et al., 2014) has brought attention to cold-water species as well. In Europe, and Norway in particular, sea cucumbers have not been considered a potential marine resource until recently, thus research and knowledge on their biology and ecology is limited (González-Wangüemert et al., 2018; Landes et al., 2019). Sea cucumbers (holothuroids) are found in all marine water bodies in Northern Europe, apart from the lower saline Baltic Sea, and more than more than 40 species have been registered in Scandinavia (Madsen and Hansen, 1994). Feeding at the lowest levels of the food web, they are of great significance in marine ecosystems, particularly in deep-water ecosystems where they can account for more than 90% of the biomass (Pawson et al., 2011). Although echinoderms such as holothuroids have a lower oxygen consumption compared to other species (Fox, 1936), they contribute significantly to benthic nutrient cycling due to their abundance in this specific ecosystem. Because of some species' unique ability to extract nutrients from particulate matter, they are also considered key candidate species for inclusion in sustainable aquaculture production systems such as integrated multi-trophic aquaculture (IMTA).

To better understand physiological responses to environmental changes and performance in captivity, in-depth studies investigating the functional metabolism of each species are required. Water temperature, for instance, is a crucial factor for ectothermic animals like sea cucumbers, as it impacts a wide range of physiological and intercellular processes (Schmidt-Nielsen, 1990; Burggren and Roberts, 1991). From temperate species, we know that temperature affects a range of behavioural and physiological parameters in sea cucumbers, such as feeding behaviour, growth, and feed utilization (Yang et al., 2005; Ji et al., 2008). An increase in water temperature leads to an acceleration of physiological processes inside the organism and thereby to higher oxygen demand and excretion of metabolic waste products, resulting in increased oxygen consumption and ammonia excretion rates (Zamora and Jeffs, 2012).

Breathing (respiration) in holothuroids primarily occurs through the respiratory tree which is a highly branched organ specialized for gas exchange and excretion. The paired respiratory tree is located close to the anal opening and breathing is performed by drawing and expelling water through the anus. If the respiratory tree has been eviscerated and internal organs are under regeneration, respiration may occur solely through the skin and tube feet (Hamel et al., 2022). However, presence or absence of a respiratory tree does not seem to have a significant effect on overall oxygen consumption as demonstrated by Dimock (1977) in the Pacific species *Stichopus parvimensis*. A third possible passage for gases and fluids is through the madreporite which in sea cucumbers is located inside the coelom (Gao and Yang, 2015, chapter 4). In addition to exhibiting an allometric relationship with body size (Astall and Jones, 1991; Kodama et al., 2015), a range of factors are known to affect the metabolic rate and thereby oxygen consumption rate (OCR) in echinoderms, e.g. salinity (Talbot and Lawrence, 2002), pH (Farmanfarmaian, 1966), partial pressure of oxygen, pO_2 (Shick, 1983; Spicer, 1995; Christensen and Colacino, 2000), seasonal variation in growth (Lawrence and Lane, 1982; Fraser et al., 2004), feeding status (Vahl,1984; Brockington and Clarke, 2001; Idrisi et al., 2003; Yu et al., 2020), nutritional quality of food (Otero-Villanueva et al., 2004) and reproductive state (Giese et al., 1966; Féral and Magniez, 1988; Ru et al., 2020). Changes in locomotory activity, such as an increase during spawning season (Ru et al., 2021), or a reduction during aestivation (Ji et al., 2008), can also impact the rate of oxygen consumption. Commonly used to determine metabolic energy use in heterotrophic organisms, the rate of oxygen consumption may also serve as an indicator for the animal's general metabolic state and is a suitable tool for uncovering potential stress responses to changing environmental conditions.

In Norway, the red sea cucumber *Parastichopus tremulus* has been identified as one of the species with the highest commercial potential for Asian markets (Kjerstad et al., 2015). *P. tremulus* has also been considered as a potential key species in Norwegian IMTA (Sunde and Christophersen, 2023). Presently only landed as by-catch, it is one of the most common sea cucumbers occurring along the Norwegian coast. Although being most abundant at depths between 100-300 m along the Norwegian west coast (Kjerstad et al., 2015), *P. tremulus* has also been recorded at shallow depths of ≤20 m and down to >3000 m in the deep sea in other areas (Ludwig, 1900; Grieg, 1921; MNHN, Chagnoux, 2023). This large range of bathymetric distribution implies that *P. tremulus* has a high natural physiological plasticity that facilitates adaptation to different environments. However, our understanding of this species' capacity to adapt to different conditions, e.g. future elevated sea temperatures linked to climate change, remains limited. Although *P. tremulus* is expected to encounter relatively minor temperature fluctuations at the depths where it is most abundant, even slight variations in temperature could potentially have significant impacts on cold-water species (e.g. Emblemsvåg et al., 2022). One of the few known physiological characteristics of *P. tremulus* from Norwegian waters is its seasonal feeding pattern, where feeding ceases and evisceration is more prevalent during winter months (Jespersen and Lützen, 1971). This phenomenon is also observed in other Northern hemisphere species (Swan, 1961; Fankboner and Cameron, 1985). However, very few studies have been carried out on the physiological and metabolic characteristics of *P. tremulus* and how these compare to those of other holothuroids (Fox, 1936; Hauksson, 1977; Hauksson, 1979).

There are many reasons as to why acquiring of more in-depth knowledge is absolutely crucial. Given the increase in anthropogenic activities in coastal areas, zones of hypoxic conditions (low dissolved oxygen) could become more widespread in the future. Hence, it is essential to determine the oxygen requirements of *P. tremulus* under different conditions to evaluate the impact of human activities on these vital components of benthic ecosystems. Subtidal species such as the red sea cucumber may have a lower tolerance to low oxygen concentrations than intertidal species that regularly experience changing conditions (Weinrauch and Blewett, 2019), and thus be more at risk when exposed to such changes. In order to enable the preservation of our coastal marine environment and an effective management of the marine resources, we urgently need to learn more about the species` basic physiological operating principles and responses to its specific environment. Furthermore, it is requisite to determine the species` environmental requirements for development, growth, and welfare in captivity, considering its potential as an aquaculture candidate (Landes et al., 2019;

Schagerström et al., 2022). However, it should be noted that the conditions conducive to growth in aquaculture may differ substantially from those in the natural habitat.

Maintaining a sea cucumber species such as *P. tremulus* under conditions that ensure welfare and facilitate growth, requires knowledge on physiological responses related to storage time and temperature. Therefore, this study aimed to quantify and observe the level of respiration and its development during short (weeks) and long (months) term storage in flow-through systems. The objective was to uncover potential changes in OCR associated with physiological stress under conditions in captivity and its metabolic consequences. Furthermore, we wanted to investigate the response of *P. tremulus* to storage at different temperatures over a period of four weeks in recirculation systems intended for storage of live seafood. We hypothesized that OCR would increase with increasing temperature, especially since 8 and 12°C diverge significantly from temperatures that are common in the species' preferred deep-sea habitat. Although *P. tremulus* may encounter temperatures up to 12°C in its natural range of distribution during the summer months, temperatures in the depth where experimental animals were retrieved from rarely exceed 7°C (Nilsen and Falck, 2006). Thereby, we expected the lowest OCR to be measured at 4°C whereas rates at both 8 and 12°C would reflect a stressed state expressed by significantly higher OCR. With regard to storage period, we expected that OCR would change with duration of storage, since laboratory conditions differ from that of the natural habitat. The direction of OCR change over time is influenced by several factors and hard to predict. For instance, metabolic depression due to the lack of appropriate food could lead to a decrease in OCR while accumulated stress under laboratory conditions could lead to an increase.

2 Materials and methods

Sea cucumbers (*Parastichopus tremulus*) used in the experiments were collected at 130-250 m depth in the fjord system south of Molde in Møre and Romsdal county, located in the north-western part of Norway (62°N-7°E) – where they have been obtained as bycatch from pot fisheries of Norway lobster (*Nephrops norvegicus*). Length and total wet weight (WW) of the sea cucumbers (n=214) ranged from 60 to 220 mm (average 126.4 mm) and from 21 to 245 g (average 72.5 g), respectively. Results are presented as the average value ± standard deviation, unless stated otherwise.

At the landing site, sea cucumbers were transferred to 1 L plastic bags filled with ambient, unfiltered seawater. The bags, each containing 1-3 individuals (dependent on size), were placed in polystyrene boxes with cooling elements distributed on top. Transportation to the Møreforsking laboratory facilities in Ålesund took about one hour. Upon arrival, the animals were directly transferred into square flow-through holding tanks (100 x 100 x 50 cm, 300 L) supplied with untreated seawater (10 L min^{-1}) pumped up from 40 m depth. During the experimental period from mid-September to mid-December 2022, sea water temperature in these tanks ranged from 10 to 12°C. Annual temperature fluctuations in incoming water peaked in October (12.5°C) and were at its minimum in March (6.4°C). Salinity varied between 31 and 32 ppt.

Three groups of sea cucumbers were collected for use in the studies: 1) obtained in November 2022 and stored for one week, 2) obtained in September 2022 and stored for 10 weeks, and 3) obtained in September-October 2021 and stored for >1 year (13-14 months) in the flow-through system prior to the experiment. Animals in group 1 and 2 were not supplied with feed other than what the incoming unfiltered seawater provided in form of organic matter. Group 3 on the other hand was fed regularly on a mixture of seaweed powder (*Sargassum thunbergii*) and sand at a ratio (volume:volume) of 50:50 (medium ration according to Sunde and Christophersen, 2023). All experiments were performed with standardized groups of sea cucumbers where one group consisted of three sub-groups (replicates) divided between three perforated plastic trays (L x W x H: 600 x 352 x 96 mm) with lids.

The effect of live-storage period was examined in sea cucumbers kept in a flow-through seawater system (FT). When investigating the effect of temperature, sea cucumber groups were placed in recirculation tanks (Adriatic Sea International Srl, San Clemente, Italy) each containing 300 L of filtered seawater (<1 µm). Three different temperatures, 4, 8 and 12°C (4.15 ± 0.14°C, 8.59 ± 0.37°C, 12.25 ± 0.35°C) were chosen for the trial. The water was circulated in a closed cycle, running through a charcoal filter and a refrigeration unit. No feed was added during the experiment. Water quality was monitored regularly and, if necessary, water was exchanged to ensure safe levels of ammonium, ammonia, nitrate, and nitrite (<0.36 mg NH_4-N L^{-1}, <0.006 mg NH_3 L^{-1}, <0.40 mg NH_3-N L^{-1}, <0.44 mg NH_2-N L^{-1}). Oxygen saturation remained high during the experiment (>9 mg L^{-1}) and the pH ranged from 7.9 to 8.3. An additional set of trays containing the same number of sea cucumbers was kept in the flow-through system as a reference group (11.11 ± 0.74°C). Initial stocking density was 3.25-5.85 kg per m^2 of tray area (4.7 ± 1.03 kg per m^2, n=12) which was considered a low density (14 individuals per tray). The total initial biomass per tank was 3 ± 0.24 kg (n=4). After two weeks (T1) half of the biomass was removed and sampled, the remaining half was taken out two weeks later, after four weeks (T2). Water temperature was logged continuously with a 15 min interval (HOBO TidbiT v2, Onset Computer Corporation, Bourne, MA, USA) in both flow through and recirculation tanks.

Oxygen consumption rates (OCR) were measured in groups of three sea cucumbers at a time. In order to investigate the impact of storage time on oxygen demand, OCR was determined after 1, 3 and 5 weeks (group 1, FT-1), after 1 and 10 weeks (group 2, FT-2) as well as after >1 year (group 3, FT-3) of storage in the flow-through system. The effect of temperature on oxygen consumption during short-term storage was examined in a separate experiment by measuring OCR for groups of 3 animals at the start of the experiment (T0), as well as after two (T1) and four (T2) weeks of storage at the three chosen temperatures (4, 8 and 12°C) in the respective recirculation systems. All measurements were conducted during September, November and December 2022.

Sea cucumbers used for initial respiration measurements were acclimated for at least a week in running seawater (flow-through system) and the technical setup was incorporated into the holding tank of each storage group to minimize disturbance and external influences such as air exposure, temperature change and physical handling. A vertical circular respiration chamber (Ø 190 mm, depth 110 mm, Loligo© Systems AS, Viborg, Denmark) was completely submerged, and filled with *in situ* water from each respective holding tank. Oxygen consumption was measured via intermittent respirometry, according to the method of Vismann and Hagerman (1996), using a computer-controlled setup that recorded oxygen partial pressure and temperature every 300 seconds. Oxygen partial pressure (pO_2) was measured with a temperature, salinity and pressure compensated optical oxygen meter (OXY-4 SMA (G3), PreSens–Precision Sensing GmbH, Regensburg, Germany) via a polymer optical fibre (POF), transferring excitation light to the oxygen sensor spot (SP-PSt3-NAU, detection limit 15 ppb; measurement range of 0 – 100% oxygen in dissolved or gaseous phase) and the sensor response back to the meter. Oxygen was measured contactless through the transparent chamber wall. Decline curves were recorded until pO_2 decreased by at least 10% (but no further than 20%) and three replicable curves with similar regression slopes were plotted, assuming optimal acclimation had occurred. Temperature constancy was ensured throughout the measurements. Oxygen consumption (MO_2) was calculated according to Equation 1:

$$MO_2 = \left(\frac{pO_2}{100}\, x\ \alpha\right)\ x\ \beta\ x\ v\ x\ 3.6$$

where pO_2 is the oxygen partial pressure at 100% atmospheric oxygen saturation (in mm Hg), α is the linear regression slope (in % oxygen per second), β is the oxygen solubility of the water at given temperature and salinity (in µg oxygen per L per mm Hg), v is the volume of water (in litres) in the respiration chamber including the tubing of the shunt, and 3.6 is the conversion factor to obtain oxygen consumption rates (OCR) in mg O_2 h^{-1} (without conversion: µg O_2 s^{-1}). Furthermore, individual oxygen consumption rates (OCR) were standardized to 1 g of dry weight sea cucumber body wall tissue using Equation 2:

$$OCR_s = \left(\frac{W_s}{W_e}\right)\ x\ OCR_e$$

where OCRs is the oxygen consumption rate of a standard-weight animal, W_s is its weight, W_e is the measured weight of the experimental animal and OCR_e is the uncorrected oxygen consumption rate of the experimental animal. OCR values are given in units of either mg oxygen per group per hour or as weight-corrected values of µg oxygen per g body wall dry weight per hour.

Individual biometric data were collected from sea cucumbers at the same sampling points as for respirometry. Body length (mouth to anus) was measured to the nearest 1 mm, using a measuring board, and animal wet weights (WW) to the nearest 0.1 g, using an SKX2202 electronic scale (Ohaus GmbH, Nänikon, Switzerland). Body wall (BW) WW was obtained after dissection from gutted (eviscerated) individuals. A standardized mid-section of the BW (ca. 30% of total WW, 8-22 g) was oven-dried at 103°C for 24-48 h to obtain dry weights (DW), and ash weight (AW) was measured after combustion at 550°C for 4 hours. The condition index (CI) was calculated based on gutted (eviscerated) sea cucumber weights according to Equation 3:

$$CI\ = \left(\frac{DW_{BW}}{WW_{BW}}\right)\ x\ 100$$

Where DW_{BW} is the dry weight of the body wall including muscle bands and mouth parts (in grams) and WW_{BW} is the wet weight of the BW including muscle bands and mouth parts (in grams). The CI is given in percent (%).

Statistical analyses were carried out using either Microsoft Excel 365 (Microsoft Corporation, Seattle, WA, USA) or Jamovi version 2.3 (http://www.jamovi.org). Comparisons between treatment groups for variables that were normally distributed were carried out using either Student's t-test for comparisons between two groups or one-way analysis of variance (ANOVA) for multiple groups. Significant differences ($p<0.05$) were followed by a *post hoc* Tukey's HSD multiple comparison test for multiple groups. For variables that failed tests for normality and homogeneity of variance, corresponding non-parametric tests were applied, either Mann-Whitney U test (two groups) or Kruskal-Wallis (multiple groups).

3 Results

3.1 Effect of live-storage period

Storage time had a significant effect on oxygen consumption rate (OCR) and condition index (CI). Weight specific OCR in animals kept for up to 5 weeks remained at similar levels (Figure 1, FT-1, one-way ANOVA, p=0.593), while OCR measurements after 10 weeks of live-storage showed a significant increase from initially ~31 µg O_2 $h^{-1}g^{-1}$ body wall dry weight (BW DW) to ~55 µg O_2 (Figure 1, FT-2, unequal variances t-test, p=0.005). Sea cucumbers, kept in the flow-through seawater system >1 year, displayed even

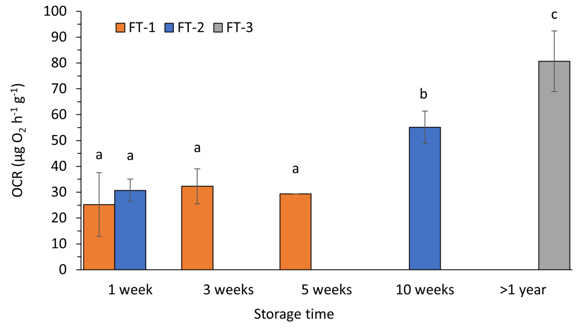

FIGURE 1
Oxygen consumption rates (OCR) per hour and gram body wall dry weight in *P. tremulus* from three sampling groups (1-3) at different live-storage time in a flow-through seawater system (FT). Data are average ± SD of three consecutive measurements per sampling point. Different letters denote significant different values.

TABLE 1 Oxygen consumption rates (OCR) per group of three individuals are average ± SD of three consecutive measurements.

			Individual measurements (n=3)			
Storage period	Temperature (°C)	OCR ($mg\ O_2\ h^{-1}$)	Length (mm)	Total WW (g)	BW WW (g)	BW DW (g)
1 week	11.7 ± 0.1	0.487 ± 0.069	146.1 ± 25.3	97.15 ± 34.47	49.75 ± 14.44	5.28 ± 1.60
	11.7 ± 0.1	0.528 ± 0.258	126.7 ± 15.3	108.35 ± 39.34	67.27 ± 13.38	7.05 ± 1.98
3 weeks	11.2 ± 0.1	0.808 ± 0.168	165.0 ± 65.4	122.69 ± 63.74	70.94 ± 34.61	8.34 ± 4.99
5 weeks	9.7 ± 0.1	0.451 ± 0.000	116.0 ± 21.6	75.64 ± 45.52	47.88 ± 22.61	5.12 ± 2.84
10 weeks	11.6 ± 0.1	0.947 ± 0.108	123.0 ± 33.9	76.10 ± 45.28	54.48 ± 30.18	5.73 ± 3.64
>1 year	11.6 ± 0.1	1.123 ± 0.163	120.7 ± 36.9	85.39 ± 6.83	47.78 ± 3.53	4.64 ± 0.35

Body length, total wet weight (WW), body wall wet weight (BW WW) and body wall dry weight (BW DW) are average ± SD of three individuals. Temperatures are average ± SD during the period of live-storage in flow-through seawater system.

higher OCR of ~81 μg O_2 $h^{-1}g^{-1}$ BW DW (Figure 1, FT-3, unequal variances t-test, p=0.045). Biometric data of individuals used for respirometry are shown in Table 1.

Group OCR (mg O_2 h^{-1} for 3 individuals) showed a similar trend to that of weight specific OCR, with the highest respiration rates in sea cucumbers after medium- and long-term storage in flow-through laboratory tanks (Table 1).

The condition index (CI) showed an opposite pattern to what was found for the OCR with the highest values in FT-1 stored 1-5 weeks (in sea cucumbers (11.0-11.4 ± ~1), compared to a significant lower CI in sea cucumbers stored 10 weeks (10.23 ± 1.03), and >1 year (9.62 ± 0.48) (unequal variances t-test, p ≤0.03) (Figure 2). Although the sea cucumbers obtained in November (group 1) had a higher mean CI compared to animals caught in September (group 2) (11.13 ± 0.87 vs. 10.71 ± 1.03), the difference was not significant between groups 1 and 2 stored for 1 week (equal variances t-test, p = 0.22). No significant change in CI was seen in group 1 during 5 weeks of storage (Figure 2, FT-1, one-way ANOVA, p=0.577), or for group 2 after 10 weeks of storage (equal variances t-test, p = 0.11). The ash content (% AW) related to total wet weight (WW) did not differ significantly between the sea cucumber groups or with storage time (ANOVA, p >0.05).

3.2 Effect of temperature during short-term live storage

Of the three temperatures investigated in the short-term live storage experiment, only measurements conducted at the lowest temperature (4°C) stood out, with significantly different oxygen consumption rate (OCR) and condition index (CI). After 2 weeks of storage (T1), animals kept at 4°C had significantly lower weight specific OCR than animals kept at 12°C (Figure 3, unequal variances t-test, p=0.011). After 4 weeks (T2), this difference became even more distinctive, with significantly higher weight specific OCR measured in individuals kept at both, 8 and 12°C (one-way ANOVA, p<0.001). While oxygen demand at 4°C had almost halved compared to initial measurements at T0, consumption at 8 and 12°C remained unchanged at about 31 μg O_2 $h^{-1}g^{-1}$ dry weight throughout the four-week period. Over the course of time, a general reduction in dispersion was observed for OCR measurements in all temperature treatments (Figure 3, Table 2).

Although there was no significant change in condition index (CI) within temperature treatments (one-way ANOVA, p>0.213) over the course of the four-week experimental period, sea cucumbers kept at 4°C were in a considerable better condition at

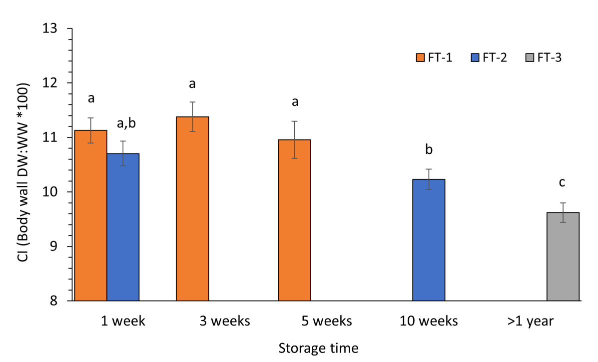

FIGURE 2
Condition index (CI) of *P. tremulus* from three sampling groups (1-3) at different live-storage time in flow-through seawater system (FT). Data are average ± SE (group 1 n=14, group 2 n= 21, group 3 n=7). Different letters denote significant different values.

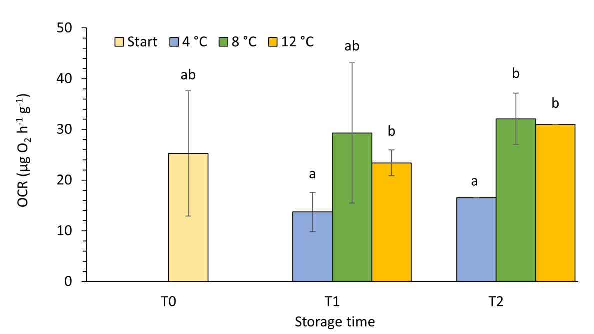

FIGURE 3
Oxygen consumption rates (OCR) per gram body wall dry weight and hour in *P. tremulus* at experimental start (T0), after 2 (T1) and 4 (T2) weeks live-storage at three different temperatures (4, 8 and 12° C). Data are average ± SD of three consecutive measurements per sampling point. Different letters denote significant different values.

TABLE 2 Oxygen consumption rate (OCR) per group of three individuals are average ± SD of three consecutive measurements.

			Individual measurements (n=3)			
Storage period	Temperature (°C)	OCR (mg O_2 h^{-1})	Length (mm)	Total WW (g)	BW WW (g)	BW DW (g)
T0	11.7 ± 0.1	0.487 ± 0.069	146.1 ± 25.3	97.15 ± 34.47	49.75 ± 14.44	5.28 ± 1.60
T1	4.3 ± 0.1	0.316 ± 0.089	170.0 ± 43.6	133.17 ± 13.02	69.12 ± 24.29	7.68 ± 4.04
	8.4 ± 0.4	0.536 ± 0.253	125.7 ± 14.0	87.22 ± 18.94	54.66 ± 12.30	6.10 ± 1.63
	12.1 ± 0.3	0.570 ± 0.062	136.7 ± 15.3	134.77 ± 52.03	73.20 ± 25.99	8.12 ± 3.02
T2	4.2 ± 0.1	0.256 ± 0.000	114.7 ± 10.0	91.42 ± 23.76	42.85 ± 4.12	5.17 ± 0.12
	8.5 ± 0.4	0.520 ± 0.082	131.3 ± 18.8	84.98 ± 39.89	49.23 ± 18.34	5.40 ± 2.51
	12.2 ± 0.3	0.613 ± 0.000	146.7 ± 25.7	127.08 ± 14.72	64.01 ± 7.99	6.59 ± 0.54

Body length, total wet weight (WW), body wall wet weight (BW WW) and body wall dry weight (BW DW) are average ± SD of three individuals. Temperatures are average ± SD during the storage time in flow-through (T0) and in recirculation (T1 and T2) seawater system.

T2 than their conspecifics kept at 8 and 12°C (unequal variances t-test, $p \leq 0.015$) (Figure 4). The condition indices of all three temperature groups did not test significantly different from each other at T1 (one-way ANOVA, p=0.710) and neither did the CI of the 8 and 12°C cohorts at the end of the experimental period at T2 (unequal variances t-test, p=0.46). Nevertheless, there is a trend suggesting an inverse correlation between CI and temperature that becomes more pronounced over time (Figure 4).

4 Discussion

Elevated oxygen consumption rates (OCR) were found in connection with prolonged storage period in the flow-through system but were also associated with higher water temperatures. The highest OCR values measured in this study (~81 μg O_2 h^{-1} g^{-1}) were associated with animals that had been fed regularly and stored for more than a year, but also animals kept without supplementary feed had a significantly higher oxygen demand (~55 μg O_2 h^{-1} g^{-1} vs. ~31 μg O_2 h^{-1} g^{-1}) after 10 weeks of storage when compared with animals just brought in to the lab. While OCR increased with storage period, a progressive decrease in condition index (CI) was observed over time, suggesting a loss of body mass, even for the animals that had been fed. This taken into account, elevated oxygen demand under otherwise identical environmental conditions may be an indicator for cumulative stress. It is however hard to determine whether elevated OCR simply represent an artifact due to the animal's increased sensitivity to acute disturbances, like handling in connection with respiration measurements, or if these are an expression of a more permanently raised metabolic state. There are many factors that may contribute and act as stressors. For instance, immune response to acute thermal stress comes at a higher metabolic cost (Gonzalez-Aravena et al., 2015). Furthermore, it is conceivable that the observed increased oxygen demand in connection with long-term storage, could be connected to undernourishment (sub-optimal nutrition), or starvation as found in other ectotherm species (e.g. Comoglio et al., 2005).

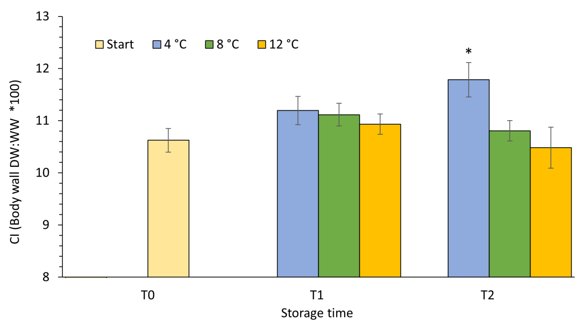

FIGURE 4
Condition index (CI) of *P. tremulus* kept at different temperatures (4, 8 and 12°C). Sampling time at experimental start (T0), and after two (T1) and four (T2) weeks in recirculation system. Data are average ± SE (n=21). Asterisk (*) denotes significant different value (4°C, T2) between temperature groups.

However, potential stressors under artificial environmental conditions in captivity often interact and are very difficult to assess, especially due to the fragmentary knowledge regarding habitat requirements and physiological mechanisms connected to internal processes and biotic and abiotic environmental factors in the wild. Previously conducted feeding experiments in our lab indicate that growth of *P. tremulus* is highest in spring-summer, whereas it is reduced or even negative during autumn-winter months (Christophersen and Sunde, 2022). Conditioning of *P. tremulus* broodstock in captivity has so far also been unsuccessful as rebuilding of the gonads after spawning has not yet been accomplished (Christophersen et al., 2023b), indicating that nutritional requirements of this species have not been met and are still largely unknown.

As sea temperatures rise due to global warming, natural cycles of nutrient availability and temperature could be disturbed and thereby have a negative effect on the fitness of wild stocks of *P. tremulus*. Since temperature may affect sea cucumber feeding behaviour and growth (Yang et al., 2005; Ji et al., 2008) habitat depth may influence oxygen demand between populations. Although most abundant in the deep-sea down to 800 m (Billett, 1988; Kjerstad et al., 2015), *P. tremulus* are also obtained from shallower parts as for example the Oslofjord (60-85 m) in Norway (Jespersen and Lützen, 1971) and the Gullmar Fjord (60 m) on the Swedish south-west coast (Holland, 1981). Since it is documented that individuals also occur at shallow depths up to 18 m (Ludwig, 1900), one could expect *P. tremulus* to be well adapted to a wide range of temperatures. Based on observations from the Gullmar Fjord, Schagerström et al. (2022) speculated that shallow areas (25-35 m) are visited in connection with spawning, potentially adding a seasonal component to the species` general temperature tolerance level. To identify temperature thresholds and the optimal range with respect to seasonal requirements is not just of great importance from a conservational standpoint, but also key for handling, holding, and rearing of a species with future economic potential.

This study showed that short-term storage of *P. tremulus* at a temperature of 4°C had a significant effect on both OCR and CI. After four weeks, sea cucumbers kept at 4°C had a significantly higher CI than individuals kept at 8 or 12°C. These observations are consistent with the fact that physiological processes run slower at lower temperatures, thereby requiring less oxygen. Body reserves are not used up as quickly, and biomass loss is reduced. Since our experiments were conducted outside the natural spawning season in June-July (Christophersen et al., 2020), we can exclude varying gonad and intestinal weight proportions as a contributing factor to differences in CI. An evaluation of the gonad status, conducted at the end of the experiments, found only small and underdeveloped gonads in most individuals. Dissection, performed at all sampling points, further showed that 77% of the experimental animals had intact intestines and respiratory trees. Gonad development, spawning or evisceration as influencing factors can therefore largely be disregarded.

As noted by Hughes et al. (2011), gaining knowledge on physiological rates, especially on a species` oxygen consumption, facilitates the modelling of its contribution to energy and carbon flow in deep-sea food webs (Rowe et al., 2008; Soetaert and van Oevelen, 2009). The measurement of OCR has long been utilized as a practical means to assess an organism's metabolic and organic carbon consumption rate (Schmidt-Nielsen, 1990; Burggren and Roberts, 1991; Brey, 2010). However, gathering such physiological data from deep-water species presents certain challenges. It is important to acknowledge that retrieving deep-sea specimens to the surface may not be entirely without consequences, as their physiological and biochemical systems are sensitive to changes in pressure and temperature (Somero, 1998). On the other hand, echinoderms, unlike other deep-water species, do not exhibit a decrease in OCR with increasing habitat depth; instead, it remains relatively constant (Hughes et al., 2011). The measurements in this study also appear to be generally comparable to OCR measurements obtained *in situ* for three different deep-sea holothuroids from the northeastern Atlantic (Hughes et al., 2011), suggesting that they may also correspond to those occurring in *P. tremulus* in nature. Measurements conducted at 4°C after two weeks (T1) of acclimation in the lab revealed a mean consumption of 0.32 mg O_2 per group of three individuals per hour (mean 133 g wet weight per individual). Hughes et al. (2011) found an *in situ* OCR of 0.93 mg O_2 per individual (130 g wet weight) in *Zygothuria lactea* at 4.2° C, and 0.31 and 0.04 mg O_2 per individ and hour at 2.6°C in *Benthodytes gosarsi* (mean 1149.8 g wet weight) and *Peniagone azorica* (mean 51.3 g wet weight), respectively.

Studies on oxygen consumption in *P. tremulus* measured in the laboratory are almost equally sparse as literature on *in situ* measurements. Fox (1936) might have been the first and one of the few to measure oxygen consumption in *P. tremulus*. He conducted measurements at 6°C and measured individual rates that ranged from 0.7 up to 2.3 µg O_2 h^{-1} g WW^{-1}. The mean OCR of the one individual (130 g wet weight) measured by Fox (1936) was 1.8 µg O_2 h^{-1} g WW^{-1}. Using total wet weight as reference, our converted values translate to average 1.65 µg O_2 h^{-1} g WW^{-1} for individuals kept at 12°C and average 0.86 µg O_2 h^{-1} g WW^{-1} for individuals kept at 4°C. Given the high intraspecific variance that occurs in our data set as well as in data sets gathered by Fox (1936) and Hughes et al. (2011) and which seems to be typical for aquatic and marine ectotherms (Patterson, 1992) – our measurements fit well within the range. Most publications available on physiological rates in holothuroids from the Atlantic report their results in relation to wet weight as standardization reference. To our knowledge, there is only one study where the authors chose to standardize OCR on a dry weight basis, arguing that otherwise any closer comparison of rates becomes impossible due to the high fluctuation of perivisceral fluid (Newell and Courtney, 1965). For the same reason, we decided to calculate OCR standardized on dry weight basis in our study. Newell and Courtney (1965) measured consumption rates of 23.14 to 66.43 µg O_2 h^{-1} g DW^{-1} in adult *Holothuria forskali* at 17°C, compared to our measurements of 15 µg O_2 h^{-1} g DW^{-1} in adult *P. tremulus* at 4°C and 27 µg O_2 h^{-1} g DW^{-1} at 12°C.

While our study only contributes a tiny piece of the puzzle to the overall understanding of *P. tremulus*' physiology, the results may still be useful in various contexts. Notably, knowledge regarding the species' oxygen demand at different temperatures is crucial within any aquaculture setting. Furthermore, these data can be a useful supplement for the modelling of dynamic energy budgets (DEB), where physiological processes of individual organisms, such as ingestion, assimilation, respiration, growth, and reproduction, are linked together in a single framework to assess the species' role in the ecosystem (van der Meer, 2006), among other things. This would also facilitate a better understanding of how climate change may impact these vulnerable yet important components of our benthic ecosystems. However, more data is needed on physiological relationships in *P. tremulus* in general and oxygen consumption in particular, both obtained in the field and laboratory, regarding its optimum range as well as tolerance levels.

Since *P. tremulus* is considered to be of commercial interest for Asian markets (Kjerstad et al., 2015), there has been increased interest in understanding the ecology and biology of the species, in order to investigate whether there is potential to develop a regulated fishery as well as an aquaculture industry in Norway (Landes et al., 2019; Christophersen and Sunde, 2022; Christophersen et al., 2023a). Worldwide, several sea cucumber species are exploited and partly overexploited with global catches exceeding 59 000 tonnes in 2019 (FAO, 2021). There are also examples for the overexploitation of wild sea cucumber species in European waters as for instance in the Mediterranean Sea and the North-East Atlantic Ocean where new target species may be at future risk (González-Wangüemert et al., 2018). The cultivation of sea cucumbers through aquaculture presents a potential solution to alleviate the strain on local wild populations. Furthermore, the inclusion of sea cucumber aquaculture in IMTA systems offers promising prospects for minimizing organic waste generated by other aquaculture practices, such as fish and shrimp farming (Dumalan et al., 2019; Neofitou et al., 2019; Jenzri et al., 2022). As per today, China dominates the global sea cucumber aquaculture industry, with *Apostichopus japonicus* accounting for an overwhelming majority (99.5%) of the production worldwide (FAO, 2021). Still, the demand is high, which increases the pressure on new target species from temperate regions (Purcell et al., 2014). Being able to understand the requirements of *P. tremulus*, will further the optimization of culture conditions and, in the future, the supply of products that are in demand on the international seafood market. The information acquired on oxygen consumption of *P. tremulus* under different conditions provides new knowledge on the biology of a yet understudied species, relevant for both ecosystem management and aquaculture.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The manuscript presents research on animals that do not require ethical approval for their study.

Author contributions

Conceptualization, resources, methodology and project administration, AL and GC. Performance of experimental work and formal analysis, AL, GC and JS. Validation, JS. Writing—review and editing, AL, GC and JS. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Corrigendum: Effect of live-storage period and temperature on oxygen consumption rate in the cold-water sea cucumber *Parastichopus tremulus*

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sea cucumber, Holothuroidea, Parastichopus tremulus, oxygen consumption, live-storage, temperature, condition index

A Corrigendum on

Effect of live-storage period and temperature on oxygen consumption rate in the cold-water sea cucumber *Parastichopus tremulus*

by Landes AM, Sunde J and Christophersen G (2023) *Front. Mar. Sci.* 10:1248840. doi: 10.3389/fmars.2023.1248840

In the published article, there was an error in Figure 1 and Figure 3 as well as in Table 1 and Table 2 as published. The authors discovered a continuous error throughout all calculations used to determine oxygen consumption rate (OCR) in *Parastichopus tremulus*. Instead of using oxygen partial pressure (as required), air pressure was erroneously used. Since air temperature was stable throughout the experiments, oxygen density in air followed the atmospheric pressure and the proportion remained the same throughout all measurements. Therefore, the overall relation between the OCR measured at different times and in different treatments remained the same, the statistical results still applied, and all general findings remained correct. In effect, only the scale (y-axis) had to be corrected in Figures 1 and 3. The corrected Figure 1 and Figure 3 and their captions appear below. In Tables 1 and 2, numerical values given for OCR had to be updated. The corrected Table 1 and Table 2 and their captions appear below.

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

In the published article, there was an error. Due to the continuous calculation error mentioned above, the numerical values of oxygen consumption rates mentioned in the text were incorrect. The text itself remained unchanged.

A correction has been made to **Results**, *3.1 Effect of live-storage period*, Paragraph 1. These two consecutive sentences previously stated:

"Weight specific OCR in animals kept for up to 5 weeks remained at similar levels (Figure 1, FT-1, one-way ANOVA, p=0.593), while OCR measurements after 10 weeks

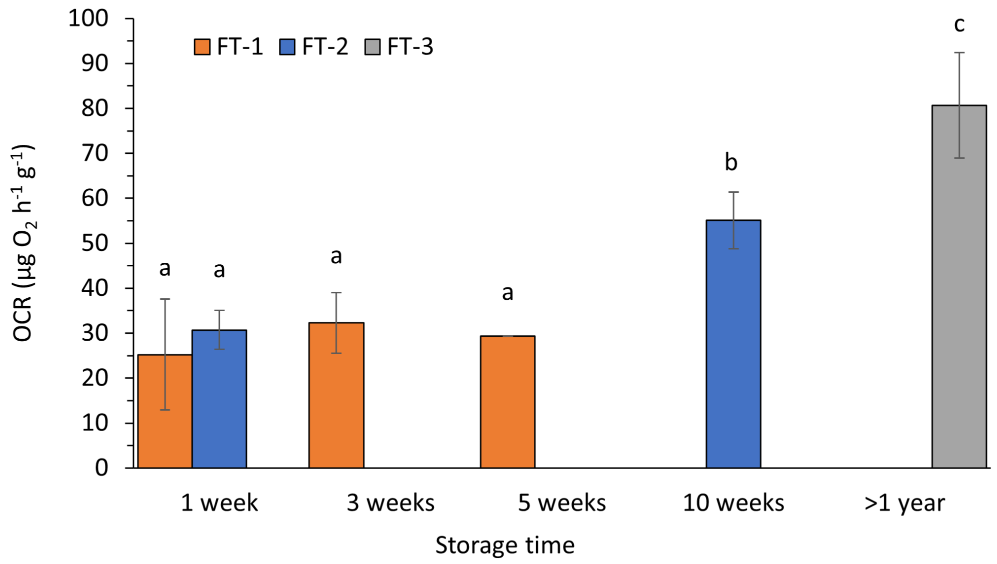

FIGURE 1
Oxygen consumption rates (OCR) per hour and gram body wall dry weight in *P. tremulus* from three sampling groups (1-3) at different live-storage time in a flow-through seawater system (FT). Data are average ± SD of three consecutive measurements per sampling point. Different letters denote significant different values.

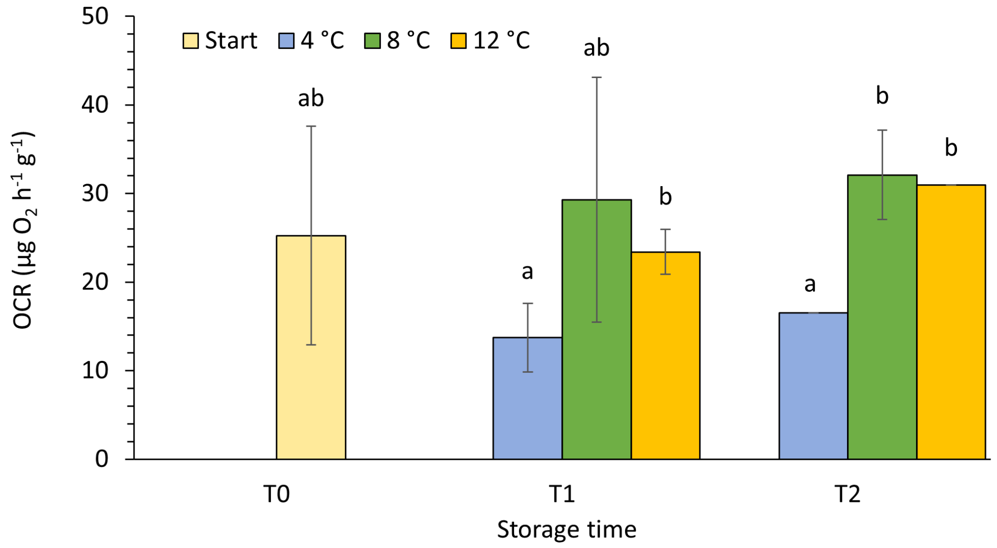

FIGURE 3
Oxygen consumption rates (OCR) per gram body wall dry weight and hour in *P. tremulus* at experimental start (T0), after 2 (T1) and 4 (T2) weeks live-storage at three different temperatures (4, 8 and 12°C). Data are average ± SD of three consecutive measurements per sampling point. Different letters denote significant different values.

of live-storage showed a significant increase from initially ~150 µg O_2 $h^{-1}g^{-1}$ body wall dry weight (BW DW) to ~270 µg O_2 (Figure 1, FT-2, unequal variances t-test, p=0.005). Sea cucumbers, kept in the flow-through seawater system >1 year, displayed even higher OCR of ~390 µg O_2 $h^{-1}g^{-1}$ BW DW (Figure 1, FT-3, unequal variances t-test, p=0.045)."

The two corrected consecutive sentences appear below:

"Weight specific OCR in animals kept for up to 5 weeks remained at similar levels (Figure 1, FT-1, one-way ANOVA, p=0.593), while OCR measurements after 10 weeks of live-storage showed a significant increase from initially ~31 µg O_2 $h^{-1}g^{-1}$ body wall dry weight (BW DW) to ~55 µg O_2 (Figure 1, FT-2, unequal variances t-test, p=0.005). Sea cucumbers, kept in the flow-through seawater system >1 year, displayed even higher OCR of ~81 µg O_2 $h^{-1}g^{-1}$ BW DW (Figure 1, FT-3, unequal variances t-test, p=0.045)."

Another correction has been made to **Results**, *3.2 Effect of temperature during short-term live-storage*, Paragraph 1. The original sentences previously stated:

TABLE 1 Oxygen consumption rates (OCR) per group of three individuals are average ± SD of three consecutive measurements.

			Individual measurements (n=3)			
Storage period	Temperature (°C)	OCR (mg O_2 h^{-1})	Length (mm)	Total WW (g)	BW WW (g)	BW DW (g)
1 week	11.7 ± 0.1	0.487 ± 0.069	146.1 ± 25.3	97.15 ± 34.47	49.75 ± 14.44	5.28 ± 1.60
	11.7 ± 0.1	0.528 ± 0.258	126.7 ± 15.3	108.35 ± 39.34	67.27 ± 13.38	7.05 ± 1.98
3 weeks	11.2 ± 0.1	0.808 ± 0.168	165.0 ± 65.4	122.69 ± 63.74	70.94 ± 34.61	8.34 ± 4.99
5 weeks	9.7 ± 0.1	0.451 ± 0.000	116.0 ± 21.6	75.64 ± 45.52	47.88 ± 22.61	5.12 ± 2.84
10 weeks	11.6 ± 0.1	0.947 ± 0.108	123.0 ± 33.9	76.10 ± 45.28	54.48 ± 30.18	5.73 ± 3.64
>1 year	11.6 ± 0.1	1.123 ± 0.163	120.7 ± 36.9	85.39 ± 6.83	47.78 ± 3.53	4.64 ± 0.35

Body length, total wet weight (WW), body wall wet weight (BW WW) and body wall dry weight (BW DW) are average ± SD of three individuals. Temperatures are average ± SD during the period of live-storage in flow-through seawater system.

TABLE 2 Oxygen consumption rate (OCR) per group of three individuals are average ± SD of three consecutive measurements.

			Individual measurements (n=3)			
Storage period	Temperature (°C)	OCR (mg O_2 h^{-1})	Length (mm)	Total WW (g)	BW WW (g)	BW DW (g)
T0	11.7 ± 0.1	0.487 ± 0.069	146.1 ± 25.3	97.15 ± 34.47	49.75 ± 14.44	5.28 ± 1.60
T1	4.3 ± 0.1	0.316 ± 0.089	170.0 ± 43.6	133.17 ± 13.02	69.12 ± 24.29	7.68 ± 4.04
	8.4 ± 0.4	0.536 ± 0.253	125.7 ± 14.0	87.22 ± 18.94	54.66 ± 12.30	6.10 ± 1.63
	12.1 ± 0.3	0.570 ± 0.062	136.7 ± 15.3	134.77 ± 52.03	73.20 ± 25.99	8.12 ± 3.02
T2	4.2 ± 0.1	0.256 ± 0.000	114.7 ± 10.0	91.42 ± 23.76	42.85 ± 4.12	5.17 ± 0.12
	8.5 ± 0.4	0.520 ± 0.082	131.3 ± 18.8	84.98 ± 39.89	49.23 ± 18.34	5.40 ± 2.51
	12.2 ± 0.3	0.613 ± 0.000	146.7 ± 25.7	127.08 ± 14.72	64.01 ± 7.99	6.59 ± 0.54

Body length, total wet weight (WW), body wall wet weight (BW WW) and body wall dry weight (BW DW) are average ± SD of three individuals. Temperatures are average ± SD during the storage time in flow-through (T0) and in recirculation (T1 and T2) seawater system.

"While oxygen demand at 4°C had almost halved compared to initial measurements at T0, consumption at 8 and 12°C remained unchanged at about 150 µg O_2 $h^{-1}g^{-1}$ dry weight throughout the four-week period."

The corrected sentence appears below:

"While oxygen demand at 4°C had almost halved compared to initial measurements at T0, consumption at 8 and 12°C remained unchanged at about 31 µg O_2 $h^{-1}g^{-1}$ dry weight throughout the four-week period."

A correction has been made to **Discussion**, Paragraph 1.

The sentence previously stated:

"The highest OCR values measured in this study (~390 µg O_2 $g^{-1}h^{-1}$) were associated with animals that had been fed regularly and stored for more than a year, but also animals kept without supplementary feed had a significantly higher oxygen demand (~270 µg O_2 $g^{-1}h^{-1}$ vs. ~150 µg O_2 $g^{-1}h^{-1}$) after 10 weeks of storage when compared with animals just brought in to the lab."

The corrected sentence appears below:

"The highest OCR values measured in this study (~81 µg O_2 $h^{-1}g^{-1}$) were associated with animals that had been fed regularly and stored for more than a year, but also animals kept without supplementary feed had a significantly higher oxygen demand (~55 µg O_2 h^{-1} g^{-1} vs. ~31 µg O_2 $h^{-1}g^{-1}$) after 10 weeks of storage when compared with animals just brought in to the lab."

A correction has been made to **Discussion**, Paragraph 5.

The sentence previously stated:

"Measurements conducted at 4°C after two weeks (T1) of acclimation in the lab revealed a mean consumption of 1.54 mg O_2 per group of three individuals per hour (mean 133 g wet weight per individual)."

The corrected sentence appears below:

"Measurements conducted at 4°C after two weeks (T1) of acclimation in the lab revealed a mean consumption of 0.32 mg O_2 per group of three individuals per hour (mean 133 g wet weight per individual)."

A correction has been made to **Discussion**, Paragraph 6.

These two consecutive sentences previously stated:

"He conducted measurements at 6°C and measured individual rates that ranged from 130 up to 427 µg O_2 $h^{-1}g$ WW^{-1}. The mean OCR of the one individual (130 g wet weight) measured by Fox (1936) was 334 µg O_2 $h^{-1}g$ WW^{-1}. Using total wet weight as reference, our converted values translate to average 790 µg O_2 $h^{-1}g$ WW^{-1} for individuals kept at 12°C and average 513 µg O_2 $h^{-1}g$ WW^{-1} for individuals kept at 4°C."

The two corrected consecutive sentences appear below:

“He conducted measurements at 6°C and measured individual rates that ranged from 0.7 up to 2.3 µg O_2 $h^{-1}g$ WW^{-1}. The mean OCR of the one individual (130 g wet weight) measured by Fox (1936) was 1.8 µg O_2 $h^{-1}g$ WW^{-1}. Using total wet weight as reference, our converted values translate to average 1.65 µg O_2 $h^{-1}g$ WW^{-1} for individuals kept at 12°C and average 0.86 µg O_2 $h^{-1}g$ WW^{-1} for individuals kept at 4°C.”

The sentence previously stated:

“Newell and Courtney (1965) measured consumption rates of 23.14 to 66.43 µg O_2 $h^{-1}g$ DW^{-1} in adult *Holothuria forskali* at 17°C, compared to our measurements of 67 µg O_2 $h^{-1}g$ DW^{-1} in adult *P. tremulus* at 4°C and 156 µg O_2 $h^{-1}g$ DW^{-1}at 12°C.”

The corrected sentence appears below:

“Newell and Courtney (1965) measured consumption rates of 23.14 to 66.43 µg O_2 $h^{-1}g$ DW^{-1} in adult *Holothuria forskali* at 17°C, compared to our measurements of 15 µg O_2 $h^{-1}g$ DW^{-1} in adult *P. tremulus* at 4°C and 27 µg O_2 $h^{-1}g$ DW^{-1} at 12°C.”

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

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Estimating age and growth parameters for three commercial NE-Atlantic sea cucumbers, *Holothuria mammata*, *H. forskali* and *H. arguinensis*, in a marine protected area

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Sea cucumbers, integral components of benthic ecosystems, have become subjects of scientific scrutiny owing to their intricate morphology and ecological importance. Due to increasing demand, several species of these echinoderms have become overexploited. As a consequence, NE-Atlantic species became new targets for the international markets. There is a vital need for comprehensive biological data to establish and enhance holothurian fisheries management. In the absence of such data, there is a risk of ineffective fisheries regulations, particularly for susceptible commercial species in the NE-Atlantic, which could lead to overexploitation. Establishing effective fisheries regulations requires a foundation of fundamental biological information, such as growth rates from target populations. This work aims to determine the growth parameters for three commercial sea cucumber species from the NE-Atlantic, in a marine protected area, *Holothuria mammata*, *H. forskali* and *H. arguinensis*. This presents a challenge, as sea cucumbers lack significant calcified structures for age determination, and assessing size is complicated because of their body size plasticity. Thus, a von Bertalanffy model was fitted to length-frequency data using the ELEFAN method with the simulated annealing procedure. Underwater length measurements of the three sea cucumber species, in a relaxed state, were systematically recorded over a span of 18 months, with measurements taken every 1.5 months. The results provide the growth parameters of each species. Models considering seasonal growth were better fits to the data. The three species had different growth rates and periods of no growth coincided with the reproductive season. Mortality was lower for the species growing slower. This is crucial information to support decision-making processes regarding stock management, such as setting limits to fisheries considering stock condition associated with environmental variability.

KEYWORDS

Holothuroidea, ELEFAN von Bertalanffy, seasonal growth model, length frequency, age at length

1 Introduction

The ecological role of sea cucumbers has long been described as vital for marine ecosystems. Through their feeding behaviour, holothurians help recycle nutrients, stimulate microalgae growth, mix the upper sediment layers and improve physicochemical processes of benthic habitats (Uthicke, 1999; MacTavish et al., 2012; Purcell et al., 2016). However, the high economic value of sea cucumbers driven by an ever-increasing demand, has led to an overexploitation of several sea cucumber species by the majority of fisheries worldwide (Purcell et al., 2013; González-Wangüemert et al., 2016). As a result of local stock depletion and closure of some regional and national fisheries (Purcell, 2014), the exploitation of natural stocks expanded to other areas endangering additional species (Purcell et al., 2013; Eriksson and Clarke, 2015). In the North-eastern Atlantic and the Mediterranean Sea, several sea cucumber species, new to the international trade markets, are now targeted for directed fisheries (González-Wangüemert et al., 2018; Vafidis and Antoniadou, 2023). The absence of time-series data on landings, biometry, and the density of natural holothurian stocks presents a significant obstacle to assess the population's health. However, in some regions, a decrease in density, abundance, genetic diversity, loss of the largest size classes and even local stock depletion are evidence of overexploitation due to fishing pressure (some occurring illegally; González-Wangüemert et al., 2014; González-Wangüemert et al., 2018). *Holothuria* (*Holothuria*) *mammata* Grube, 1840 and *Holothuria* (*Holothuria*) *arguinensis* Koehler and Vaney 1906 are commercially relevant and targeted species in the in the NE-Atlantic and Mediterranean (González-Wangüemert et al., 2014; González-Wangüemert et al., 2018; Dereli and Aydın, 2021; Vafidis and Antoniadou, 2023), while *Holothuria (Panningothuria) forskali* Delle Chiaje, 1824 has seen an increasing interest (González-Wangüemert et al., 2016) with values going for over $ 100 USD *per* dry kg on the market (Sousa et al., 2020). Illegal captures of sea cucumbers, mainly *H. arguinensis* and *H. mammata*, have increased for the past years with populations showing local signs of overexploitation (González-Wangüemert et al., 2018).

For these species, there is a lack of knowledge on their ecology and biology which could ultimately lead to misguided or unsound decision-making (Lovatelli et al., 2004; Purcell et al., 2010). Individual growth is hard to estimate since sea cucumbers' length and weight vary and are easily influenced by manipulation, due to physiological responses to environmental *stimuli* (e.g. muscle contraction and release of viscera or water). Length measurements should be standardized to avoid measurement errors and this is more easily achieved either by measuring individuals underwater, avoiding any contact, or by taking post-*mortem* measurements (Azevedo e Silva et al., 2021). Individual recognition techniques such as tagging, are usually shed by holothurians (Conand, 1981; Shelley, 1982) and have only been relatively successful in suspension feeders (Gianasi et al., 2015). Determining growth through the identification of annual growth rings in calcified structures has recently been described for sea cucumbers and this method could facilitate the determination of growth rates for holothuroids (Sun et al., 2019).

Generally, modelling approaches have provided successful growth estimations, through growth parameters, for sea cucumber populations. Understanding growth parameters is crucial not only for the preservation and control of a particular species, but also for mitigating the impact of fishing on natural populations. This knowledge enables us to establish fishery regulations grounded in biological information. A von Bertalanffy model applied with the ELEFAN_SA (simulated annealing optimization) procedure allows for an unconstrained search of parameters within the multi-dimensional space. This is an advantage since there is no need for an *a priori* fixing of parameters during search routines (Mildenberger et al., 2017), which could lead to biased results. Still, ELEFAN requires users to provide search conditions for each parameter, as well as length-class size and the number of length-class for calculation of the moving average (Zhou et al., 2022). This procedure is an analysis toolbox compiling single-species stock assessment methods specifically designed for data-limited fisheries analysis using length-frequency data (Mildenberger et al., 2017), which is an advantage.

Growth parameters of a population allow us to understand its populational dynamics, which will fundamentally lead to a better management of natural stocks. Estimating growth parameters is an important step that, not only guarantees correct assumptions for stock management, but also leads to a better understanding of the species ecology and biology. The aim of this study was to determine

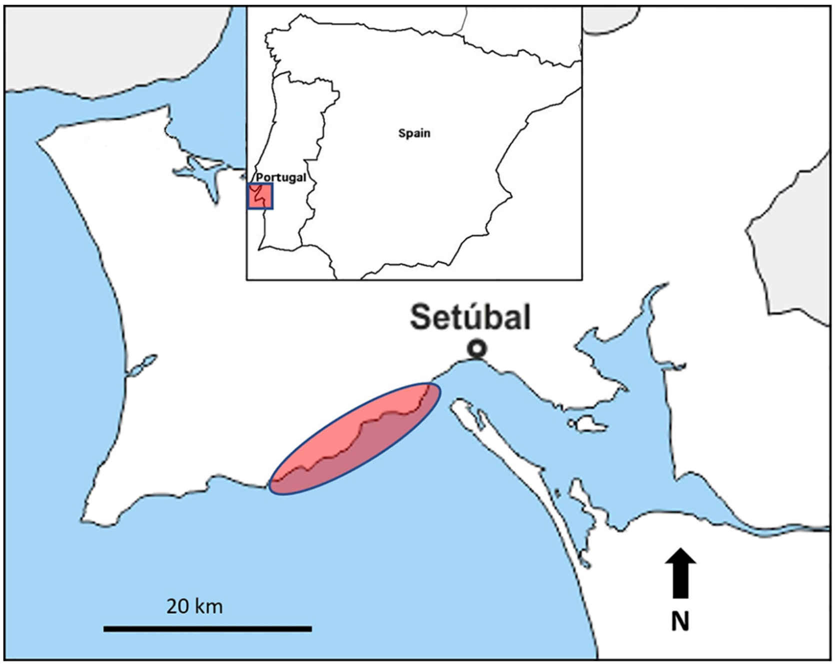

FIGURE 1
Map of the study region, with indication of the sampled area (in red).

the growth parameters and natural mortality through length-frequency data for the three commercial NE-Atlantic sea cucumbers, *H. forskali*, *H. mammata* and *H. arguinensis*, in a rocky reef habitat, filling this knowledge gap. This will allow us to understand these species different growth strategies, providing crucial information that will support decision-making policies regarding stock management and the conservation of these ecologically important species.

2 Methods

2.1 Study area

The study was focused on a NE-Atlantic coastal area in the southwest of Portugal, within a marine protected area (MPA), the Arrábida Marine Park (38°26'50.4"N; 9°01'58.7"W; Figure 1), with regulation that bans most fishing activities (none of which susceptible of capturing sea cucumbers) and restricts human presence. Most places are in fact inaccessible unless with maritime transportation, which is also forbidden in some areas. Although fisheries are mostly banned in this MPA and local marine authorities display a regular surveillance for illegal activities, sea cucumbers are not yet a fishing target in this region. So, at the moment, these are unexploited populations. Different habitats compose this MPA, but it is mostly dominated by rocky reefs that end in a sandy floor (Gonçalves et al., 2002; Félix et al., 2021). For these reasons, this area allows the study of sea cucumbers' population parameters in the most pristine-like conditions possible in the Portuguese coastline.

2.2 Sampling

Starting in February 2018 and until March 2019, a visual census sampling was done every one and a half months, in 30 m x 3 m transects, to record length measurements (to the nearest 0.1mm) of the three local sea cucumber species with commercial interest (*H. forskali*, *H. mammata* and *H. arguinensis*). Sampling was done through scuba diving, in depths ranging from two to ten meters, depending on the depth of the sampling site. Since at least *H. arguinensis* and *H. mammata* have displacement behaviours between adjacent habitats (Félix et al., 2021; Azevedo e Silva et al., 2023), sampling was conducted considering full heterogeneity of habitats, in order to ensure the distribution of the species in the region was covered and to guarantee the growth functions would be adequate for the population of the three species. Length of sea cucumbers is variable and easily influenced by manipulation, so length measurements were carefully done underwater with a measuring tape by a team of two divers, where one measures the sea cucumber and the other registers the species and length. The diver measuring length remained the same, ensuring there were no errors attributable to user input. The measurements were carried out in sea cucumbers in a relaxed state, avoiding any handling related stress and following the proposed standardized methodology for comparability (Azevedo e Silva et al., 2021).

2.3 Data analysis

All length data obtained from sampling were grouped in different size classes according to the species analysed. *H.*

arguinensis was grouped in three cm classes, *H. mammata* in two cm classes and *H. forskali* was grouped in one cm classes. While smaller bin sizes can provide more information, the sample size for *H. arguinensis* was only 177, resulting in modes with only 1 individual in several months when the bin size was two cm. Therefore, to avoid fitting the model to several modes with only 1 individual, a larger bin size was selected.

All analyses were conducted using the statistical computing software "R" (version 3.5.1). Non-seasonal and seasonal von Bertalanffy Growth Models (VBGF) were fitted to the restructured length frequency data with the ELEFAN (Electronic Length Frequency Analysis) procedure (Pauly, 1987) from the TropFishR package (Mildenberger et al., 2017). Data is restructured through a process that emphasizes the cohorts through peaks and troughs in the data set, calculating a count score for each length bin based on count deviations as compared to the selected Moving Average - MA (Pauly et al., 1992).

The non-seasonal VBGF (Equation 1) is described by the following function (Von Bertalanffy, 1938):

$$L_t = L_\infty\left(1 - \mathrm{e}^{-K(t-t_0)}\right) \tag{1}$$

where: L_t is length at age; L_∞ is the asymptotic length or the mean length that the species of a given population would reach if they were to grow forever; K is the rate at which asymptotic size is approached; t_0 is the age at which length is zero. Instead of the parameter t_0, ELEFAN uses a yearly repeating temporal starting point for each cohort's growth curve (time of the year when length of cohort equals zero), which TropFishR returns as "anchor time" (t_{anchor}). This value ranges between 0 and 1.

The seasonal VBGF (2) was modelled with a *Hoening* function (Somers, 1988), described by the following:

$$L_t = L_\infty\left(1 - \mathrm{e}^{-\left(k(t-t_0)-\left(c\frac{k}{2\pi}\right)\sin 2\pi(t-t_s)+\left(c\frac{k}{2\pi}\right)\sin 2\pi(t_0-t_s)\right)}\right) \tag{2}$$

where, the new parameter C is the relative amplitude of the seasonal oscillations and t_s is the phase of the seasonal oscillations denoting the time of year corresponding to the start of the convex segment of sinusoidal oscillation (when growth turns positive). The *Hoening* function includes seasonality in growth, determining the amplitude of the effect of the season in growth and the beginning of the seasonal oscillation (Pauly et al., 1992).

The time of the year when the growth rate is slowest, known as the winter point (WP), was calculated according to Equation 3:

$$\mathrm{WP} = \mathrm{ts} + 0.5 \tag{3}$$

Growth performance (Equation 4) comparisons were made using the growth performance index (Φ') described by the following function (Pauly and Munro, 1984):

$$\phi' = \log(k) + 2\log(L_\infty) \tag{4}$$

The R package TropFishR (Mildenberger et al., 2017) performs the restructuring procedure within the ELEFAN functions. The VBGF models were fitted with the "ELEFAN_SA" optimization procedure, a method based on simulated annealing that offers significant improvements to the estimation of the VBGF parameters while also searching over all parameters simultaneously (Mildenberger et al., 2017; Taylor and Mildenberger, 2017). The algorithm searches for the best fit through a series of iterations (consecutive curve fitting attempts) and allows for unconstrained search for optimum combinations of growth parameters, including seasonality (Schwamborn et al., 2019).

Moving Average (MA) was set based on three different scenarios for both *H. forskali* and H. *mammata*. First scenario considers a general rule of thumb where an MA setting that approximates the number of bins spanning the width of the smallest mode, which should reflect the youngest cohort following recruitment to the fishery (Taylor and Mildenberger, 2017). Second setting is the value immediately bellow. The third setting was the value immediately above. For *H. arguinensis*, since in the first scenario MA = 3, the other two scenarios were adapted to MA = 5 and MA = 7. Different scenarios were tested since the MA settings can significantly affect the scoring of the growth curve (Taylor and Mildenberger, 2017). The remaining settings for the functions were chosen, ensuring the widest possible search for overall maximum and a large number of iterations (SA temp = 6e5;maxit = 1000; SA_time =2 min), to ensure full convergence of iterations towards one best fit value (Schwamborn et al., 2018; Schwamborn et al., 2019). The search space used during fit optimization was constant for all species with t_{anchor} = 0-1, K = 0.1-2, C = 0-1 and t_s = 0-1, varying only in the L_∞ parameter, where the search space was defined by the bounds L_{max} * 0.8 and L_{max} * 1.2 (where L_{max} is the maximum length registered for the species throughout the entire sampling period). Then local maxima were analysed in order to overcome the possibility of the curve fitting algorithms being captured by local maxima (Schwamborn et al., 2019). Fitting scores (Rn) were recorded for comparison, estimated according to Equation 5:

$$R_n = \frac{10^{ESP/ASP}}{10} \tag{5}$$

where the Estimated Sum of Peaks (ESP) is the sum of peak values crossed by the growth curves, with the caveat that positively crossed bins are only counted once, while negatively crossed bins are counted every time they are encountered (Pauly, 1985). The Available Sum of Peaks (ASP) is the sum of all positive peaks, which represents a maximum possible score (if negative bins are crossed).

Natural mortality (M) was estimated with the two VBGF parameter (K, L_∞) based on the method from Then et al. (2015) [33] with the TropFishR package (Mildenberger et al., 2017). Age-at-length was back calculated from length using the seasonal estimated VBGF function, according to Equation 2 (Ramírez-González et al., 2020) and finally, mean monthly growth rate was calculated as averages over the previous 12 months for age groups (Wilhelm et al., 2017; Ahmed et al., 2020).

3 Results

A total of 2019 *H. mammata*, 3590 *H. forskali* and 177 *H. arguinensis* were sampled between February 2018 and March 2019,

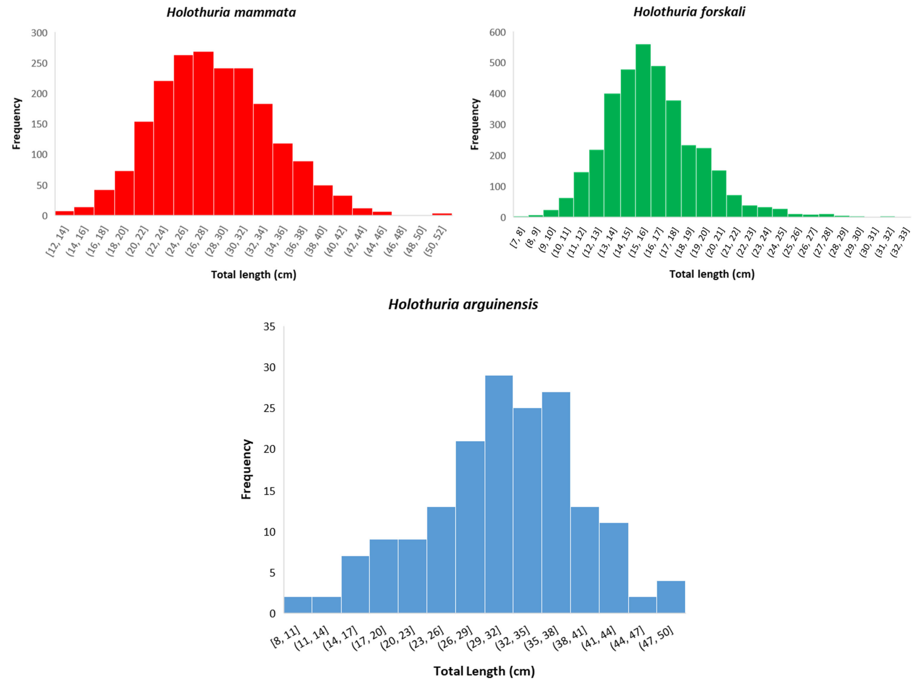

FIGURE 2
Length class distribution of *Holothuria mammata*, *Holothuria forskali* and *Holothuria arguinensis* sampled in the Arrábida coastline, SW of Portugal from February 2018 to March 2019.

all included in the VBGF models. The three species presented unimodal length distribution where the total length ranged from 11.9 to 51 cm for *Holothuria mammata*, 7 to 33 cm for *Holothuria forskali* and 8 to 49 cm for *Holothuria arguinensis* (Figure 2).

The different Moving Averages resulted in different model fitting scores (R_n) for the three species, where models selected were the ones with the highest fit and reasonable parameters (Table 1). Non-seasonal models presented worse fits to the data than seasonal models and this difference was more evident for *Holothuria forskali*.

Among the models the one with a MA width of 5 bins had the highest Rn for *H. mammata* and *H. arguinensis*, while for *H. forskali*, the MA that generated the best fit was 9. *H. forskali* revealed strong seasonal oscillation effect (C = 0.994) while this factor was lower for *H. mammata* and *H. arguinensis* (0.5999 and 0.7790, respectively; Table 1). The natural mortality rate (M) was higher for *H. forskali*.

Both *H. mammata* and *H. arguinensis* models show a good overall fit as reflected by the intersection of growth curves with positively scored bins (Figure 3) and the higher Rn scores, while for *H. forskali* this is only evident for younger cohorts.

The Winter point (WP) was 0.6 (July) for *H. mammata*, 0.17 (February) for *H. forskali* and 0.35 for *H. arguinensis* (April; Figure 4). Growth coefficient (K) was the lowest for *H. mammata*

TABLE 1 Parameters for the seasonal VBGF models fitted with the simulated annealing optimization procedure.

VBGF models								
Description	L∞	K	t_anchor	C	Ts	ϕ'	Rn	M
NS : HM, MA=5	53.471	0.227	0.472	–	–	2.812	0.730	–
NS : HF, MA=9	36.133	0.688	0.191	–	–	2.953	0.304	–
NS : HA, MA=7	53.153	0.674	0.774	–	–	3.280	0.460	–
S:HM, MA=5	54.967	0.209	0.311	0.600	0.100	2.801	0.748	0.350
S:HF, MA=9	34.339	0.659	0.231	0.994	0.671	2.891	0.397	0.946
S:HA, MA=5	53.029	0.354	0.816	0.779	0.849	2.998	0.519	0.520

NS, Non-seasonal; S, Seasonal. HM, Holothuria mammata; HF, Holothuria forskali; HA, Holothuria arguinensis. MA, Moving Average; L∞, Asymptotic length (cm); K, Growth rate; t_anchor, time of the year when length of cohort equals zero (in a scale from 0 to 1); C, Seasonality factor; Ts, Time of the year when growth accelerates (in a scale from 0 to 1); ϕ', Growth performance index; - Rn, Fitting score; M, Natural mortality (year^{-1}).

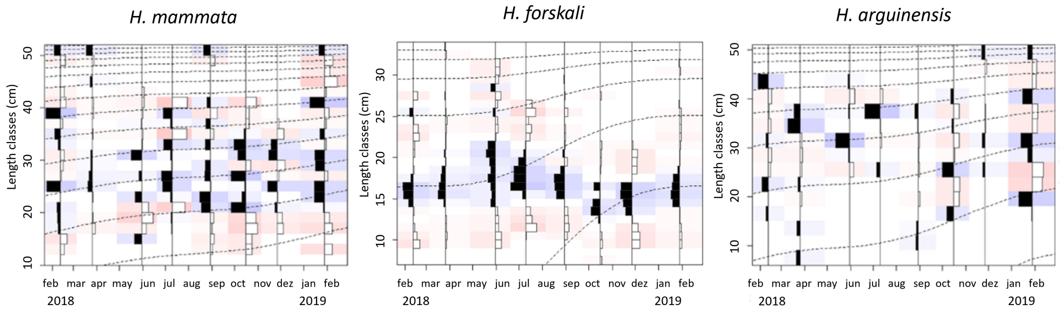

FIGURE 3
Age cohorts (dashed lines) estimated by the VBGF models run with the optimized simulated annealing ELEFAN procedure, fitted to the length-frequency data of *Holothuria mammata*, *Holothuria arguinensis* and *Holothuria forskali*. Black and white bars represent positively and negatively scored bins, respectively. Moving average settings considered were 5 for *H. mammata* and *H. arguinensis* and 9 for *H. forskali*.

and higher for *H. forskali* and *H. arguinensis* and this is notable on the monthly mean growth rate, where *H. forskali* reaches asymptotic length faster.

4 Discussion

The studied region allowed the study of unexploited sea cucumber populations, which proved advantageous in accurately estimating natural growth parameters. A common occurrence in natural populations under severe fishing pressure is the absence of large individuals (Pauly et al., 1998). This leads to an underestimation of L_∞ (Schwamborn et al., 2018) and an overestimation of K, due to the known interaction between the growth constant K and L∞. Consequently, an overestimation of K leads to an underestimation of a population's ability to recover from fishing pressure (Schwamborn et al., 2019). The growth parameters provided by this study are therefore considered to be essential for these species since they are closer to a pristine condition.

To the best of our knowledge there are no reported growth parameters for *H. mammata*. The population of *H. mammata* in Arrábida's coast is the healthiest found up to this moment (Félix et al., 2021), showing the largest individuals and the highest densities so far. It also has unimodal size-class distributions (Félix et al., 2021), as opposed to what has been reported by other studies (González-Wangüemert et al., 2014; Aydin and Erkan, 2015; González-Wangüemert et al., 2018). This is a reflection of good environmental conditions, successful recruitment and an unexploited population (Félix et al., 2021). Mortality rate of *H. mammata* was the lowest between the three species from this study. However, its growth rate is also the lowest. *H. mammata* is usually present in rocky reefs, hiding in crevices, and they either need to move to sandy patches to feed or they present diel feeding behaviour (Félix et al., 2021). These behaviours are size dependent and despite increasing survival, which is an adaptation against predation or disturbances from hydrodynamics, they represent a limitation to feeding opportunities and consequently, to the growth rate. This is an indication of a trade-off between growth and survival, as suggested for temperate species (Morgan, 2012), highlighting that while this species exhibits a lower mortality rate, representative of enhanced survival mechanisms, its growth rate is comparatively lower. The trade-off concept suggests that the allocation of resources to enhance survival, such as increased adaptations against predation or disturbances from hydrodynamics, may come at a cost to growth opportunities.

Holothuria forskali is an understudied species with few studies focusing on its biology and ecology (e.g. Tuwo and Conand, 1992; Santos et al., 2017; David et al., 2020; Azevedo e Silva et al., 2021; Ballesteros et al., 2021; Félix et al., 2024). To the best of our knowledge, this is the first study to report growth parameters for this species. *H. forskali* is the species with the highest growth rate between the three species, indicating that its asymptotic length is achieved faster. Considering this is the only species of the three with a defensive mechanism against predators (Cuvierian tubules; Muller et al., 1970; Azevedo e Silva et al., 2021), our findings indicate an evolutionary strategy in *H. forskali*, suggesting accelerated growth during the early stages: Given the reduced predation risk of this species, this growth pattern is inferred as an adaptive response to mitigate higher environmental stress conditions, since larger individuals would be more likely to remain stable in the substrate (Olaya-Restrepo et al., 2018). In fact, *H. forskali* is the more resilient species of all three to environmental stressors like hydrodynamics (Félix et al., 2024). However, it should be noted that K is relative to L∞ so a higher K does not necessarily mean that it is growing faster in absolute terms. In fact, according to the monthly growth rates, *H. forskali* and *H. arguinensis* seem to grow at around the same rate for the first year, after which *H. forskali*'s growth declines abruptly. Apart from having the highest K between the three species of this study, mortality rate of *H. forskali* is also the highest. In temperate coastal regions, where food may be seasonally abundant, the trade-off between achieving fast growth and reducing mortality explains a higher mortality in faster growing sea cucumbers (Hammond, 1982; Sonnenholzner, 2003; Morgan, 2012). Mortality could be due to predation of lower size class individuals or their inability to deal with physical stressors. A faster growth rate could be considered as an adaptive advantage since faster growth may have positive implications on mortality for some species (Ahmed et al., 2020). The models fit particularly well for the younger cohorts, however, this is not evident for older cohorts. The sample size is big enough to be considered representative, but it could be difficult to

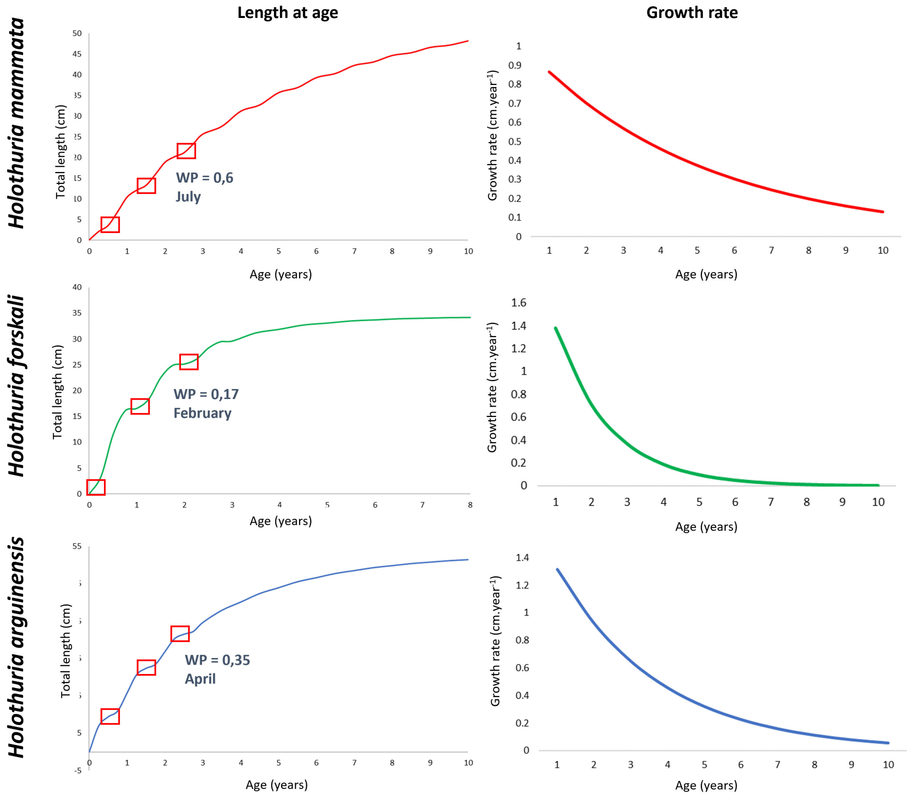

FIGURE 4
Length-at-age for seasonally oscillating Von Bertalanffy growth curve depicting Winter Point (WP) and Monthly mean (12 months) growth rate for *Holothuria mammata, Holothuria forskali* and *Holothuria arguinensis*.

distinguish between the older, bigger cohorts. Since *H. forskali* eviscerates the Cuvierian tubules as a defensive mechanism, regenerating them comes at an energy cost (Vandenspiegel et al., 2000). The release of Cuvierian tubules could be a relevant factor contributing to the variability in growth rates. It can also be hypothesized that, while regenerating the Cuvierian tubules, this species may become more vulnerable to predation. Furthermore, in this region, *H. forskali*, shows evidence of two consecutive yearly reproductive peaks, which may have an added impact on somatic growth (unpublished data from the project at seacucumber.eu/en/projecto).

H. arguinensis presented a higher growth rate (K = 0.35 and L∞ = 53.03) than what was already reported for the Atlantic coast of Morocco (K = 0.17 and L∞ = 25.96; 0.29 and L∞ = 25.44; Haddi et al., 2021), but revealed a much slower growth rate than the populations at Ria Formosa, south of Portugal (K = 0.88 and L∞ = 69.90; Olaya-Restrepo et al., 2018). It is noteworthy, however, that the study for the Atlantic coast of Morocco used a recalculated length, based on both length and width, and this should be borne in mind when making a comparison. In the present study area, this species occurs exclusively on subtidal habitats. Nutrient rich subtidal environments provide more stable conditions for sea cucumber populations (Félix et al., 2021), particularly for *H. arguinensis* (Azevedo e Silva et al., 2023), but nutrient increases are seasonal and very dependent on upwelling events (Wooster et al., 1976). Moreover, faster growth rates in sea cucumbers under high stress conditions and in disturbed environments may help reducing mortality in younger holothurians (Morgan, 2012), so this could be considered as an adaptative advantage. In fact, the growth rate reported in that study (Olaya-Restrepo et al., 2018) is among the highest reported for sea cucumbers, even when considering tropical sea cucumbers and this could be linked to the high disturbance of the habitats coupled with the productivity of that ecosystem (Brito et al., 2009; Olaya-Restrepo et al., 2018). Nevertheless, the high fishing pressure in Ria Formosa showed already a significant reduction of the larger size classes (González-Wangüemert et al., 2018), which may have an influence in the estimation of the growth rate, increasing it. The combination of all factors may add up to justify the unusually high growth rate of *H. arguinensis* in Ria Formosa.

Assuming high growth rates and its advantages on the sustainability of the stocks can lead to neglecting a conservative management of the species and to a depletion of the stocks. This oversight could potentially lead to a slow, decade-long recovery, as pointed out for *Holothuria nobilis* (Uthicke et al., 2004). Slower growth rates have fundamental implications on the health of populations, primarily under high fishing pressure. It is important to ensure a highly conservative management for this species, guaranteeing an efficient monitoring of the populations. Even though Ria Formosa is a protected area, easy access to sea cucumbers facilitates illegal harvesting and implementation of restrictive measures should be reinforced.

Seasonal VBGF functions revealed a better fit to the data for the three species than non-seasonal VBGF functions. These results confirm seasonality in the growth of *H. mammata*, *H. forskali* and *H. arguinensis*, with seasonality being more evident in *H. forskali* species. In fact, *H. forskali* reveals a period of no growth during winter (February), while *H. mammata* and *H. arguinensis* have slower growth during early summer (June-July) and spring (April), respectively. Seasonal growth has been described for most temperate sea cucumber species (Morgan, 2012; Olaya-Restrepo et al., 2018), where annual cycles may limit food availability, drive reproductive behaviour and increase metabolism, leading to a fluctuation of body weight (Morgan, 2012) and consequently, body length (Azevedo e Silva et al., 2021). Indeed, *H. forskali*'s period of no growth coincides with the reported reproductive period for this species (Tuwo and Conand, 1992; Santos et al., 2017; unpublished data from the project at seacucumber.eu/en/projecto) and *H. mammata* and *H. arguinensis*' slower growth periods also coincide with their reported reproductive periods (Marquet et al., 2017; Venâncio et al., 2022). The energy input into reproduction during breeding season (Poot-Salazar et al., 2014; Olaya-Restrepo et al., 2018) is consistent with our results that reveal growth during this period is slower or halted. Moreover, considering that sea cucumbers invest more energy into reproduction than growth (Ramírez-González et al., 2020), it should also be highlighted that most relative growth of the three species occurs during somatic growth, when sea cucumbers are not yet adult or ready to reproduce.

In this region, the productivity increases in the summer (Wooster et al., 1976; Costa et al., 2013), which coincides with the period when *H. forskali's* growth increases. *H. forskali* presents a more selective feeding behaviour than the other two species, foraging on hard surfaces and selectively choosing food items, mostly composed of algae (Azevedo e Silva et al., 2021). Therefore, *H. forskali* may be more susceptible to seasonality that influences food quantity and quality. Temperature has been known to be a factor regulating the reproductive cycle of holothurians (Ahmed et al., 2020) and increase metabolism. In the study area, the temperature began increasing in January, peaking in October, but being generally higher during summer months (Félix et al., 2021; Venâncio et al., 2022). A higher temperature only seems to impact positively the growth of *H. forskali* as it likely increases its metabolism and positively affects food supply. On the other hand, it seems to negatively affect the growth of *H. arguinensis* and *H. mammata*. Since temperature seems to offset reproductive events for both these species (Marquet et al., 2017; Venâncio et al., 2022) this relationship between growth and temperature is explained by an allocation of resources into reproduction, a phenomenon described for sea cucumbers (Ru et al., 2018; Ahmed et al., 2020).

In the future, validating length-at-age model results with age determined from growth rings (Sun et al., 2019) will lead to more confidence in the models and more robust results. Growth studies provide valuable information that can help implement and improve management measures. However, studied populations should be monitored since a change in growth parameters could be the result of a change in the health of a population. The implications that incorrect assumptions can have on models are greatly enhanced by management decisions based on inflated growth estimates. The population traits of the species in the present study, in conjunction with the important ecological role of sea cucumbers, emphasize the need for conservative regulatory measures.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements because there was no impact on the animals studied. Sea cucumbers were measured with a tape underwater without handling. All permits required were obtained from the national entity.

Author contributions

FA: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Software, Visualization, Writing – original draft, Writing – review & editing. AB: Formal analysis, Funding acquisition, Project administration, Supervision, Visualization, Writing – review & editing. TS: Investigation, Methodology, Resources, Validation, Writing – review & editing. AP: Formal analysis, Funding acquisition, Investigation, Resources, Supervision, Validation, Visualization, Writing – review & editing. JS: Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – review & editing. EV: Investigation, Methodology, Visualization, Writing – review & editing. PF: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Transcriptome analysis of purple and green *Apostichopus japonicus* reared under different breeding environments

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Sea cucumber (*Apostichopus japonicus*) is one of the cultured species with the highest production value to a single species in China's marine agricultural industry with high nutritional and medicinal value. Body color influences numerous biological functions in sea cucumbers, and it has become a key trait in selective breeding. It is found that a cultivation environment with strong light intensity leads to a lighter body color of *A. japonicus* compared to dark conditions. However, little research has been conducted on the molecular mechanisms regulating body color in different breeding environments. In this study, we used Illumina sequencing to examine gene expression patterns in green and purple *A. japonicus* exposed to different culture conditions. Overall, the genes in the body wall of purple individuals were more affected by the environment. We also identified pathways that were potentially influenced by the breeding environment, such as "Drug metabolism - cytochrome P450", "Porphyrin and chlorophyll metabolism", "Phosphatidylinositol signaling system", "TGF-beta signaling", and "mTOR signaling". The aim of this study was to determine the light conditions and breeding environment that are more favorable to the requirements of body color in *A. japonicus* and apply them to the selection of high-quality breeds. The results obtained will support the breeding of *A. japonicus* with specific traits and the production of seedlings to promote the development of this maricultural industry.

KEYWORDS

***Apostichopus japonicus*, transcriptome, body color, breeding environment, differentially expressed genes**

1 Introduction

Factors associated with the breeding environment, such as light, water temperature, water depth, salinity, dissolved oxygen, pH, and culture cycle, significantly affect sea cucumbers (Jiang et al., 2016). Light in particular is essential for various biological activities and is an important environmental factor for the survival and proliferation of sea cucumbers (Yaguchi and Yaguchi, 2021). Moreover, light intensity, as an important environmental factor for the growth of aquatic organisms, can directly or indirectly affect animal feeding, phototaxis, clustering and diurnal activity rhythms (Li et al., 2020). Light intensity (lx) refers to the luminous flux of visible light received per unit area. It has been shown that *Tridacna crocea*, which is characterized by blue outer coat membranes, exhibits different color changes at different light intensities, with the color variation produced at 15,000 lx. being more significant than that at 5,000 lx and 10,000 lx. (Liu et al., 2021). Graham and Hutchison (1979) investigated diurnal activity rhythms in three freshwater turtle species (*Chrysemys picta*, *Clemmys guttata*, and *Sternotherus odoratus*) under different light cycles and showed that they became diurnal under long photoperiods (16L:8D) and nocturnal under short photoperiods (8L:16D). Luo et al. (2022) showed that shortening the photoperiod reduced the magnitude of circadian activity rhythms in water snakes. Animals in nature exhibit circadian rhythms that are related not only to their organism, but also to the photoperiod in the breeding environment. Overall, light is an important factor influencing circadian rhythms, and many freshwater and marine species are exposed to optimal light conditions.

Light is an important factor in the development, survival, growth, and reproduction of animals. Numerous studies, conducted both in China and internationally, have investigated the responses of fish, shrimp, and sea cucumber larvae in light fields as well as their characteristics and body color (Wurts and Stickney, 1984; Primavera and Caballero, 1992; Wang et al., 2003; Moller and Naylor, 2009). However, only a few studies have been conducted on the influence of different light environments on body color formation in sea cucumbers, and they mostly consisted in exposing individuals to different artificially simulated light conditions to observe variations in their body color. Some investigations have shown that light affects body color in fish; for example, light was revealed as one of the most important causes of induced whitening in *Paralichthys olivaceus* (Guan et al., 2008; Wang et al., 2008a; Wang et al., 2008b; Wang, 2017). In *Siniperca scherzeri*, body color change was also shown to be related to light: the stronger the light intensity, the darker the black of the body surface, but when light intensity was weak, the black color faded and yellow emerged, and under dark conditions, both black and yellow disappeared (Wang, 2003; Miao et al., 2013). Black-eyed goldfish reared in dark conditions was shown to exhibit the slowest growth rate along with a non-obvious body color change, while they grew fastest and had significant body color change under natural light conditions (Wang et al., 2002). The body color of *A. japonicus* was shown to be directly dependent on the growing environment and to be specifically affected by light intensity and duration of light exposure in different regions and seasons (Xue et al., 2007; Jiang, 2011; Wang et al., 2012; Wang et al., 2016). At present, sea cucumbers inhabiting natural environments are mainly distributed in dark or light reef sides or other types of cover. In contrast, indoor reared environments are mainly dominated by less intense light or dark conditions. However, there is no literature to clarify the optimal light intensity for sea cucumber. Throughout evolution, sea cucumbers have developed a series of adaptive characteristics to variations in light. Studying the response mechanism of these organisms to light is of great scientific value for further research on breeding.

The key genes regulating the response of *A. japonicus* to light have not yet been examined. In the present study, we carried out the transcriptome analysis of RNA obtained from the body wall tissues of green and purple *A. japonicus* cultured under different breeding environments, and analyzed and verified the differentially expressed genes. The aim was to determine which breeding conditions are more favorable to the growth and development of *A. japonicus* and changes in body color so that they can be applied in the selection of high-quality breeds. The results of this study to the selection and breeding of good varieties of *A. japonicus* will contribute to promoting the development of the *A. japonicus* industry.

2 Materials and methods

2.1 Experimental animals and tissue collection

The experiment was carried out in the Rushan Branch of Shandong Orient Ocean Co., Ltd., using a total of four *A. japonicus* specimens with different colors. Two of them, one green (G) and one purple (P), were obtained from dark conditions and cultured on pellet feed mixed with seaweed and sea mud, while the other two, one light green (LG) and one light purple (LP), were obtained from natural environment. and fed on fermented pellet feed for sea cucumbers during the culture period. The experimental body wall was frozen in liquid nitrogen and stored at −80°C.

2.2 RNA extraction, cDNA preparation, and illumina sequencing

Total RNA was extracted using the Trizol kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. RNA quality was assessed on an Agilent 2100 bioanalyzer (Agilent, Palo Alto, CA, USA) and verified via agarose gel electrophoresis without RNase. After extraction, eukaryotic mRNA was enriched by Oligo (dT) magnetic beads, while prokaryotic mRNA was enriched by removing rRNA using the Ribo ZeroTM magnetic kit (Epicentre, Madison, WI, USA). The enriched mRNA was then broken into short fragments using fragmentation buffer and reverse transcribed into cDNA using random primers. cDNA was extracted using DNA polymerase I, RNase H, dNTPs, and buffer. It was then used for second-strand cDNA synthesis. The cDNA fragments were purified and end-repaired, and poly(A) was added. Then, the fragments with lengths ranging from 250 to 300 bp were screened using AMPure XP beads, PCR amplified, and the PCR products were purified again using the above-mentioned beads (NEBNex® Ultra^{TM} RNA Library Prep Kit for Illumina®). Finally, the fragments were sequenced using an Illumina NovaSeq 6000 platform, and the raw reads were deposited into the Sequence Read Archive (SRA) database at the National Center for Biotechnology Information (NCBI) (TaxID: 307972).

2.3 Mapping reads

The raw data is further filtered in order to obtain high quality clean reads. In addition, to ensure the quality and reliability of the data analysis, the reads with adapters, N (which indicated that the base information could not be determined), and low quality (reads with Qphred ≤ 20 bases accounting for more than 50% of the entire read length) were removed from the raw data. At the same time, Q20, Q30, and GC contents were calculated for the clean data. Then, the reference genome was obtained and the paired-end clean reads were mapped to it using HISAT2 v2.0.5.

2.4 Identification of differentially expressed genes and their functional annotation

Based on the results of principal component analysis (PCA), the 12 samples were divided into four groups (i.e., P, LP, G, and LG). Then, differential expression analysis was performed for P vs LP, G vs LG, and P vs G to identify DEGs between different colored *A. japonicus*. Genes with adjusted a value of P <0.05 found by DESeq2 were assigned as differentially expressed. Genes with a false discovery rate (FDR) ≤ 0.001 and a fold change ≥ 2.0 or ≤ 0.5 in pairwise comparisons were considered as DEGs and were functionally annotated in the NCBI Non-redundant protein (NR), Kyoto Encyclopedia of Genes and Genomes (KEGG), Swissprot, the protein family database (Pfam), Gene Ontology (GO), EuKaryotic Orthologous Groups (KOG), and Clusters of Orthologous Groups of proteins (COG) databases.

2.5 Real-time PCR validation

To validate the RNA-sequencing results, nine genes were randomly selected for real-time PCR. Primers were designed for optimal performance using primer 5 (Supplementary Table S3). Using the Prime Script^{TM} RT Reagent Kit with genomic DNA Eraser (Takara), the RNA was reverse transcribed into single-stranded cDNA and adopted as a template for qRT-PCR. Actin was used as the reference gene. Supplementary Table S3 lists the primers (AJ-GEN F and AJ-GEN R) and actin primers (actin F and actin R) for the gene of *A. japonicus*. The mRNA expression levels of differently colored *A. japonicus* individuals were assessed using the SYBR® Premix ExTaq^{TM} (Takara) kit. The reaction system was set up according to the instructions, with pre-denaturation at 95°C for 5 seconds, followed by 40 denaturation cycles at 95°C for 10 seconds each, denaturation at 60°C for 20 seconds, and extension at 72°C for 30 seconds. Finally, the melting curve was added. The expression level of mRNA was measured using the $2^{-\Delta\Delta Ct}$ method.

2.6 Statistical analysis

The results of the experiments were expressed as mean ± standard deviation (SD). Data were analyzed using one-way analysis of variance (ANOVA) with multiple comparisons for significant differences in gene expression between groups, and the normal distribution of raw data was assessed via T-test. Statistical analyses were performed using GraphPad Prism version 8.0. P values < 0.05 were considered significant. Genes with P ≤ 0.05 and an absolute fold change of ≥ 2 were considered DEGs.

3 Results

3.1 Transcriptome assembly and annotation

To understand the transcriptional regulation of different colors in *A. japonicus*, the three samples examined for each group, i.e., LP, P, G, and LG, were subjected to RNA sequencing (RNA-seq). A total of 12 cDNA libraries were constructed in this study. To ensure data quality, the raw data were filtered before information analysis to reduce the interference of invalid data. First, fastp (Chen et al., 2018) was used to perform quality control on the raw reads and filter low-quality data to obtain clean reads (Supplementary Table S1). RNA-seq generated 40, 141, 262-47, 613, and 400 high-quality clean reads. Then, the clean reads were compared to the ribosomal database for the species examined using the short read comparison tool HISAT2. The reads mapped to ribosomes in the comparison groups without allowing mismatches were removed and the unmapped reads were retained for subsequent transcriptome analysis. A reference genome-based comparison analysis was carried out in HISAT2. In total, 28, 534, 702-35, 058, 298 reads (67.9–76.98% of clean reads) were mapped to the *A. japonicus* genome (Supplementary Table S2).

3.2 Identification of DEGs

To reveal the effect of breeding conditions on the body color of *A. japonicus*, the samples of LG and LP individuals grown in the

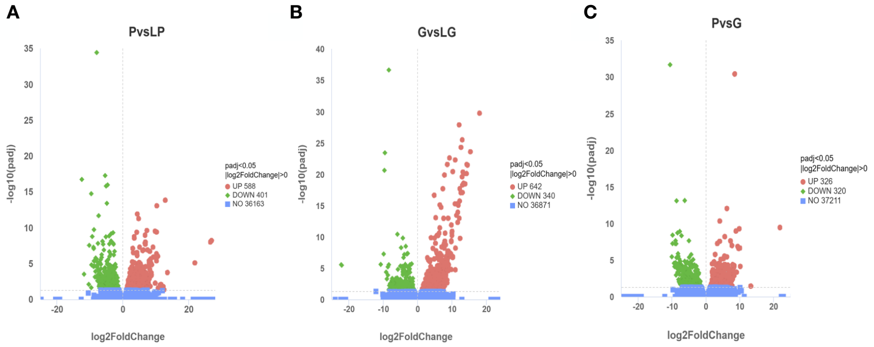

FIGURE 1
Identification of differentially expressed genes (DEGs) in the body wall of *A. japonicus* after exposure to different breeding conditions. **(A–C)** represent the volcano plots of DEGs in P vs LP, G vs LG, and P vs G, respectively. The red and green dots indicate upregulated and downregulated genes, respectively. P, purple; LP, light purple; G, green; and LG, light green.

natural environment and those of G and P individuals cultured under artificial breeding conditions were subjected to DEG analysis ($P < 0.05$). Overall, 989, 982, and 646 DEGs were detected in P vs LP, G vs LG, and P vs G, respectively. A total of 588 upregulated genes and 401 downregulated genes were differentially expressed between P and LP, and a total of 642 upregulated genes and 340 downregulated genes were differentially expressed between G and LG. In addition, 326 upregulated genes and 320 downregulated genes were differentially expressed between P and G individuals (Figure 1). To verify the expression of the identified DEGs, nine

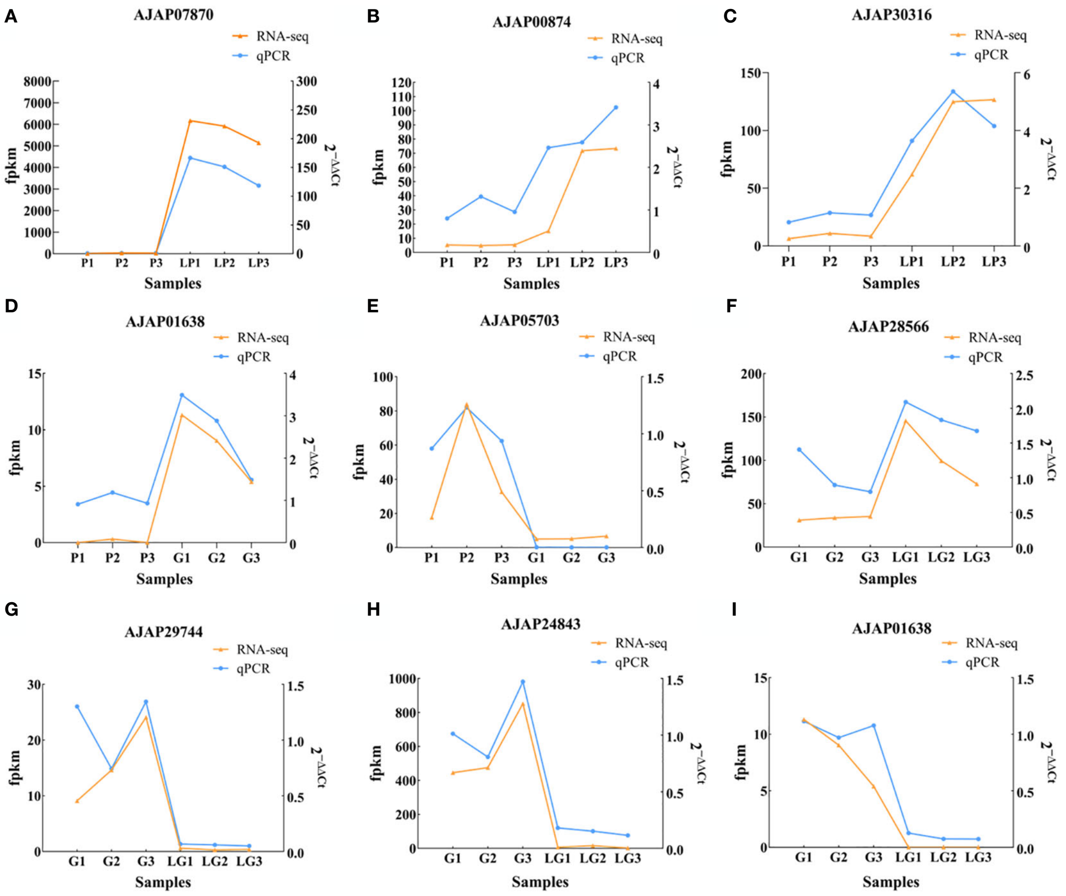

FIGURE 2
Graphs illustrating the differences in the relative expression of nine selected DEGs. **(A–C)** Differential expression of *AJAP07870*, *AJAP00874*, and *AJAP30316* in the P vs LP group; **(D, E)** Differential expression of *AJAP01638* and *AJAP05703* in the P vs G group; **(F–I)** Differential expression of *AJAP28566*, *AJAP29744*, *AJAP24843*, and *AJAP01638* in the G vs LG group.

TABLE 1 Selection of DEGs in purple (P) and light purple (LP) *A. japonicus* for RNA-seq and RT-PCR analysis.

Gene ID	P vs LP			
	Exp	RNA-seq	qRT-PCR	Same Trend
AJAP07870	↓	-7.9713	-7.19****	Y
AJAP00874	↓	-3.37254	-1.48**	Y
AJAP30316	↓	-3.61494	-1.78***	Y

Genes with the same trend (up or down regulation) as shown by both RNA-seq and RT-PCR analysis are indicated by “Y” (yes). **, ***, **** indicate P < 0.01. Exp, expression trend; “↑” upregulated expression; “↓” downregulated expression.

TABLE 2 Selection of DEGs in green (G) and light green (LG) *A. japonicus* for RNA-seq and RT-PCR analysis.

Gene ID	G vs LG			
	Exp	RNA-seq	qRT-PCR	Same Trend
AJAP28566	↓	-1.66193	-0.89**	Y
AJAP29744	↑	5.20066	4.03**	Y
AJAP24843	↑	6.202915	2.77**	Y
AJAP01638	↑	8.41421	1.74****	Y

Genes with the same trend (up or down regulation) as shown by both RNA-seq and RT-PCR analysis are indicated by “Y” (yes). **, **** indicate P < 0.01. Exp, expression trend; “↑” upregulated expression; “↓” downregulated expression.

TABLE 3 Selection of DEGs in purple (P) and green (G) *A. japonicus* for RNA-seq and RT-PCR analysis.

Gene ID	P vs G			
	Exp	RNA-seq	qRT-PCR	Same Trend
AJAP01638	↓	-6.30039	-1.07*	Y
AJAP05703	↑	2.993407	8.55****	Y

Genes with the same trend (up or down regulation) as shown by both RNA-seq and RT-PCR analysis are indicated by “Y” (yes). * P < 0.05, **** P < 0.01. Exp, expression trend; “↑” upregulated expression; “↓” downregulated expression.

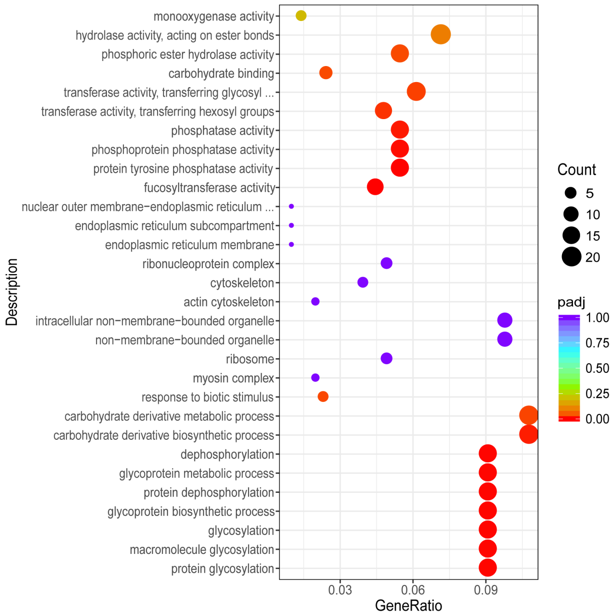

FIGURE 3
Scatterplot illustrating the GO enrichment of DEGs in P vs LP (P, purple; LP, light purple).

genes were selected for qRT-PCR. The RNA-seq results reported in Figure 2 are well in line with the qRT-PCR results (Tables 1–3).

3.3 Functional enrichment of DEGs

To further understand the function of DEGs, GO term and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed. After GO annotation, the DEGs in P vs LP were shown to be significantly enriched with 18 GO terms, including 10 biological processes and 8 molecular functions (Figure 3). The three most significantly enriched functional categories were "fucosyltransferase activity", "protein tyrosine phosphatase activity", and "protein glycosylation". The DEGs in G vs LG were significantly enriched with 12 GO terms, including 2 biological processes and 10 cellular components (Figure 4). The three most significantly enriched functional categories were "tetrapyrrole binding", "antioxidant activity", and "peroxidase activity". The DEGs in G vs P were significantly enriched in 241 GO terms, including 114 biological processes, 13 cellular components, and 114 molecular functions. However, the GO terms in these two groups were not significantly enriched in DEGs (Figure 5).

To further investigate their functionality, these DEGs were all mapped to the KEGG database. A hypergeometric test with a P value of 0.05 was used as the criterion for pathway detection. After the mapping, the 989 DEGs in the P vs LP group were divided into 54 pathways, of which two were significantly enriched (Table 4). The 982 DEGs in the G vs LG group were divided into 58 pathways, of which two (Table 5) were significantly enriched ($P < 0.05$). The 646 DEGs in the P vs G group were divided into 32 pathways, but none of them were significantly enriched (Figure 6). Two significantly enriched pathways were detected in P vs LP, i.e., "Drug metabolism - cytochrome P450" and "Arachidonic acid metabolism" and in G vs LG, i.e., "Oxidative phosphorylation" and "Arachidonic acid metabolism".

To further understand the molecular mechanism of body color and growth in green and purple *A. japonicus* cultured in different breeding environments, the following enrichment pathways were selected for subsequent analysis: "Phosphatidylinositol signaling system", "Wnt signaling", "Drug metabolism - cytochrome P450", "Porphyrin and chlorophyll", "TGF-beta signaling", and "mTOR signaling" (Tables 6–8).

4 Discussion

4.1 "Wnt signaling", "drug metabolism - cytochrome P450", and "Porphyrin and chlorophyll metabolism"

Pigment cells develop from the neural crest in the embryo and play an important role in the production of body color in animals (Streelman et al., 2007). However, in some species, these cells can rapidly change color by changing the position of pigments and reorienting reflective structures (Yu, 1996). The mechanisms of color change vary among species. For example, *Chamaeleonidae* where shown to use cell signals to change color (Stuart-Fox et al., 2007). *Misumena vatia* (Thomisidae) body color variation is related to the formation and location of ocular pigment granules (Insausti and Casas, 2008). Wnt signaling plays an important role in many developmental processes, including neural crest-derived melanocyte development (Cho et al., 2009). This pathway was shown to promote the differentiation of neural crest cells to melanocytes in mice (Dunn et al., 2000), while its inhibition was shown to reduce pigment cells in zebrafish (Dorsky et al., 1998). The family of macrophage migration inhibitory factors (e.g., FMO) are ancient and

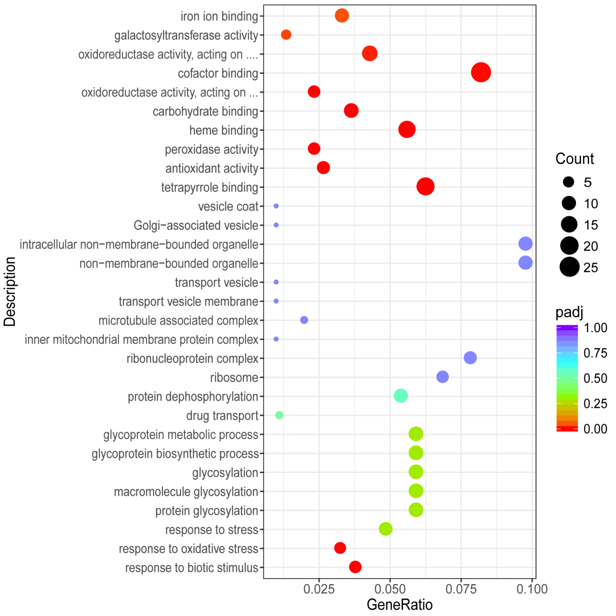

FIGURE 4
Scatterplot illustrating the GO enrichment of DEGs in G vs LG (G, green; LG, light green).

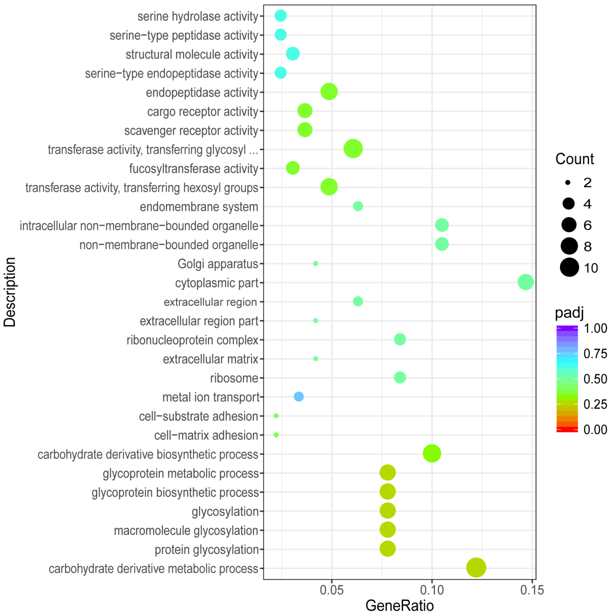

FIGURE 5
Scatterplot illustrating the GO enrichment of DEGs in P vs G (P, purple; G, green).

have been identified in bacteria, plants, and animals (Huang et al., 2016). These genes encode an inflammatory cytokine with reciprocal isomerase activity (Lubetsky et al., 2002; Calestani et al., 2003; Wessel et al., 2020; Spurrell et al., 2023). Dimethylaniline monooxygenase [N-oxide-forming] 2 (FMO2) (Bickel, 1971) is a mitochondria-specific factor involved in pigment cell expression but not in pigment biosynthesis. UDP-glucuronosyltransferase is an intracellular membrane protein that catalyzes the transfer of glucuronide from UDP glucuronic acid to lipophilic receptors. Glucuronidation plays an integral role in the metabolism of various endogenous substances, such as bilirubin and steroid hormones (Mackenzie, 1986; Mackenzie and Rodbourn, 1990; Stuart-Fox et al., 2007; Mroz et al., 2018). In the present study, UDP-glucuronosyltransferase in G vs LG was upregulated in the “Drug metabolism - cytochrome P450” and “Porphyrin and chlorophyll metabolism” pathways, while in P vs LP and G vs LG it was downregulated in the “Drug metabolism - cytochrome P450” pathway. FMO2 in both P vs LP and G vs LG was downregulated in “Drug metabolism - cytochrome P450”. These results indicate that both the upregulation of UDP-glucuronosyltransferase and the downregulation of FMO2 may lead to a decrease in the pigmentation of sea cucumbers in natural farming environments. These findings are more conducive to the light conditions and breeding environments required by the body color of the *A. japonicus*, and are applied to the selection of high-quality varieties.

4.2 “Phosphatidylinositol signaling system”, “TGF-beta signaling pathway”, and “mTOR signaling pathway”

The epidermal cells of sea cucumbers secrete mucus, and the epithelium serves to protect the body. Myc proteins (c-Myc, L-Myc,

TABLE 4 Enriched DEG pathways in P vs LP (P, purple; LP, light purple).

Pathway ID	Pathway	DEGs with pathway annotation (44)	All genes with pathway annotation (3166)	P-value
spu00982	Drug metabolism - cytochrome P450	5 (11.36%)	55 (1.74%)	6.53×10^{-4}
spu00590	Arachidonic acid metabolism	4 (9.09%)	36 (1.14%)	3.67×10^{-2}

TABLE 5 Enriched DEG pathways in G vs LG (G, green; LG, light green).

Pathway ID	Pathway	DEGs with pathway annotation (67)	All genes with pathway annotation (3183)	P-value
spu00190	Oxidative phosphorylation	8(11.94%)	93(2.92%)	6.21×10^{-4}
spu00590	Arachidonic acid metabolism	5(7.46%)	35(1.10%)	7.08×10^{-4}

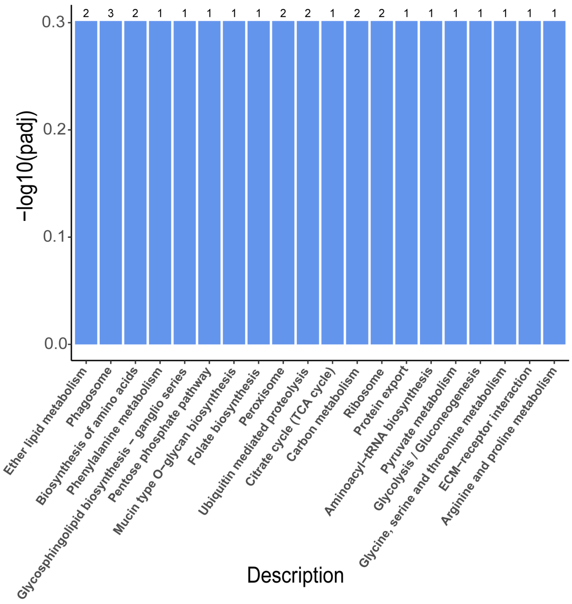

FIGURE 6
Histogram illustrating the KEGG enrichment of DEGs in P vs G. (P, purple; G, green).

TABLE 6 Selected DEGs between purple (P) and light purple (LP) *A. japonicus* and associated enriched pathways.

Gene id	Nr/ Swissprot description	Ratio (P vs LP)	Probability
Drug metabolism - cytochrome P450			
AJAP26268	UDP-glucoronosyl and UDP-glucosyl transferase	6.971938	0.977931
AJAP30316	flavin-containing monooxygenase 2 (FMO2)	-3.62494	0.999286
AJAP02070	sigma class glutathione-s-transferase 2	-2.95374	0.999001
AJAP14503	glutathione S-transferase-like	-2.64661	0.989788
AJAP00070	GST_pi-like protein	-3.59377	0.954734
Porphyrin and chlorophyll metabolism			
AJAP29651	Ceruloplasmin; Ferroxidase	5.032656	0.99999
AJAP26268	UDP-glucuronosyltransferase 2B7	6.971938	0.977931
TGF-beta signaling pathway			
AJAP04605	repulsive guidance molecule A isoform X3	-1.98093	0.96745
Phosphatidylinositol signaling system			
novel.3376		-2.26693	0.950922
Wnt signaling pathway			
novel.2754	Cell migration-inducing gene 5 protein	5.532099	0.999976

S-Myc, and N-Myc) are a class of transcription factors that regulate growth and cell cycle entry by inducing the expression of genes required for these processes (Zhu and Thompson, 2019). The mTOR protein kinase belongs to a major eukaryotic signaling network that regulates cell growth depending on environmental conditions and plays a fundamental role in cellular and organismal physiology (Dowling et al., 2010; Hoeffer and Klann, 2010; Laplante and Sabatini, 2012). Phosphatidylinositol 3-kinases (PI3Ks) are a protein family involved in the regulation of a variety of cellular functions, including cell survival, growth, metabolism, and glucose homeostasis (Fruman et al., 2017). TGF-beta superfamily ligands play an important role in the development and physiology of different animal species, among which TGF-beta signaling may be closely related to tumor progression (Gigante et al., 2012; Larson et al., 2020). TGF-beta signaling plays an important role in early embryonic development as well as in tissue and organ formation, immune surveillance, and the balance between tissue repair and homeostasis in the adult body (Heldin et al., 1997; Feng and Derynck, 2005; Qin et al., 2022; Li et al., 2023). The results of this study showed that in G vs LG, Myc protein genes were up-regulated in the "TGF-beta signaling" and "Wnt signaling" pathways. Myc gene products, especially c- Myc, play an important role in the induction of apoptosis and regulation of cell growth (Lourenco et al., 2021; Patange et al., 2022).

5 Conclusion

This is the first study to investigate the effects of different breeding environments on body color-related genes in green and purple *A. japonicus* using RNA-seq analysis. We identified five

TABLE 7 Selected DEGs between green (G) and light green (LG) *A. japonicus* and associated enriched pathways.

Gene id	Nr/ Swissprot description	Ratio (G vs LG)	Probability
Drug metabolism - cytochrome P450			
AJAP30296	UDP-glucuronosyltransferase 2B1	3.588473	0.999638
AJAP30316	flavin-containing monooxygenase 2 (FMO2)	-3.62494	0.999286
Porphyrin and chlorophyll metabolism			
AJAP30296	UDP-glucuronosyltransferase 2B1	3.588473	0.999638
Wnt signaling pathway			
AJAP02429	Protein notum homolog	4.848749	0.998568
AJAP11986	Myc protein	1.205439	0.959865
TGF-beta signaling pathway			
AJAP11986	Myc protein	1.205439	0.959865
mTOR signaling pathway			
AJAP21010	V-type proton ATPase subunit F	-1.55491	0.982506

TABLE 8 Selected DEGs between purple (P) and green (G) *A. japonicus* and associated enriched pathways.

Gene id	Nr/ Swissprot description	Ratio (P vs G)	Probability
Phosphatidylinositol signaling system			
novel.3376		-2.52012	0.962446
Wnt signaling pathway			
novel.2754	Cell migration-inducing gene 5 protein	4.818737	0.998793

pathways involved in the regulation of pigmentation genes. Moreover, based on the number of identified DEGs, it was revealed that purple individuals were more susceptible to the breeding environment. Those grown in natural conditions were lighter in color compared to those grown in the dark, in which flavin-containing monooxygenase 2 (FMO2) (*AJAP30316*) and polyketide synthase (*AJAP13983*) showed significant downregulation. In particular, the former gene was also significantly enriched in "Drug metabolism - cytochrome P450". In addition, GO and KEGG analyses revealed the presence of possible DEGs enriched in the following pathways: "Wnt signaling", "Drug metabolism - cytochrome P450", "Porphyrin and chlorophyll metabolism", "Phosphatidylinositol signaling system", "TGF-beta signaling", and "mTOR signaling". The results of this study provide important clues to better understand the effects of different breeding environments on green and purple *A. japonicus* and will contribute to the development of optimal culture strategies.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA989434.

Ethics statement

The animal study was approved by Laboratory Animal of National Institute of Health. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

BL: Writing – original draft. LX: Conceptualization, Writing – review & editing. SL: Methodology, Supervision, Writing – review & editing. LS: Supervision, Writing – review & editing. FS: Data curation, Writing – review & editing. WC: Data curation, Writing – review & editing. CJ: Data curation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2024.1334761/full#supplementary-material

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Microplastics pollution in genetically connected populations of *Holothuria forskali* from south Bay of Biscay

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Introductio: Sea cucumbers are increasingly demanded as seafood in different cultures around the world. Holothurians could be potentially exploited in southern Bay of Biscay (Iberian Cantabrian coast) where they are not fished yet. Recent studies show relatively high pollution by emerging pollutants like microplastics (MPs) in the region; perhaps the exposure of holothurians to those MPs may put them at risk.

Material and methods: Here we analysed 60 samples of *Holothuria forskali* taken from the west and east of the Cape Peñas transition zone in different seasons. MPs were quantified from body wall tissue, and mitochondrial DNA sequences (16S and COI genes) were employed to determine population connectivity and infer replenishment capacity.

Results: Much higher MPs content in holothurian tissue than in water and sediments confirmed bioconcentration in this species. Significant differences between sampling locations were found, holothurian pollution being consistent with the occurrence of MPs sources like rivers, fishing ports and aquaculture. Genetic connectivity and no signals of diversity reduction in the most polluted samples suggest population replenishment capacity in *H. forskali* in the studied region. Results for a few samples of *H. mammata* from the same sampling points confirmed a higher MPs pollution at the west of Cape Peñas.

Discussion: According to these exploratory results, sea cucumbers seem to accumulate MPs especially in the western Cantabrian Sea. Further studies are recommended to understand the possible MPs threat for these holothurian species.

KEYWORDS

sea cucumbers, microplastics, marine pollution, mitochondrial DNA, population connectivity, Bay of Biscay

1 Introduction

Holothurians (Phylum Echinodermata, Class Holothuroidea) are epibenthic echinoderms, omnivorous non-selective sediment feeders (Hamel and Mercier, 1998; Hartati et al., 2017). They ingest the underlying substrate and recover edible materials (detritus, eggs, phytoplankton, etc.) by filtration, as well as floating plankton (Dar and Ahmad, 2006). Holothurians have been traditionally exploited in the central Indo-Pacific since 1700 (Conand, 1990; Purcell, 2014), and their fisheries are expanding all around the globe (Purcell et al., 2023). According to FAO (https://www.fao.org/3/cc5230en/cc5230en.pdf) 57,700 tonnes of wild sea cucumbers were harvested globally in the decade of 2011-2020. Tropical fisheries are the largest contributors to global catches (Conand, 2018; Conand et al., 2022), with 64 species commercialised so far. Sea cucumber prices are highly variable depending on the species, size, and origin. Mean price of final dried product is around 500 $/kg in Chinese retail markets (González-Wangüemert et al., 2018).

Due to the increasing demand from international markets, together with high price and easy harvesting, new holothurians markets target new species in temperate waters, such as the Mediterranean Sea (Antoniadou and Vafidis, 2011; González-Wangüemert et al., 2014; González-Wangüemert and Godino, 2016). Systematic holothurian fisheries started in Turkey in 1996, followed by Greece, Spain, France, and Italy, and focus on a few species (Hamel and Mercier, 2008; González-Wangüemert et al., 2014). FAO has just started to officially record sea cucumber landings per country in the Mediterranean fishing area (i.e., FAO 37), where they were previously undocumented (Vafidis and Chryssanthi, 2023). Currently, FAO recommends size and gear limitations (https://www.fao.org/3/i1384e/i1384e05a.pdf) that are followed by different countries. In Turkey, fishery regulation measures have been implemented since 2007, but due to an increase of illegal fishing new regulations were brought into force by the Ministry of Agriculture and Forestry in 2019 (Dereli and Aydin, 2021). Portuguese legislation about sea cucumber fisheries includes the species *Holothuria forskali*, *Parastichopus regalis* and *Mesothuria intestinalis* (Diário da República, 1.a série — N.o 235 — 6 de Dezembro de 2010, Annex I), catch being forbidden in protected areas such as Ria Formosa (South Portugal) (González-Wangüemert and Godino, 2016). In Spain, sea cucumber fisheries are established in the Mediterranean area, where more than 10 companies export to China sea cucumbers from different species (*Holothuria tubulosa, H. forskali, H. mammata*, and *H. arguinensis*) caught from Spanish waters (González-Wangüemert and Godino, 2016) according to the current export policies of fishery products of the Ministry of Agriculture, Fisheries and Food (https://www.mapa.gob.es/es/ministerio/servicios/analisis-y-prospectiva/informecomextanual2022_tcm30-657480_tcm30-507752.pdf). A recent legislation to control the *H. forskali* catches has been published in Galicia (NW Spain), but in the rest of regions sea cucumbers fisheries are not regulated, illegal catch being suspected in Andalusia (South Spain) (González-Wangüemert et al., 2018). In the southern Bay of Biscay, Exploitation of sea cucumbers in this region has not been explored in depth yet, despite the quantity of potential target species: 35 species of the seven orders of class Holothuroidea have been described from that region (Fernández-Rodríguez et al., 2019).

Sea cucumbers play a significant role in the reduction of organic load in the ecosystems, influencing actively nitrogen and phosphor cycles while modifying the substrate and the surrounding environment (Simões et al., 2019). Due to their feeding behaviour, they introduce environmental pollutants into their digestive system that can be transposed to other organs, being therefore bioindicators of environmental pollution (Grossmann, 2014; Zhu et al., 2018; Mohsen et al., 2020; Bulleri et al., 2021; Marrugo-Negrete et al., 2021). Emerging pollutants like microplastics (MPs thereafter), i.e., small plastic particles <5mm (Andrady, 2011), have been found in different holothurian species worldwide (for example, Plee and Pomory, 2020, in Florida; Iwalaye et al., 2020, in South Africa, or Tejedor-Junco et al., 2021, in the Canary Islands). Sea cucumbers have been proposed as suitable biomarkers of sediment contamination by MPs (Rios-Fuster et al., 2022; Fagiano et al., 2023), and not only for their non-selective sediment feeding strategy. Three pathways of MPs uptake are recognised in the genus *Holothuria*: by tentacles or direct feeding, by respiration (respiratory tree) and by the vascular system (access through madreporite) (Iwalaye et al., 2020).

Plastic particles may produce both chemical and physical damage to marine organisms when they are ingested, being considered a risk for natural life (Rochman, 2018). For this reason, the abundance and composition of MPs in species of fishing interest are increasingly studied (Daniel et al., 2021). MPs can affect holothurian species in different ways, such as increased oxidative stress and altered immune response (Mohsen et al., 2021). It is important to analyse the exposure of natural populations to these pollutants to identify potentially vulnerable populations before starting new exploitations. In the south Bay of Biscay, MPs containing polymers harmful to health and aquatic life, such as polyethyleneimine (PEI), polyethylene glycol (PEG), acrylic (PAN/PAA), polyester aniline derivatives or chloroquine, have been detected from organisms as diverse as algae (Bilbao-Kareaga et al., 2023), gastropod and bivalve molluscs (Janssens & Garcia-Vazquez, 2021; Masiá et al., 2022), cnidarians (Janssens & Garcia-Vazquez, 2021) or fish (Menéndez et al., 2022). Holothurians could be equally polluted in the region, perhaps putting natural populations at risk.

The distribution of MPs pollution in the region is quite patchy because beaches and organisms are differentially polluted by plastic and MPs depending on local sources like rivers, fishing ports, and even malfunctioning wastewater treatment plants (WWTP) (e.g., Masiá et al., 2021; Menéndez et al., 2022). Connectivity is essential for populations to respond to environmental stresses, since when a population is highly disturbed and disappears from a deme, it can be replenished with individuals from neighbouring demes (e.g., Klingbeil et al., 2022), as happens in top shells on the Cantabrian coast (e.g., Muñoz-Colmenero et al., 2015). Determining population connectivity patterns and replenishment levels is critical to understand the resilience of species (Steinberg et al., 2016), being essential for marine spatial planning and species management (Carr et al., 2017). Marine species that spend part of their life cycle as planktonic larvae have a high dispersal potential, exhibiting a weaker genetic structure and higher gene flow than species with low dispersal potential (Cowen and Paris, 2006; Galarza et al., 2009). This is the case of holothurians. In other regions, restrictions to gene flow between relatively distant populations have not been found in *Holothuria poli* (Valente et al., 2015), and the West Mediterranean *H. mammata* is considered a single panmictic metapopulation (Borrero-Pérez et al., 2011). Genetic differentiation has been found only on a large scale for *H. poli* (Gharbi and Said, 2011), *H. mammata* (Borrero-Pérez et al., 2011) and *H. scabra* (Uthicke and Benzie, 2001). However, the gene flow in marine species can be constrained by physical and ecological accidents (Hellberg et al., 2002). In the south Bay of Biscay, the Cape Peñas is a main hydrographic feature located in the middle of the coastline (Domínguez-Cuesta et al., 2019) that could represent a barrier for the connectivity of the species. The connectivity between sea cucumber populations in this region is still untested, and is necessary to understand the potential for population replenishment in cases of environmental disturbances.

The objective of this study was to analyse the potential risk of contamination by MPs in *H. forskali* inhabiting the central south Bay of Biscay, as well as the population connectivity between differentially polluted zones. A smaller sample of *H. mammata* from the same sampling points was also analysed to confirm the results obtained for *H. forskali*.

Departure hypotheses are:

A. For the patchy distribution of MPs in south Bay of Biscay coast, differences in MPs concentration between sea cucumbers from relatively close areas are expected (Masiá et al., 2019; Menéndez et al., 2022). Higher MPs concentration will occur in zones with more sources of MPs.
B. The presence of harmful chemicals such as PEI, Polyester, PEG and PAN is expected in holothurians, as in other organisms from the study region (Janssens and Garcia-Vazquez, 2021; Menéndez et al., 2022). For the regional patchiness, the MPs composition will be different in different zones.
C. Connectivity between populations of holothurian species is expected at a short scale (Borrero-Pérez et al., 2011; Valente et al., 2015); thus signals of genetic depletion in more polluted areas are not expected.

2 Materials and methods

2.1 Ethic statement

The specimens analysed were collected by professional divers and anaesthetised with magnesium chloride ($MgCl_2$) at 5% to prevent animal suffering. The project was approved by the competent research ethics committee of the Government of Asturias Principality, General Directorate of Maritime Fisheries, project code IDI-2018-00201, approval date 5 of February of 2020.

2.2 Samples and sampling areas

H. forskali adults were sampled from two different points (Figure 1, Table 1): Palo Verde (Ribadesella) and Cape Blanco (El Franco), located at East and West, respectively, of the Cape Peñas. Cape Peñas is the main geomorphological feature situated in the middle of the coastline, where the main component in the coastal drift (North Atlantic Drift: NAD) goes eastward (Marquina et al., 2015; Domínguez-Cuesta et al., 2019), moving water from west to east. Rivers, fishing activities, and wastewater discharges of large urban concentrations, are principal contributors to marine MPs (e.g., Browne, 2015; Lebreton et al., 2018; Masiá et al., 2021; Meijer et al., 2021; Menéndez et al., 2022). Wastewater treatment plants (WWTPs) and main rivers in the study region are marked in Figure 1. Information on the hydrology of the Asturian coast is published and available on the official website of the Hydrological Confederation of the Cantabrian Sea (https://www.chcantabrico.es/organismo/las-cuencas-cantabricas/marco-fisico/hidrologia). The location of WWTPs in Asturias can be found on https://consorcioaa.com/saneamiento.

The coordinates of the sampling points, main influencing rivers and potential MPs sources are presented in Table 1. Cape Blanco is located 14 km east of the Eo River which is 99 km long and has an annual average flow of 19.61 m^3/s; in addition, the smaller Porcia River (31 km length, 3.1 m^3/s annual mean flow) is 1.6 km west of Cape Blanco. The WWTP El Franco is 1.11 km west, and the fishing ports of Viavelez and Tapia are located respectively 1.4 km east and 7.5 km west of Cape Blanco, in addition to the fishing ports of Figueras and Ribadeo in the Eo estuary. The Eo estuary concentrates the oyster aquaculture in the region. On the other hand, Palo Verde is located 4 km east of the Sella River, of 66 km length with annual average flow of 18.07 m^3/s. The fishing port and the WWTP of Ribadesella are 5 km west of this sampling point (Figure 1). Tourism visiting beaches and recreational areas occurs in the two zones. From the data summarised in Table 1, total river discharges and the number of fishing ports are higher at the west (Cape Blanco) than at the east (Palo Verde), while the other potential sources of plastic and MPs are apparently similar in the two locations.

Sampling was done through three consecutive seasons: summer 2020, winter 2021 and spring 2021. Ten adults of *Holothuria forskali* were sampled from each sampling point each season (Table 1), making a total of N = 60 *H. forskali* individuals. In spring 2021 10 adults of *Holothuria mammata* were taken from the same sampling points and processed as *H. forskali*, for comparison of the results with a second species living in the same conditions (N = 20 *H. mammata*). Holothurians were identified *de visu* by experts of the Asturias Fisheries Research Centre, where they were gutted, then they were transported to the laboratory of Natural Resources of the University of Oviedo in clean labelled bags for further processing.

Samples of water (10L) and sediment (50g) were taken together with sea cucumbers in winter and spring (Table 1). Water (two replicates of 5L each, being 10L in total per sampling point each season) was taken in bottles carefully cleaned with filtered distilled water. One replicate of 50g of sediment was taken manually with

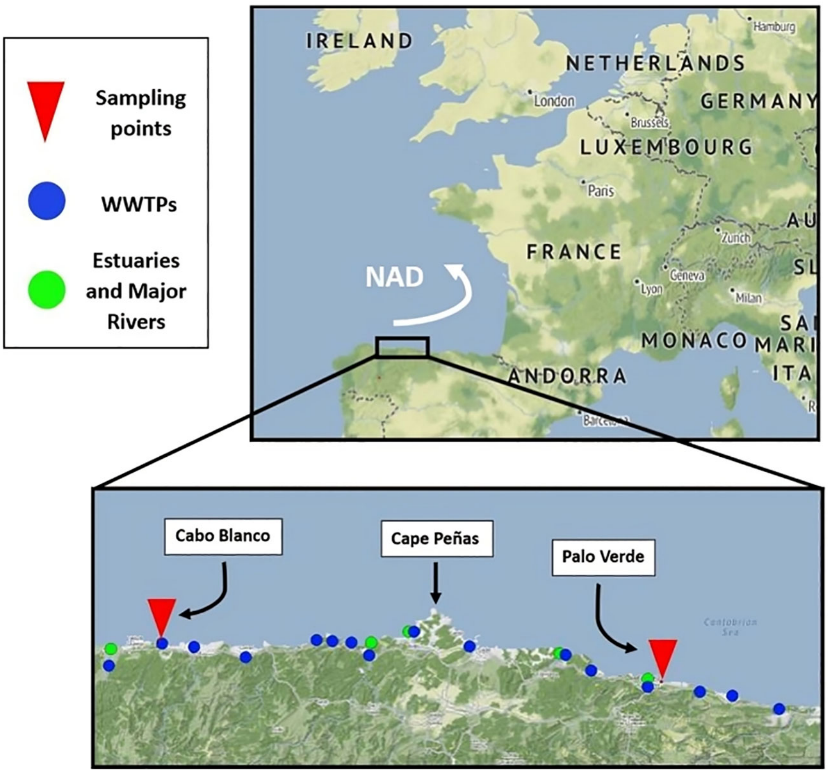

FIGURE 1
Study region in the Cantabrian Sea. Top, location of the sampling area in Europe showing the North Atlantic Drift (NAD) as a white arrow. Bottom, sampling points (red triangles): Cape Blanco, left (west) and Palo Verde, right (east). Wastewater treatment plants (WWTPs) are marked as blue dots. Main estuaries (green dots) are, from west to east: Eo, Nalon, Aviles, Villaviciosa and Sella.

small glass jars just below or by each sample of holothurians and stored in the same jars. Unfortunately, environmental samples could not be taken in summer 2020 for logistic limitations due to bad weather conditions during the scheduled sampling time.

TABLE 1 Summary of sampling sites and samples taken by season (Summer 2020, Winter 2021 and Spring 2021).

	Cape Blanco (West)	Palo Verde (East)
Coordinates	43.570671, -6.854390	43.460441, -5.013347
Sampling landscape	Islet	Rocky cape with cliffs
Nearby rivers	Eo, Porcia	Sella
Other MP sources	Eo and El Franco WWTPs, fishing ports of Tapia, Viavelez, Figueras, Ribadeo.	Ribadesella and San Jorge WWTPs, fishing port of Ribadesella.
Samples		
Summer 2020	10 *H.f.*	10 *H.f.*
Winter 2021	10 *H.f.*, water, sediment	10 *H.f.*, water, sediment
Spring 2021	10 *H.f.*, 10 *H.m.*, water, sediment	10 *H.f.*, 10 *H.m.*, water, sediment

H.f., Holothuria forskali; H.m., Holothuria mammata.

2.3 MPs extraction and processing

Water samples were filtered immediately after arrival in the laboratory through 0.45µm Polyethersulphone (PES) sterile filters (PALL Corporation®, 47 mm diameter), following the protocol described by Masiá et al. (2019). One litre was filtered through each filter to facilitate MPs counting. MPs extraction from sediment was carried out by flotation, following the protocol described by Besley et al. (2017). Briefly, 50g of previously dried sediment were added to 200mL of a hypersaline solution (358.9g of NaCl per litre of filtered distilled water), manually shaken for two minutes three times (Laglbauer et al., 2014), then left at room temperature for 24 hours. The final solution containing potential MPs was filtered through 0.45 µm pore PES filters. This step was carried out as carefully as possible to avoid sand falling over the filter (Menéndez et al., 2022). Two filters per sample were necessary due to saturation while filtering.

For holothurians, approximately 10g of edible tissue (body wall from gutted individuals) were taken longitudinally (mouth-anus axis, from the inner muscle to the skin) to ensure a reliable representation of the MPs accumulation along the organism, per individual. The material was cut with a clean scalpel in a laminar flow cabinet. Digestion was performed adding 20 mL of H_2O_2 (30% v/v) per gram of tissue. This solvent is not expected to degrade or destroy MPs (Liebezeit and Dubaish, 2012; Riani et al., 2023). The digestion mixture was stored in a 400 mL glass jar (covered with aluminium foil to avoid plastic contamination) at 65°C for 7 days to ensure that all organic material was digested (modified from Renzi et al., 2018). Daily shakes were performed. After digestion, the sample was diluted to 1L with filtered distilled water to facilitate the filtering process (Menéndez et al., 2022). During the filtration, the jar was rinsed with filtered distilled water to recover all potential material stuck in the walls. In total, five 0.45 µm pore PES filters were used per individual.

Filters were directly observed in a Leica 2000 Stereomicroscope (40x magnification) in closed and cleaned petri dishes. Only potential plastic particles smaller than 5 mm length were considered (Andrady, 2011; Andrady, 2017). MPs were classified by shape and colour. Shapes were fibres (cylindrical, length at least 3:1 width, continuous colouration), fragments (irregular shapes and colourations, sharp angles) and beads (spherical, commonly aggregated in pellets) (Hidalgo-Ruz et al., 2012; Cole, 2016; Lorenzo-Navarro et al., 2021; Monira et al., 2022). Colours were recorded as described by other authors; expectations are mostly black, blue, transparent, and other marginal colours (Abayomi et al., 2017; Gago et al., 2018; Wang et al., 2019; Ugwu et al., 2021).

For the chemical analysis, 15-20% of the main MPs found, principally blue and black fibres, were randomly selected (Uurasjärvi et al., 2021). They were picked from the petri dishes under laminar flow cabin. µFTIR analysis (Fourier-Transform Infrared Spectroscopy) was done (FTIR Varian 620-IR and Varian 670-IR, using a wavelength between 4000 and 500 cm^{-1} and a germanium glass, Varian 620-IR and Varian 670-IR cm^{-1}), in the Autonomous University of Madrid. Results with a bibliographic search score over 60% were used. Polymers were checked in the ECHA (European Chemicals Agency) website (https://echa.europa.eu/es/home) for their potential toxicity for the aquatic life and/or for humans.

2.4 Contamination prevention

To prevent potential contamination from airborne MPs, all procedures were carried out into a vertical laminar flow cabinet. Researchers wore cotton lab coats all time. All liquids (reagents, water, and hypersaline solution) were filtered through 0.2 µm pore size PES filters. Materials in contact with samples/reagents (tweezers, vacuum pump recipients, petri dishes, iron plates, glass jars and aluminium foil) were carefully cleaned with filtered distilled water following instructions from Lusher et al. (2015). Blanks (n =) consisting in the digestion reagent were run simultaneously with the samples to control the contamination during manipulation, dilution, and filtration. All filters were stored after filtration in clean petri dishes into a closed paperboard box for, at least, 48 hours before starting the MPs counting.

2.5 DNA extraction, PCR, and sequencing

For all the individuals, a small piece of longitudinal inner muscle was taken (approx. 0.5g) for DNA extraction using Chelex-based protocol (Estoup et al., 1996). In order to increase the PCR success, DNA was previously diluted 1:10 in bidistilled water.

Two fragments of mitochondrial DNA, cytochrome oxidase I (COI) and 16S rDNA genes, were analysed. They have been employed in Valente et al. (2015) to determine the population genetic structure of *Holothuria poli* in the Mediterranean Sea. Within 16S rRNA gene a fragment of 486 base pairs (bp) was amplified following the modified protocol described by Valente et al. (2015), using the primers developed by Palumbi (1996): 16Sar-L 5'-CGCCTGTTTATCAAAAACAT-3' and 16SB 5'-CTCCGGTTTGAACTCAGATCA-3'. The PCR protocol was an initial denaturation at 95°C for 3 min, then 40 cycles of denaturation at 94°C for 20 s, annealing at 45°C for 20s and extension at 72°C for 20 s. Finally, 72°C for 10 min for a final extension.

A fragment of 313 bp within cytochrome oxidase a subunit I (COI) gene was amplified using Leray et al. (2013) primers (mICOIintF 5'-GGWACWGGWTGAACWGTWTAYCCYC-3' and jgHR 5'-TAIACYTCIGGRTGICCRAARAAYCA-3'). PCR protocol consisted of an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 15 s, annealing at 46°C for 15 s and extension at 72°C for 10 s, and a final extension at 72°C for 3 min.

For the amplification of both genes, PCR reactions contained 10 µmol of each primer, 2.5 mM $MgCl2$, 0.25 mM dNTPs, 1X Buffer GoTaq Promega, 0.15 µl of GoTaq Polymerase (5u/µL) and 2µL of diluted DNA, in a total final volume of 20 µL. PCR products were visualised using 2% agarose gel stained with 10 mg/mL Symple-Safe^{TM} (2.5 µL, EURx, Gdansk, Poland). Amplicons were sent for Sanger sequencing at Macrogen Spain, Inc. (Madrid, Spain).

Sequences were edited and trimmed with BioEdit Sequence Alignment Editor software (Hall, 1999). Finally, the outcome sequences were checked for correct species and gene identity in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/, accessed in July 2023) using the BLAST algorithm and best match criterion.

2.6 Data analysis

2.6.1 Microplastics

MPs counts were transformed to MPs/g in all the elements (water, sediment, and holothurian tissue) to make the results comparable, using a density of 1.025 g/l for Cantabrian Sea water. First, considering the variables MPs/g for holothurians, season (1, 2 and 3 for the three consecutive seasons), location (0 for east and 1 for west), and species (0 for *H. mammata* and 1 for *H. forskali*), exploratory principal component analysis (PCA) was run to visualise the relationships between variables. The following settings were applied: correlation option; variables with $r<0.8$ pairwise correlation; significant components with >0.7 eigenvalue.

Due to small sample sizes, nonparametric tests were preferred for comparisons between samples. Chi-squared analyses were performed to compare samples for the proportion of MPs types (shape and colour) and composition (chemical), between locations or seasons for *H. forskali* and between locations for *H. mammata*.

For the quantitative variable MPs/g, normality was checked using Shapiro-Wilk test, and homogeneity of variance using

Breusch–Pagan test. To test the effect of season (three levels: summer, winter, spring) and sampling location (two levels: west, Cape Blanco, and east, Palo Verde sampling points) on *H. forskali* means, two-way PERMANOVA analysis was performed, with 9,999 permutations and Euclidean distance. To test the effect of the location on the *H. mammata* mean of MP/g, one-way PERMANOVA was employed with the same settings.

Standard significance level of $p<0.05$ was adopted. Analyses were done on free software PAST v.2.17 (Hammer et al., 2001).

2.6.2 Genetic diversity and population differentiation

Haplotype diversity (Hd), number of haplotypes, exclusive haplotypes and polymorphic sites were calculated for each sample using DnaSP software v.6 (Rozas and Rozas, 1995). Relationships between haplotypes and their geographical distribution were analysed in haplotype networks constructed with the software Network version 10.2.0.0. (http://www.fluxus-engineering.com) with default settings.

Population genetic differentiation was estimated from F_{ST} value based on haplotype frequencies, and its significance was calculated by performing 2000 random permutations using ARLEQUIN software v.3.5.1.3 (Excoffier and Lischer, 2010). The level of significance adopted was standard $p < 0.05$.

3 Results

3.1 Microplastic content

Blanks exhibited a mean MPs concentration of 0.0076 ± 0.0079 MPs/g, which was two and even three orders of magnitude lower than those of sea cucumbers (see below), thus it can be assumed that no external contamination occurred during the digestion process. MPs were found from all types of samples, accounting for 1,063 particles in total: 683 from holothurians (Supplementary Table 1), 15 from sediment and 365 from water samples (Supplementary Table 2). The majority was fibres that represented the 95.7%, 100%, and 94.2% of all particles in sea cucumbers, sand and water, respectively.

In general, water had the lowest concentration of particles (maximum 0.012 ± 0.01 MPs/g in spring), followed by sediment (mean 0.05 ± 0.352 MPs/g). Holothurians exhibited much higher MPs concentrations than environmental samples, being *H. forskali* from the west in summer the highest (1.54 ± 1.135 MP/g) (Table 2). Sediment results were excluded from further analysis due to the small number of samples taken, with no replicates per location. According to a higher number of potential MPs sources, MPs concentration was apparently similar or higher at the west, especially in holothurians.

The principal component analysis (PCA) scatter plot provides a visual interpretation of the relationships between the variables considered (Figure 2). PC1 and PC2 explained the 40% and 37% of the variance respectively, being the season and location the factors with higher loads. The diagonal representing MPs concentration in holothurians was in the second quadrant together with the location (1 for west and 0 for east, corresponding to more potential sources of MPs pollution at the west) that was the closest diagonal. The diagonal representing the season (1-summer 2020, 2-winter 2021, 3-spring 2021) was in the quadrant II, opposite to the diagonal representing the species that was in the quadrant IV. *H. forskali* and *H. mammata* were segregated, with *H. mammata* concentrated in the quadrant II. For the two species, individuals sampled from the west (yellow signs) were clearly separated and above of those sampled from the east (blue signs), suggesting a strong effect of the location on the MPs concentration – as expected from a higher number of MPs sources near the west sampling point.

In *H. forskali*, that was the main focus of this study, location was highly significant in the two-way PERMANOVA, unlike the season that was not significant, nor the interaction (Table 3); this means that individuals sampled at the west exhibited significantly higher MPs concentration than those sampled at the east independently of the season, as seen in Table 2.

Regarding MPs types in *H. forskali* (n = 530 particles in total), most of them were mainly black and blue fibres, fragments being much less abundant (Supplementary Table 1). Chi-squared test comparing the seasonal profiles was not significant for the east (χ^2=12.01; 8 d.f.; p=0.15 > 0.05, not significant) nor for the west location (χ^2=12.47; 8 d.f.; p=0.13). Pooling together the seasonal samples of each location, the difference between east and west locations was highly significant (χ^2=19.75; 4 d.f.; p=0.0006), with a higher proportion of fragments and black fibres at the west

TABLE 2 Mean concentration (standard deviation in parentheses) of MPs in the different samples studied by season, species (or species associated) and location.

		Summer		Winter		Spring	
		East	West	East	West	East	West
Water		nd	nd	0.005 (0.003)	0.008 (0.004)	0.012 (0.01)	0.011 (0.009)
Sediment*	By *H. forskali*	nd	nd	0.08	0.08	0	0.02
	By *H. mammata*	nd	nd	nd	nd	0.04	0.08
Holothurians	*H. forskali*	0.618 (0.359)	1.54 (1.135)	0.636 (0.378)	0.941 (0.609)	0.559 (0.279)	0.953 (0.609)
	H. mammata	nd	nd	nd	nd	0.611 (0.325)	0.952 (0.405)

Results are given in MPs/g for sea cucumbers and sand, MPs/individual for sea cucumbers and MPs/L for water as measurement unit. For sediment there is not standard deviation because it was only one replicate per site and by species. nd, no data available.
* means there is not standard deviation because of the number of replicates.

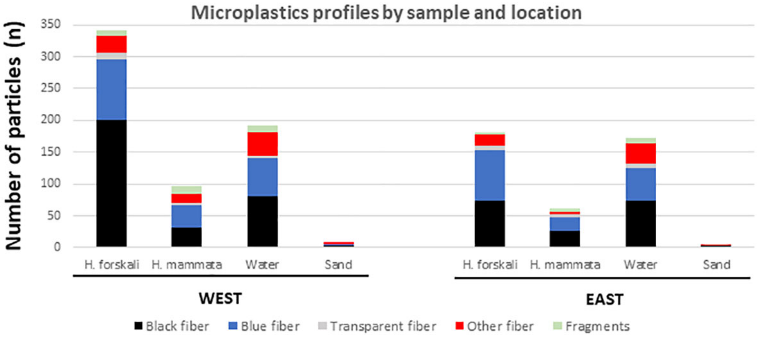

FIGURE 2
PCA scatter plot representing the relationships between the MPs concentration in holothurians (MPs/g) and the other variables considered: location, season, and species.

(Figure 3, first and third columns at left). The MPs types obtained from water samples (n = 365, sampled in winter and spring; fifth and seventh columns in Figure 3) were not significantly different between locations (χ^2=1.38; 4 d.f.; *p*=0.84).

For the composition of the 151 MPs analysed from all the samples (the two holothurian species and water from the west and east locations), 18.8% were natural compounds, such as cotton and cellulose; the rest were plastics and other substances (Supplementary Table 3). The most abundant material was Rayon (45%) followed by Polyethyleneimine cellulose - PEI (15%), Polyester (9.7%), Polyethylene terephthalate - PET (5.8%), Polyacrylonitrile - PAN (2.6%), Nylon (1.3%), Polyethylene glycol - PEG (0.65%) and Urethane Alkid (0.65%) (Supplementary Table 3). The hazardousness of the compounds was checked in the ECHA website (http://echa.europe.eu). Four of them are recognised as harmful for aquatic life and/or the human health: PEI, Polyester, PAN, and PEG that summed a total of 42 particles (27.8% of the total). These compounds were expected in the second departure hypothesis.

Focusing on the artificial compounds found in *H. forskali* (Figure 3, second and fourth columns; Supplementary Table 3), the clear difference between east and west found for the quantity and types of MPs was not found for particles composition. The profile was relatively similar at east and west, with a relatively high proportion of rayon followed by PEI and polyester and no significant differences among the two locations (χ^2=4.53; 3 d.f.; *p*=0.21). For the water the difference between west and east samples was also not significant (χ^2=3.82; 3 d.f.; *p*=0.28). PEI particles were not found in water samples taken at the east (Figure 3, column at right), but this was not enough for statistical significance, being probably due to small sample size (= sampling effect, with only 17 artificial particles in east water sample; Supplementary Table 3).

In the second species considered, *H. mammata*, the results were similar to those found in *H. forskali* although the number of MPs detected was much smaller (n = 160 particles), because sampling for this species was done only in the spring. As shown in Table 2, the individuals sampled from the west location exhibited more MPs than those sampled at the east (means 0.952 with SD 0.405, versus 0.611 with SD 0.325, respectively). As in *H. forskali*, the difference of means was statistically significant (one-way PERMANOVA with 9,999 permutations gives a total sum of squares of 3.005 and within-group sum of squares of 2.423, F = 4.324, *p* = 0.047).

In *H. mammata*'s profile by type of MPs (Figure 4), the majority of particles were also black and blue fibres. As in *H. forskali*, fragments were more abundant at the west and transparent fibres at the east; however, the proportion of black and blue fibres was quite similar and the difference between west and east locations was not significant (χ^2 = 3.10; 4 d.f.; *p*=0.54). For the composition, in *H. mammata* from the west two particles were PAN, which is a harmful substance according to the ECHA. This substance was not found in *H. forskali*. The composition profile of west and east locations looked quite different (Figure 4, second and fourth columns), but the difference was not significant (χ^2 = 4.64; 4 d.f.; *p*=0.33); in this case samples sizes were very small, since only 10 and 12 particles from west and east location respectively were analysed with FT-IR.

TABLE 3 Two-way PERMANOVA (9,999 permutations) testing the effect of the factors "Season" and "Location" on the variation of MPs concentration in *Holothuria forskali* (d.f. = degrees of freedom).

Factor	Sum of squares	d.f.	Mean square	F	*p*
Season	1.263	2	0.632	1.592	0.211
Location	**4.380**	**1**	**4.380**	**11.037**	**0.0005**
Interaction	1.113	2	0.556	1.402	0.259
Residual	21.432	54	0.397		
Total	28.189	59			

Significant factor marked in bold.

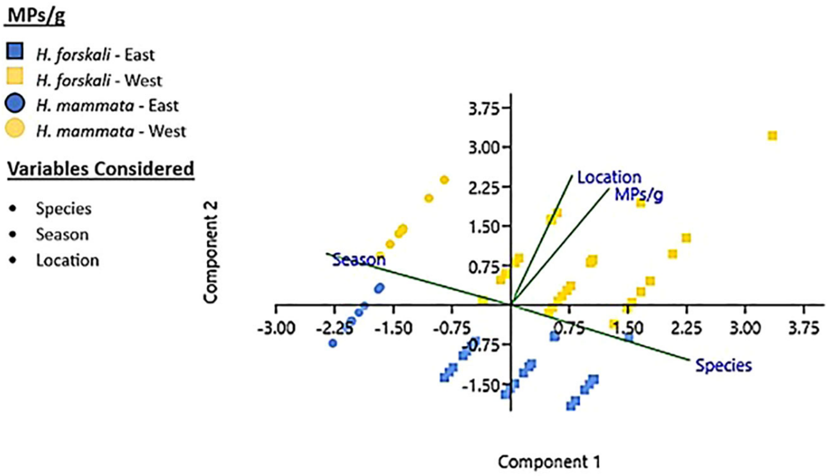

FIGURE 3
Profile of microplastics found from *Holothuria forskali* (Hf) and water samples in this study, for type (shape and colour) and chemical composition.

3.2 Genetic diversity and estimated population connectivity

Not all the individuals could be genetically analysed, possibly due to procedural issues. In total 72 sea cucumbers were successfully sequenced for the 16S rRNA gene, and 56 for COI gene. After editing the sequences, alignments of equal length were obtained for the two genes and species: 321 and 373 bp for 16S in *H. forskali* and *H. mammata*, respectively; and 237 and 313 bp for COI in *H. forskali* and *H. mammata*, respectively. BLAST analysis of the DNA sequences obtained confirmed the species visually assigned in all the cases (*H. forskali* or *H. mammata*), with more than 99% of identity for both genes. The sequences obtained were submitted to GenBank, where they are available with the accession numbers OR681936-OR681967 and OR682161-OR682170 for 16S gene, and OR681876-OR681886 and OR690923-OR690929 for COI gene.

3.2.1 Genetic diversity

For the gene 16S a total of 32 haplotypes among 54 individuals of *H. forskali*, and 10 haplotypes among 18 individuals of *H. mammata*, were detected (Table 4). For COI gene, the number of haplotypes found was 11 haplotypes among 36 *H. forskali* individuals, and 7 haplotypes among 20 *H. mammata* individuals (Table 4). High haplotype diversity was found for the two genes in the two species, ranking from 0.96 for 16S gene in *H. forskali* to 0.75 for COI gene in *H. mammata*. The haplotype diversity was higher for *H. forskali* for both markers. Many exclusive haplotypes (unique of a location) were found, especially in *H. forskali*. In *H. forskali* the number of haplotypes was similar for the west and the east locations, being respectively 20 and 19 for 16S gene, and 6 and 8 for COI gene; thus, signals of depleted diversity were not found with the present data.

In the case of *H. mammata* diversity was somewhat lower, especially in the east location: the number of haplotypes was 8 in the

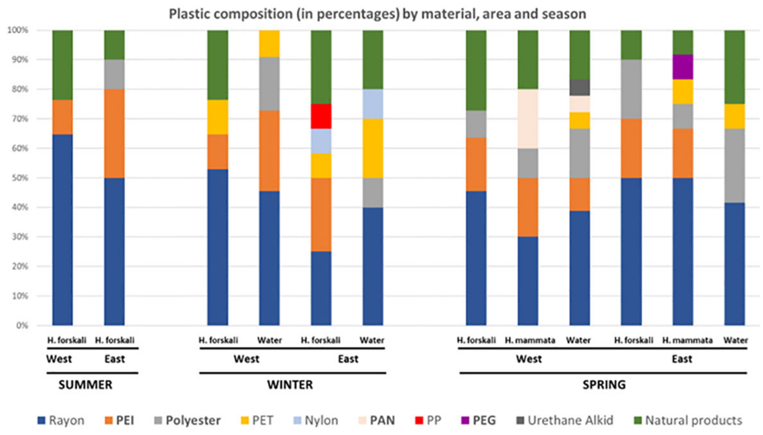

FIGURE 4
Composition of particles found from *H. mammata* (Hm) samples in west and east sampling points.

TABLE 4 Genetic diversity of 16S rRNA and COI genes for *H. forskali* and *H. mammata* in the two sampling locations considered in this study.

Gene	Species	N	h	Hd	F_{ST}	*p-value*		N	Shared h	Exclusive h
16S	***Holothuria forskali***	54	32	0.96	0.001	0.387	**West**	27	7	13
							East	27		12
	Holothuria mammata	18	10	0.85	0.158	**0.009***	**West**	9	2	6
							East	9		2
COI	***Holothuria forskali***	36	11	0.77	-0.032	0.919	**West**	13	3	3
							East	23		5
	Holothuria mammata	20	7	0.75	0.198	**0.018***	**West**	10	3	3
							East	10		1

N, number of individuals analysed; h, number of haplotypes; Hd, Haplotype diversity. Significant FST values are in bold marked with an asterisk. h, Haplotypes; Hd, haplotype diversity; N, sample size.

west and 4 in the east location for 16S gene; 6 in the west and 4 in the east location for COI gene (Table 4). As for MPs, the results obtained for *H. mammata* must be taken with caution due to small sample sizes (18 and 20 individuals, for 16S and COI genes, respectively).

3.2.2 Population differentiation

Pairwise F_{ST} results did not show any significant difference between regions in *H. forskali* for any gene (Table 4), thus limitations to gene flow and connectivity were not found with these genes. In contrast, the two *H. mammata* samples exhibited significant F_{ST} suggesting gene flow restrictions for both genes (Table 4). Again, these results should be taken with caution due to small sample sizes for this species.

Parsimony haplotype networks for both genes are shown in Figure 5. They were consistent with lack of differentiation between the two locations, the haplotypes with the higher frequencies being all shared among locations (Figure 5). The networks exhibited irregular shapes, quite similar in the two species with many singletons and exclusive haplotypes and generally more complex for 16S than for COI gene, according to the relative diversity of these two genes in this study. Differences between west and east locations were not evident in these networks for *H. mammata*, suggesting that the observed F_{ST} significance could be simply due to small sample sizes.

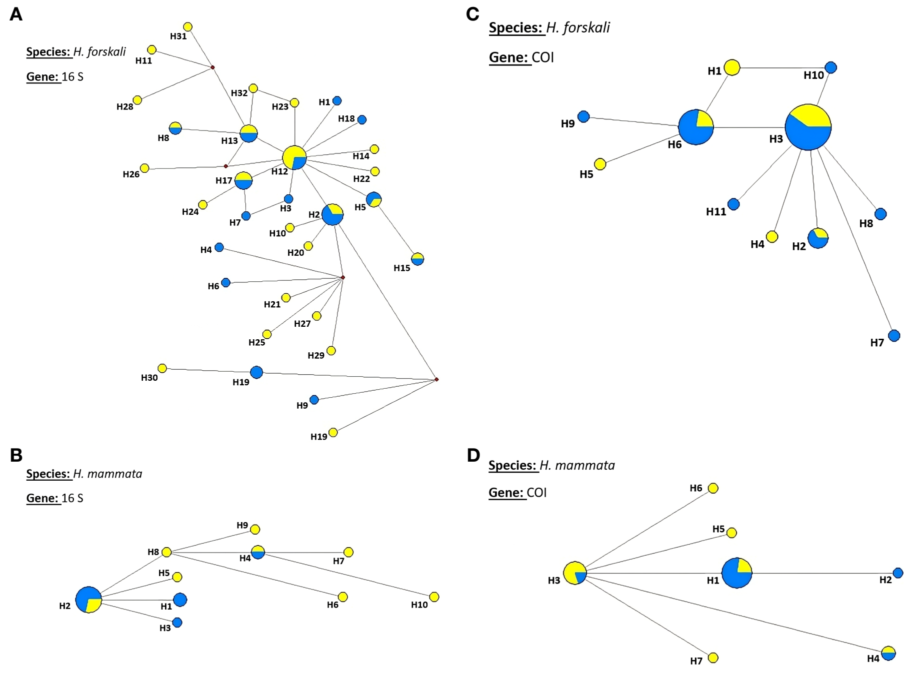

FIGURE 5
Parsimony haplotype networks for 16S and COI genes in *H. forskali* and *H. mammata*. **(A)** *H. forskali*- 16S; **(B)** *H. mammata*-16S; **(C)** *H. forskali*-COI; **(D)** *H. mammata*-COI. East location: blue, West location: yellow. The size of the circle is proportional to the number of individuals with each haplotype.

4 Discussion

To the best of our knowledge, this is the first study on MPs content and population connectivity of *Holothuria forskali* in the Bay of Biscay. The results obtained were consistent with previous studies from Greece and Italy reporting MPs in this species (Seary et al., 2013; Deudero and Alomar, 2015). Supporting Hypothesis 1, the level of MPs pollution was higher in the west, where more MPs sources occur, than in the east. Fishing and aquaculture, more concentrated near the west location, are important sources of MPs marine pollution (Xue et al., 2020; Chen et al., 2021) and would explain at least partially the observed difference. In this region, Masiá et al. (2021) reported a higher abundance of macro- and microplastics in beaches near ports and areas with intense fishing activity. Greater river discharge at the west would also explain a higher MPs pollution, since rivers are well known MPs sources (Campanale et al., 2020; Lestari et al., 2020). These MPs would accumulate in holothurians, which are sentinels of MPs pollution (Rios-Fuster et al., 2022; Fagiano et al., 2023).

From the perspective of the potential consumption of these holothurians - if they are harvested in a future – the MPs concentrations found in our study were considerably higher than those published in other studies. For example, Mohsen et al. (2023) found an average of 0.081 MPs/g in *Apostichopus japonicus* commercialised in Chinese markets, which is about ten times smaller than that found in our study on the Cantabrian Sea. Relatively high MPs concentrations have been found in other marine species from the same coast (in comparison with other regions), for example in mussels suggesting problematic accumulation of MPs in the area.

As expected in Hypothesis 2, the type and composition of those MPs was consistent with that reported for other organisms and water from the Cantabrian Sea (La Daana et al., 2017; Masiá et al., 2022; Menéndez et al., 2022), dominated by black and blue fibres while the particles of other colours are relatively scarce. The types of MPs were significantly different in the two locations, according to Hypothesis 2 and the patchiness found in the region in previous studies (Masiá et al., 2019; Masiá et al., 2021). However, Hypothesis 2 was not totally accomplished, since the expected difference in holothurians MPs composition between these locations was not found. Higher sample sizes analysed with FT-IR would increase the statistical power of this observational study, but perhaps the composition is not so different because the ultimate sources of pollution (single use plastics, fishing materials, industrial wastes, microbeads in cleaners and cosmetics etc.) are relatively similar across the region, as they are in other marine waters (Frias et al., 2016; Bråte et al., 2018; Zhang et al., 2019; Wu et al., 2020).

A (relative) novelty of this study was finding much higher MPs concentration in the holothurians than in the water samples. This is the first evidence of bioaccumulation of MPs in the edible tissue of sea cucumbers of the genus *Holothuria*. The presence of MPs in the body wall suggests they were translocated from the gut, or from the respiratory system as seen in other species (Brennecke et al., 2015; Jovanović et al., 2018; Elizalde-Velázquez et al., 2020). Bioaccumulation in sediment feeders is not strange. Fagiano et al. (2023) found that marine sediments hold two-fold microplastics than sea surface waters and are dominated by fibers. Bioconcentration of MPs is supported by previous studies in marine invertebrates (Sfriso et al., 2020; Álvarez-Ruiz et al., 2021; Kuehr et al., 2022). In the case of sea cucumbers, bioconcentration has been hypothesised by other authors (Iwalaye et al., 2020; Lombardo et al., 2022; Martines et al., 2023) and confirmed in the genus *Apostichopus* (Mohsen et al., 2022). *H. forskali* was also proposed as a bioindicator of MPs pollution (Rios-Fuster et al., 2022; Fagiano et al., 2023); however, these studies focused on MPs ingestion analysing the gastrointestinal tract, so direct comparisons with our results are not reliable. From our results, *Holothuria* joins the list of genera that bioaccumulate MPs in tissues other than the gut.

For the third hypothesis, it was apparently accomplished for *H. forskali*, or at least we found no evidences of lack of connectivity from our data. The connectivity inferred in this study for *H. forskali* is supported by its long planktonic larval duration (Ivy & Giraspy, 2006), which favours the panmixia and suggests the capacity to recover from population disturbance through recruitment from other areas (Valente et al., 2015). For *H. mammata*, although F_{ST} was significant, very small sample sizes prevent robust conclusions about connectivity. Historically, *H. mammata* has been described only in the Mediterranean Sea, the southern Portuguese coast and the Azores Islands (Mercier and Hamel, 2013). Recent eastwards dispersion along the Cantabrian basin could explain some differences in new colonised areas; however, *H. mammata*'s haplotype networks relatively similar to those of *H. forskali* would suggest a similar connectivity. Moreover, the occurrence of shared haplotypes might indicate that gene flow between populations is happening in *H. mammata*. This is consistent with Valente et al. (2015) and Gharbi and Said (2011) results for Mediterranean *H. poli*, where population genetic structure can be detected only in some cases at longer distances. Interestingly and pointing at a good capacity of population replenishment, the level of diversity was not lower in the more polluted deme at west. On the contrary, it was similar or higher that the diversity found at the cleaner east location in the case of *H. mammata*. This result would point at a good recovery capacity of *Holothuria* populations in case of environmental disturbances, as it happens for other marine organisms of high larval dispersal capacity (e.g., Muñoz-Colmenero et al., 2015; Klingbeil et al., 2022).

The preliminary genetic results obtained in this study point to greater haplotypic diversity of the 16S gene than that of the COI gene, for the two species and in the two locations examined (see Table 4 above). It is generally assumed that the 16S gene is highly conserved evolutionarily, more than COI (Jo et al., 2016). However, a few other studies show a higher Hd in 16S than in COI in holothurians and other marine invertebrates (Borrero-Pérez et al., 2011; Soliman et al., 2016 in *H. mammata* and *H. edulis* respectively). This issue has not been discussed in depth yet and here we cannot give a proper explanation for this observation. It would deserve further exploration with many more data and other species, to understand the evolutionary patterns of these genes in marine invertebrates.

5 Conclusions

The species *Holothuria forskali*, as well as *H. mammata*, are able to bioaccumulate MPs in edible tissues. In South Bay of Biscay samples, *H. forskali* exhibits a higher concentration of MPs in the west that in the east of Cape Peñas, according to the respective number of MPs sources in each area. Differences in the distribution of MPs types were found between sites, supporting previously observed patchiness of MPs pollution along the coast. Toxic compounds such as PEI, Polyester, PEG, and PAN, also found from other species and water on this coast, were confirmed in these holothurians. High diversity at mitochondrial genes and low F_{ST} values suggest population connectivity and replenishment capacity between polluted and unpolluted demes in *H. forskali*. Similar diversity results suggest a similar situation for *H. mammata*, although these results should be taken with caution due to limited sample size.

Data availability statement

The original contributions presented in the study are publicly available. The datasets generated for this study can be found in the GenBank, GenBank Accession numbers OR681936-OR681967, OR682161-OR682170, OR681876-OR681886, and OR690923-OR690929.

Ethics statement

The animal study was approved by General Directorate of Fisheries, Asturias, Spain. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

DM: Investigation, Methodology, Visualization, Writing – original draft. AÁ: Formal analysis, Investigation, Methodology, Resources, Writing – review & editing. AA: Formal analysis, Methodology, Project administration, Supervision, Visualization, Writing – review & editing. EG: Conceptualization, Data curation, Formal analysis, Funding acquisition, Supervision, Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2024.1333141/full#supplementary-material

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Gene-expression patterns during regeneration of the multi-organ complex after evisceration in the sea cucumber *Eupentacta quinquesemita*

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Many species of sea cucumbers (Echinodermata, Holothuroidea) perform evisceration, i.e., ejection of internal organs including digestive tracts when responding to environmental stresses. After evisceration, they also show a high regenerative capacity, in which all the eviscerated organs regenerate. Especially in anterior evisceration species, the oral complex structure consisting of multiple organs, including the digestive tract and central nervous system, are reconstructed. The detailed developmental mechanisms underlying the process remains unclear, and therefore, in this study, focusing on *Eupentacta quinquesemita*, gene expression analyses in the regenerating tissues were carried out. For the formation of the gut tube, genes involved in mesenchymal-epithelial transition were upregulated consistently with the histological changes. Upregulation of Hox and Parahox genes along the anterior-posterior axis was observed, implying the involvement of these genes in the spatial differentiation of the digestive tract. In addition, the expression of *otx*, *six* and *pax*, i.e., transcription factors patterning anterior nervous tissues, was upregulated during the regeneration of the central nervous system. Taken together, these results suggest that conserved genes are co-opted to the internal organ regeneration after evisceration in sea cucumbers.

KEYWORDS

sea cucumber, evisceration, regeneration, digestive tract, central nervous system, mesenchymal-epithelial transition

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Introduction

Sea cucumbers possess high capacity of regeneration that enables them to reconstruct whole internal organs, including the digestive tract. They perform evisceration and eject internal organs as a type of autotomy in response to various extrinsic stimuli such as attacks by predators (Byrne, 2001). A small sea cucumber, *Eupentacta quinquesemita*, the species used in this study, is known to perform seasonal evisceration in addition to the response to physical stimulation (Byrne, 1985). Patterns of evisceration in sea cucumbers are classified into two major types, i.e., anterior and posterior evisceration (Mashanov and García-Arrarás, 2011). In contrast to posterior evisceration, in which only the digestive tract between the esophagus and cloacal stump is discarded, anterior evisceration seen in Dendrochirotida species (including *E. quinquesemita*) ejects the entire anterior structures, including the oral complex (Mashanov and García-Arrarás, 2011). The oral complex contains the nerve ring that is thought to function as a part of the central nervous system (CNS) in echinoderms (Mashanov et al., 2009).

Intense studies have been conducted for years, revealing the histological changes and molecular mechanisms of drastic regeneration in holothurians after evisceration. Both in anterior and posterior evisceration species, spatial and temporal dynamics of cellular processes such as mitosis, apoptosis and cell migration have been described in detail based on histological research (reviewed in Quispe-Parra et al., 2021b) and gene expression analyses suggested that remodeling of extracellular matrix (ECM) has key roles in the regeneration of eviscerated tissues (e.g., Ortiz-Pineda et al., 2009). In addition, RNA-seq analyses implied the involvement of various signaling factors in regeneration process (e.g., Sun et al., 2013). However, in contrast to the extensive knowledge about the cellular basis of intestinal regeneration in holothurians, the molecular mechanisms of larger scale, i.e., mechanisms which organize the drastic and complicated reconstruction of multiple organ complexes, are not fully understood.

During the regeneration, genetic programs used in embryonic development are often re-employed for reconstruction and repatterning of organs (Goldman and Poss, 2020). Thus, for understanding the regulation of complicated regeneration in holothurians, it should be important to investigate to what extent the conserved molecular mechanisms of embryonic development are co-opted in the fundamental process of regeneration, i.e., the reconstruction of the tubular structure of the digestive tract, regional differentiation of the digestive tract along the anterior-posterior (AP) axis, and regeneration of the central nervous system in the oral complex.

Regarding the reconstruction of the tubular structure of the digestive tract, histological observations in. *E. quinquesemita* (Figure 1A) suggested that the regeneration of the digestive tract after evisceration involves mesenchymal-epithelial transition (MET) in the anterior regenerating tissues (Figure 1B, Thiery and Sleeman, 2006; Chaffer et al., 2007; Okada and Kondo, 2019). MET refers to a phenomenon in which mesenchymal cells change their adhesiveness to acquire epithelial characteristics (Pei et al., 2019), and is histologically the opposite of the epithelial-mesenchymal transition (EMT) (Figure 2A). MET and EMT are known to contribute to epithelial regeneration and embryonic development (Ekblom, 1989; Kalluri and Weinberg, 2009; Rousselle et al., 2019). In both EMT and MET, a similar molecular mechanism is suggested to be activated, in which E-cadherin plays the major roles (Liu et al., 2016). In this mechanism, two key transcription factors, *snail* and *twist*, are known to be involved (Kang and Massagué, 2004; Barrallo-Gimeno and Nieto, 2005; Lamouille et al., 2014).

A holothurian individual possesses a digestive tract differentiated into specific parts such as the esophagus, stomach and intestine along the A-P axis (Hyman, 1955). In the sea cucumber *Apostichopus japonicus* (Holothuroidea), during embryogenesis, Hox genes are suggested to provide the spatial information, leading to the differentiation of digestive tract parts (Kikuchi et al., 2015). The expression patterns of Hox and Parahox genes during development have been investigated in some species of

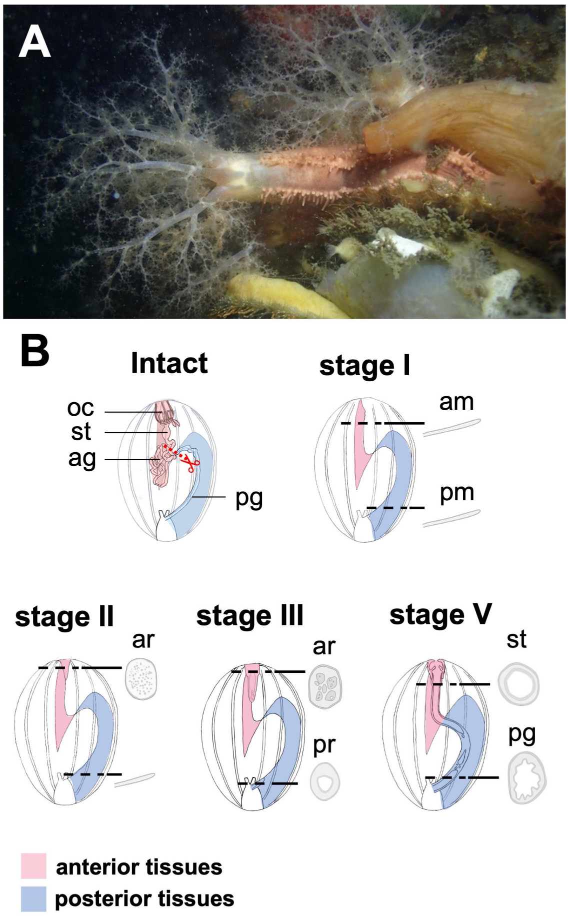

FIGURE 1
Regeneration process of digestive tract in a sea cucumber, *Eupentacta quinquesemita*, **(A)** The material species focused on in this study, an anterior-eviscerating sea cucumber, *E. quinquesemita*. **(B)** Schematic diagram of regeneration of the digestive tract during stages I and IV based on Okada and Kondo, 2019. MET occurs during stages II and III, and a tubular structure consisting of epithelium is newly formed inside mesenchyme. Ag, anterior gut; am, anterior mesentery; ar, anterior rudiment; oc, oral complex; pg, posterior gut; pm, posterior mesentery; pr, posterior rudiment; st, stomach.

echinoderms and shown to have a spatially colinear pattern along the A-P axis, indicative of their involvement in regional differentiation (reviewed in Byrne et al., 2016). In addition, RNA-seq analysis revealed that some Hox genes are upregulated during intestinal regeneration in a posterior eviscerating sea cucumber, *Holothuria glaberrima* (Quispe-Parra et al., 2021a), although the detailed spatial and temporal expression patterns of Hox and Parahox genes during the intestinal regeneration remain unclear.

In the case of anterior evisceration, not only the digestive tract but also the nerve ring, which constitutes the central nervous system in sea cucumbers, together with radial nerves in the body wall, is ejected (Byrne, 2001; Mashanov et al., 2005, 2009). The nerve ring is then regenerated at the same time as the digestive tract (Dolmatov, 1992). In various echinoderms, genes such as *otx*, *six3/6*, and *pax6*, which are involved in anterior neuronal patterning in deuterostomes (Hirth and Reichert, 1999), are expressed in the nerve ring and oral nervous system during development (e.g., Morris and Byrne, 2005). These three genes are known to play important roles in anterior neural patterning and sensory-organ formation. In addition, conserved neural factors such as *elav*, *mushasi* and *neuroD* are involved in the neurogenesis in the sea cucumber *Holothuria glaberrima* (Mashanov et al., 2015). However, the expression dynamics of these factors during the regeneration of the nerve ring have not yet been reported.

In this study, therefore, we conducted spatial and temporal expression analyses during regeneration after anterior evisceration using *E. quinquesemita*, focusing on MET-related genes, Hox and Parahox genes and neurogenesis- related genes *in situ*. We investigated the anterior regenerating tissues and the posterior regenerating tissues separately to clarify the spatial expression patterns, since the regeneration occurs independently in the anterior part and the posterior part, which then fuse to complete the regeneration (Figure 1B, Okada and Kondo, 2019). To obtain orthologous gene sequences in *E. quinquesemita*, transcriptome analysis was carried out to construct a gene database. In addition, in order to reveal the regeneration process of the nerve ring in this species, histological observations focusing on the nerve-ring regeneration were also carried out.

Materials and methods

Collection of *E. quinquesemita*, acclimatization, and experimental treatment

The sea cucumber, *E. quinquesemita*, was collected by diving at the depth of about 3 m in Tokyo Bay, near the pier of Hakkeijima Sea Paradise in Yokohama, Kanagawa Prefecture, or at the depth of about 5–10 m near Aquamarine Fukushima in Onahama, Fukushima Prefecture. Animals were kept in tanks until used for experiments. Methods for induction of evisceration and regeneration were based on Okada and Kondo, 2019. The day of evisceration was designated as 0 days post evisceration (dpe). Briefly, evisceration was induced by injection of approximately 100 μL of 0.45 M KCl into the coelom and the eviscerated animals were kept in aquaria with sea water at 13–18°C without feeding, up to 26 dpe. Embryos were collected after spontaneous

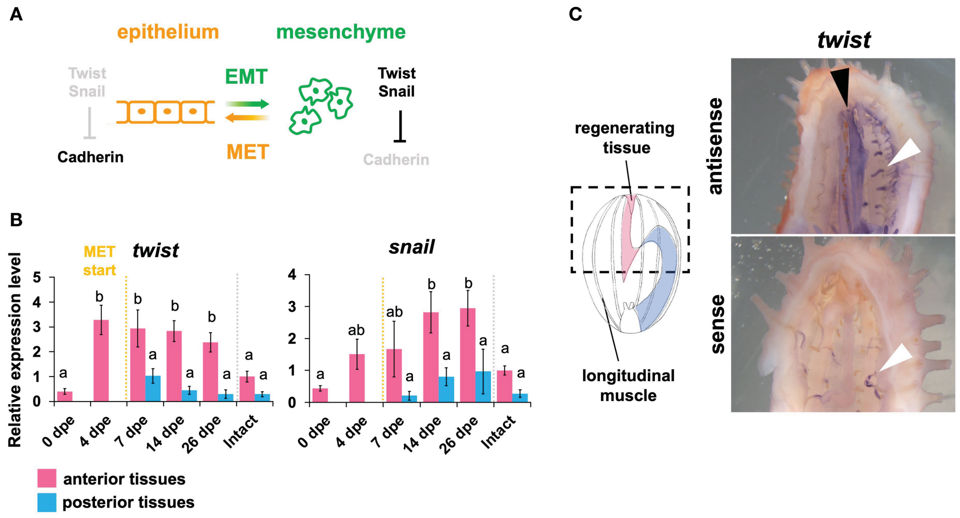

FIGURE 2
Expression pattern of MET- related genes in the regeneration of the digestive tract. **(A)** Schematic illustration of MET (mesenchymal-epithelial transition) and EMT (epithelial-mesenchymal transition) pathway. Two important transcription factors, i.e., Twist and Snail, play important roles in the regulation of Cadherin expression. At the time during which MET occurs and lasts, it is considered that the twist and snail expressions are down-regulated, while the cadherin expression is up-regulated. Based on Foroni et al. (2012). **(B)** Expression patterns of MET-related genes, i.e., *twist* and *snail*. Relative expression levels (mean ± S.D., n = 3) to the mean expression were calibrated using an internal control (EF1α). Horizontal axes show days post evisceration (dpe). The results were subjected to one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons tests ($p < 0.05$). Different letters above the bars indicate significant differences between groups. **(C)** *in situ* hybridization of *twist* at 4 dpe. Dotted line in schematic illustration indicates the region shown in right panels. The black arrowhead indicates signal detected in dorsal mesentery of the anterior regenerating part. Sense probes were used as controls. The white arrowheads indicate the ampullae of the water vascular system and they were also stained in the sense control.

spawning in laboratory tanks and kept in 2 L of filtered sea water in a plastic container at 20°C without feeding.

RNA-sequencing analysis

To identify MET-related genes, Hox and Parahox genes and neuronal patterning genes in the focal sea cucumber species, RNA sequencing was performed. Total RNAs were extracted from 2 embryonic stages (early and late gastrula) and juveniles of *E. quinquesemita* using QIAzol lysis reagent (Qiagen), and subsequently the RNA samples were pooled together. The pooled sample was then subjected to library preparation for RNA sequencing and sequenced on a Hiseq 4000 platform (Illumina) at a commercial service provider, Eurofins Genomics (Tokyo). The resulting 100-bp (base pairs) paired-end reads were deposited in the Sequence Read Archive of the DNA Data Bank of Japan (DDBJ) under the accession number DRA017482. Adapter and low-quality sequences were removed from the paired-end reads using Trimmomatic v0.39 (Bolger et al., 2014) with the following options: ILLUMINACLIP : TruSeq3-PE.fa:2:30:10, LEADING:20, TRAILING:20, SLIDINGWINDOW:4:20, MINLEN:25. Using the filtered reads, *de novo* transcriptome assembly was performed using Trinity v2.8.5 (Grabherr et al., 2011) with default options. To obtain the target orthologous genes, BLAST searches against the assembly were carried out with target gene orthologs in *Strongylocentrotus purpuratus* and *Apostichopus japonicus* (Tu et al., 2012; Zhang et al., 2017). The top-hit sequences from the *E. quinquesemita* transcriptome database were defined as putative orthologs of the target genes. To confirm the orthologs, reciprocal BLAST searches and phylogenetic analyses were performed (Supplementary Figures 1–7).

Real time quantitative PCR analysis

To analyze gene expression patterns by quantitative PCR, total RNAs were firstly extracted from the stages during regeneration after the evisceration in the focal sea cucumber species (Table 1). Since the regeneration requires about 3 weeks, samples were prepared at 0, 4, 7, 14, and 26 dpe. Regenerating digestive tracts were isolated by dissection and tissues from 2–4 individuals were pooled as each sample to obtain enough total RNAs. Separately from anterior and posterior digestive tracts, total RNAs were extracted by using QIAzol lysis reagent (Qiagen) and the RNA purification was performed with Agencourt AMPure XP (Beckman Coulter). Intact digestive tracts from mature individuals were also used for comparison. The extracted RNAs were reverse-transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantifications of target transcript levels were performed using Fast SYBR Green Master Mix and an ABI Prism 7500 instrument (Applied Biosystems). To evaluate endogenous control levels of constitutive expressions of putative reference genes, i.e., elongation factor 1 alpha (EF1α), tubulin beta chain (TUBB), NADH dehydrogenase (NADH) and ribosomal protein S18 (RPS18), were evaluated using geNorm (Vandesompele et al., 2002) and Normfinder (Andersen et al., 2004). The results indicated that EF1α was the most appropriate reference gene for comparisons among stages and body parts. Primers for qPCR (Supplementary Table 1) were designed using Primer Express software (ver. 3.0.0, Applied Biosystems). Data acquisition and analyses were performed using ABI Prism 7500 software ver. 2.0.4 (Applied Biosystems) with the relative standard curve method. For statistics, biological triplicates were subjected to one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons tests ($p < 0.05$) using R ver. 4.0.2.

In situ hybridization

To investigate the localization of target genes, whole mount *in situ* hybridizations (WISHs) were carried out, based on previous studies with minor modifications (Mashanov et al., 2010; Omori et al., 2011; Kikuchi et al., 2015). The PCR products were used as templates to transcribe riboprobes with a DIG RNA Labeling Kit (Roche). After dissection, the tissue samples were fixed with 4% paraformaldehyde in 0.5 M NaCl, 0.1 M 3-(N-morpholino) propanesulfonic acid (MOPS pH 7.0) overnight at 4°C. The samples were washed with phosphate buffered saline (PBS), and then decalcified with 0.5 M ethylenediaminetetraacetic acid (EDTA) (pH 8.0) in PBS for 3–4 days at room temperature. After decalcification, the samples were kept in 99.5% ethanol at –20°C until use. The samples were washed in PBS containing 0.1% Tween-20 (PBST), treated with 1/50 volume of

TABLE 1 List of tissues included in the samples for qPCR analysis.

		intact	0 dpe	4 dpe	7 dpe	14 dpe	26 dpe
	stage of regeneration		stage I	stage II	stage III	stage III	stage IV
anterior	dorsal mesentery	○	○	○	○	○	○
	anterior rudiment	–	–	○	○	○	–
	oral complex, stomach, anterior intestine	○	–	–	–	–	○
posterior	ventral mesentery	○			○	○	○
	posterior rudiment	–			○	○	–
	posterior intestine	○			–	–	○

Shaded columns were not sampled due to the small amount of tissues.

proteinase K (TaKaRa) in PBST for 20 min at 37°C, acetylated sequentially in 0.25% and 0.5% acetic anhydride in 0.1 M triethanolamine, 5 min each. After the samples were washed twice with PBST for 5 min each, prehybridization was performed at 58°C for 2 h or longer in hybridization buffer containing 50% formamide, 5× saline-sodium citrate (SSC), 100 µg/mL yeast RNA, 5× Denhardt's solution, 0.1% Tween 20. The riboprobes were diluted in hybridization buffer at 58°C to a final concentration of about 400 ng/ml and denatured at 80°C for 5 min. The hybridization was carried out at 58°C overnight. After hybridization, the samples were washed with 50% formamide in 5× SSC at 58°C for 20 min, 5× SSC at 58°C for 50 min, 2× SSC at 58°C for 50 min, 0.1× SSC at 58°C for 15 min and then PBST at room temperature for 15 min twice. Subsequently, the samples were incubated in 0.1% blocking reagent (Roche) in PBST (blocking buffer) at room temperature for 30 min, followed by an incubation in 1/2000 volume of anti-DIG-AP (Roche) in blocking buffer at 4°C overnight. Following a wash with PBST (15 min, 8 times), immunodetection was performed using BM purple (Roche) at room temperature. After detection, the samples were washed with PBST and then kept in 10% formalin/PBS.

Histological observations on the regeneration of nerve ring

Animals were anesthetized in 72 g/L $MgCl_2$ in sea water for approximately 15 minutes to 1 hour before fixation. An individual body was dissected along the anterior-posterior (oral-aboral) axis of the body at the right ventral interambulacral zone, exposing the whole-body cavity, and dissected into anterior and posterior parts. The dissected bodies were fixed in Bouin's Fixative or 4% paraformaldehyde in 0.5 M NaCl, 0.1 M 3-(N-morpholino) propanesulfonic acid (MOPS pH 7.0) overnight at 4°C. The specimens were washed with PBS, and then decalcified with 0.5M EDTA (pH 8.0) in PBS for 3–4 days at room temperature. After decalcification, the specimens were kept in 99.5% ethanol at −20°C until embedding in paraffin (Paraplast X-TRA, SIGMA) for histological observation. Serial sections of 6- µm thickness were produced and were stained with hematoxylin-eosin (HE) or toluidine blue (TB) and observed under a light microscope (ECLIPSE TE300, Nikon). Sections were mostly made perpendicular to the anterior-posterior axis of the body. In total, 4 animals were used for histological observations.

Results

RNA-sequencing and ortholog searches

RNA-sequencing analysis of the transcriptome derived from gastrula embryos and juveniles of *E. quinquesemita* yielded 28,686,525 pairs of 100-bp reads. After filtering, 26,497,155 paired reads were retained. *De novo* transcriptome assembly of the sequence reads generated 350,742 contigs with a total of 296,553,944 bases and N50 of 1,513 bp. Using the transcriptome data, a gene database was constructed to search target genes, i.e., MET-related genes, Hox genes and neural patterning genes. As a result, the gene orthologs from the focal sea cucumber species were obtained by reciprocal BLAST searches and by constructing phylogenetic trees (Supplementary Figures 1–3). Based on the obtained gene sequences from the RNA-sequencing result, primers for qPCR were designed (Supplementary Table 1).

Expressions MET-related genes

Firstly, the expression patterns of MET-related genes, i.e., *twist* and *snail*, during the regeneration after anterior evisceration in *E. quinquesemita* were investigated. The results clearly showed that both of these genes were highly expressed in the anterior tissues of the regenerating digestive tract, in comparison with the posterior ones (Figure 2B). At 0 dpe, the expression level of *twist* in the anterior part was still low, but the *twist* expression became highly upregulated at 4 dpe, and was gradually decreased at the later stages. In the case of *snail*, the expression level was gradually upregulated, reaching the highest expression level at around 14–26 dpe, at the time when the *twist* expression started to decrease. The results of whole mount *in situ* hybridization showed that *twist* was expressed in anterior mesenteries (Figure 2C). On the other hand, *in situ* signals were not detected for *snail*.

Expressions of Hox genes

Anterior Hox genes such as *hox1*, *hox5*, and *hox8* were highly expressed in the anterior tissues, while posterior Hox genes such as *hox9/10*, *hox11/13/a*, and *hox11/13b* were highly expressed in the posterior ones (Figure 3A). No difference between anterior and posterior tissues was detected in the expression levels of *hox7*. Temporal expression peaks varied among the Hox genes. *hox5* showed the highest expression level at 0 dpe in anterior tissue, while *hox 8* showed it at 4 dpe in anterior tissue. For *hox1, hox9/10, hox11/13a*, and *hox11/13b*, the temporal expression peak occurred at 14–26 dpe.

Expressions of Parahox genes

Like Hox genes, Parahox genes, i.e., *gsx*, *xlox*, and *cdx*, also showed colinear expression patterns: *xlox* was expressed in anterior tissues, while *cdx* was expressed exclusively in posterior tissues (Figure 3B). These genes were expressed at a later stage of regeneration, after 14 dpe. Among the three examined Parahox genes, *gsx* showed a distinctive pattern, in which no difference was seen between anterior and posterior tissues, and the expression level was highest at 7 to 14 dpe.

Regeneration of nerve ring

Histological observations clearly revealed the process of nerve ring regeneration (Figures 4B–E). At the time when anterior evisceration occurred (0 dpe), the anterior end of the sea cucumber body shrank to close the wound, so the radial nerve tissues came close to each other. At the early stage (4 dpe), neural tissues showing histological characteristics of cell proliferation extended from wounded radial nerves in the body wall, entering the regenerating tissues at the center of the body trunk (Figure 4B). At 6 and 12 dpe, the apical ends of regenerating nerves were elongated in the perpendicular direction, forming ring structures by connecting to each other (Figures 4C, D). At 12 dpe, an almost complete ring-like structure was formed by the elongated neural tissues connecting to each other (Figure 4D). At 21 dpe, the formation of the nerve ring was completed (Figure 4E). In addition, at 12 dpe, tentacular canals of the water vascular system were apparent (Figure 4D) and at 21 dpe, regenerating tentacles were observed (Figure 4E).

Expressions of neural patterning genes

Orthologous genes of 6 neural patterning genes, i.e., *otx, six3/6, pax6, elav, musashi*, and *neuroD*, were obtained from the gene database in *E. quinquesemita* (Supplementary Figures 4–7). Based on the sequences of the gene orthologs, primers for qPCR were designed (Supplementary Table 1). F or *otx, six3/6*, and *pax6*, the expression levels were higher in the anterior tissues, and almost no expression was detected in the posterior tissues (Figure 5). For *otx* and *six3/6*, the expression peaks were seen at 4 dpe, and then the expression levels were decreased. For *pax6*, the expression peak was seen at 26 dpe. A neural marker gene, *elav*, showed a strong expression level immediately after the evisceration (0 dpe) in the anterior tissue (Figures 3, 4). The expression level of *musashi*, which is involved in neural differentiation was slightly higher in the anterior tissues, but it was relatively constant over the entire regeneration period. For *neuroD*, no difference in expression levels was detected between anterior and posterior tissues, or among regeneration stages (Figure 5).

Discussion

MET factors involved in digestive-tract regeneration

In general, *twist* and *snail* suppress cadherin expression (Foroni et al., 2012) and the downregulation of cadherin genes leads to the maintenance of mesenchyme (Figure 2A, Kang and Massagué, 2004; Barrallo-Gimeno and Nieto, 2005; Lamouille et al., 2014; Pei et al., 2019). Thus, our results suggest that the expression level of cadherin could be low in anterior regenerating tissues, that is, the number of mesenchyme cells should be increased, along with the upregulation of *twist* and *snail*. This process is consistent with the histological observations showing that mesenchymal cells proliferated at the earlier stages of digestive-tract regeneration in this species (Okada and Kondo, 2019). In addition, the timing of *twist* expression was earlier than that of *snail* expression (Figure 2B), which is congruent with a report showing that *snail* expression is regulated downstream of *twist* (Smit et al., 2009). Therefore, this time difference between the expressions of these two factors is suggested to reflect the epistatic relationship between the two factors.

Recent RNA-seq analysis in the related species *Eupentacta Fraudatrix*, which also shows anterior evisceration, revealed the upregulation of EMT-related factors such as *snai2* and *id2* during regeneration after evisceration (Boyko et al., 2020). It was also suggested in a posterior eviscerating species that EMT was involved in the intestinal regeneration (García-Arrarás et al., 2011). Therefore, it is possible that induction of EMT at the onset of intestinal regeneration via regulators such as *twist* and *snail* is widely shared among holothurians.

On the other hand, histological observations suggest that MET is involved in the digestive tract regeneration of anterior tissues of *E. quinquesemita*, in which epithelial tissues appeared among mesenchymal tissues (Okada and Kondo, 2019). Although the qPCR results in this study suggest that EMT occurs in anterior regenerating tissues as a whole, down regulation of *twist* and *snail* should occur locally at the tissues where the epithelial formation occurs after 7 dpe. Histological investigation suggested that MET does not occur in the anterior regenerating tissues of *E. fraudatrix* (Mashanov and Dolmatov, 2001, Mashanov et al., 2005), so the dynamics of gene expression related to MET might be different between species.

Expressions of Hox/Parahox genes during the digestive-tract regeneration

The results of this study revealed a tendency for anterior Hox genes to be expressed anteriorly while posterior ones were expressed posteriorly in regenerating tissues (Figure 3A). It is known that, in sea urchin larvae, *xlox* is expressed at the border between stomach and gut (Arnone et al., 2006). This is consistent with our result in which *xlox* was expressed in the anterior region, since the border in *E. quinquesemita* is located at a relatively anterior portion of the digestive tract (Byrne, 2001; Okada and Kondo, 2019). As shown by histological observations, the developmental speed in posterior regenerating tissues is a bit slower than that in anterior tissues (Okada and Kondo, 2019). Therefore, the expression of *cdx*, which is required for formation of the posterior digestive tract, was also seen at later stages of the regeneration process (Figure 3B). The expression of another Parahox gene, *gsx*, is known to be localized at a part of the neural tube in the development of amphioxus (Brooke et al., 1998; Arnone et al., 2006). It is generally believed that, in ancestral animals, *gsx* is expressed at the anterior (or oral) side of the digestive tract (Samadi and Steiner, 2010), although there are many animal species that do not exhibit *gsx* expression in their digestive tracts (Wollesen et al., 2015). In this study, *gsx* expression was shown to peak during the middle period of regeneration

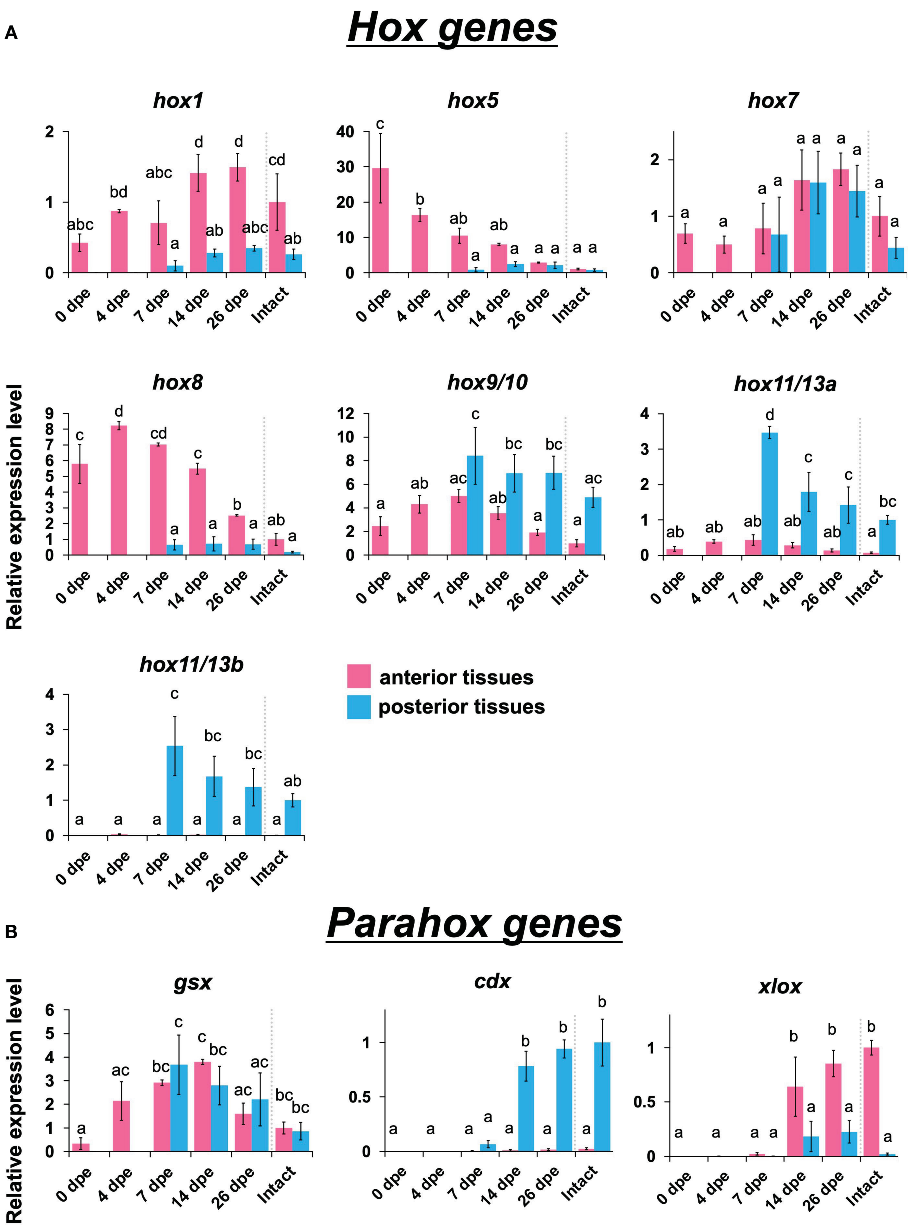

FIGURE 3
Expression profiles of Hox and Parahox genes in the regeneration after evisceration. Expression levels of Hox and Parahox genes were quantified by qPCR. Relative expression levels (mean ± S.D., n = 3) to the mean expression were calibrated using an internal control (EF1α). Horizontal axes show days post evisceration (dpe). The results were subjected to one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test ($p < 0.05$). Different letters above the bars indicate significant differences between groups. **(A)** Expression profiles of *hox1, hox5, hox7, hox8, hox9/10, hox11/13a* and *hox11/13b*. **(B)** Expression profiles of three neural patterning genes, i.e., *gsx, cdx* and *xlox*.

(Figure 3B), suggesting that this gene shows diverse expression patterns depending on the animal species.

Overall, our results suggest that Hox and Parahox genes provide spatial information along the A-P axis for digestive-tract differentiation. Time differences of the expression peaks among Hox/Parahox genes indicate that the timepoints for determination and differentiation vary among digestive-tract parts. Upregulation of Hox genes in the regenerating intestine has been reported in several holothurians (e.g., Méndez et al., 2000), warranting further investigation of the conservation and diversification of detailed gene expression patterns and histology among species, especially anterior eviscerating species and posterior eviscerating species.

Regeneration of central nervous system

Our histological observations focusing on the nervous tissue regeneration showed that the nerves were regenerated from the anterior ends of the remaining radial nerve cords, and later connected to each other to form the ring structure (Figure 4F). A

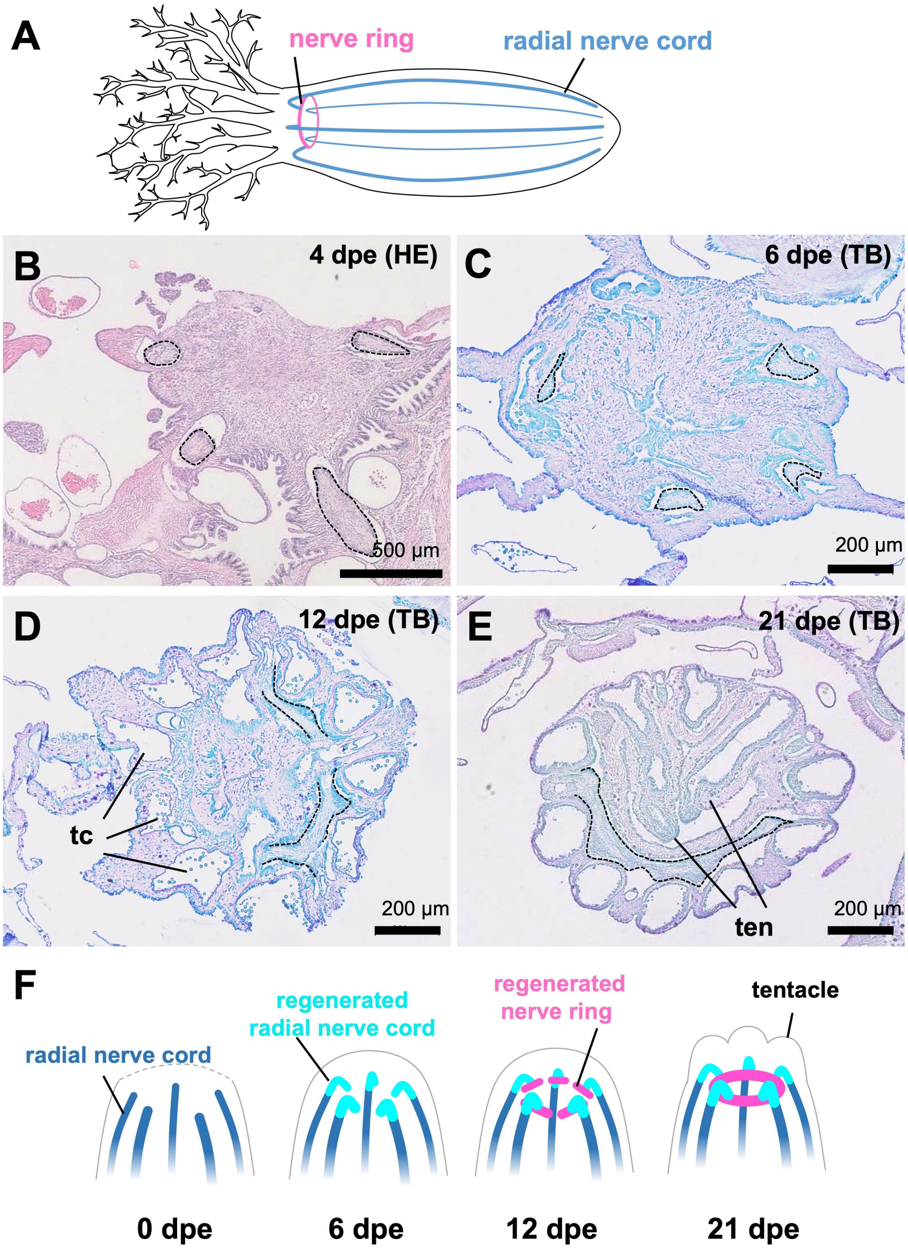

FIGURE 4
Regeneration process of nervous system in *E. quinquwsemita.* **(A)** Nervous system of *E. quinquesemita*. It consists of the nerve ring (also known as the circumoral nerve ring), tentacular nerves and radial nerve cords. **(B–E)** Histological images showing the regeneration process of circumoral nerve ring. Cross sections of the anterior regenerating tissues of *E. quinquesemita* were stained with hematoxylin-eosin (HE) or toluidine blue (TB). Dotted line indicates the outline of nervous tissue. **(F)** Schematic illustration of nervous system regeneration based on histological observation. Anterior parts in regenerating individuals are presented. tc, tentacular canal; ten, tentacle.

similar regeneration pattern is known in the closely related species *E. fraudatrix* (Dolmatov, 1992). Since seasonal evisceration is known in *E. quinquesemita* (Byrne, 1985), the complete renewal of the nerve ring observed in these species might play a key role in their lifecycle.

Gene expression analyses by real time qPCR showed that the neural patterning genes *otx, six3/6* and *pax6* were upregulated in the anterior body part during the regeneration process. In particular, *otx* and *six3/6* were upregulated at a relatively early stage of regeneration (4 dpe, Figure 5). The timing of upregulation corresponds to the timing when the regenerating nerve tissues start to enter the regenerating tissues composed of mesenchymal cells that later form the digestive tract. This may suggest that *otx* and *six3/6* are required for the differentiation and elongation of nerve tissues around the digestive tract. It was previously shown that, in the echinoderm species *Holopneustes purpurescens* (Echinoidea), the *otx* gene is

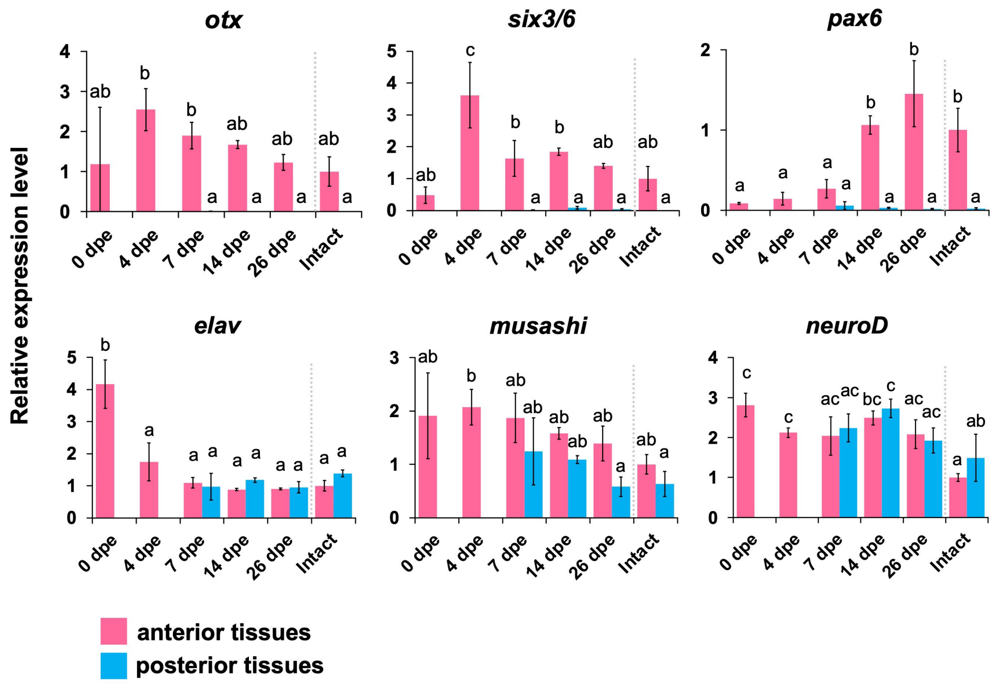

FIGURE 5
Expression profiles of genes that are known to play important roles in the development of the nervous system. Expression profiles of genes that are known to play important roles in the neuronal patterning (*otx, six3/6, pax6*) and neurogenesis (*elav, musashi, neuroD*) in regeneration stages, quantified by qPCR. Relative expression levels (mean ± S.D., n = 3) to the mean expression were calibrated using an internal control (EF1α). Horizontal axes show days post evisceration (dpe). The results were subjected to one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons tests ($p < 0.05$). Different letters above the bars indicate significant differences between groups.

expressed in the nerve ring during larval stages (Morris et al., 2004; Morris and Byrne, 2005). In a feather star, *Anneissia japonica*, *otx* and *six3* are expressed in oral nervous tissues (Omori et al., 2020). Therefore, it is suggested that neural patterning genes such as *otx* and *six3/6* are co-opted from normal development to the re-formation of the nerve ring after anterior evisceration.

On the other hand, *pax6* was upregulated later in the regeneration process (14–26 dpe). Around at 12 dpe, histological observations showed that the morphological characters of tentacles became apparent (Figures 4D, E). As it is known that *pax6* is expressed in tentacles in adults of the sea cucumber *Apostichopus japonicus* (Liu et al., 2020), the upregulation of *pax6* associated with the tentacular formation is consistent with previous knowledge. In other holothurians, RNA-seq analyses suggest that conserved paracrine factors involved in embryonic development such as *wnt6, wnt9*, and *bmp1* are upregulated during intestinal regeneration (Ortiz-Pineda et al., 2009; Sun et al., 2013; Yuan et al., 2019; Auger et al., 2023) and *notch* is upregulated in nerve cord regeneration (Mashanov et al., 2014). It is known that, in regeneration processes in vertebrates, expressions of transcription

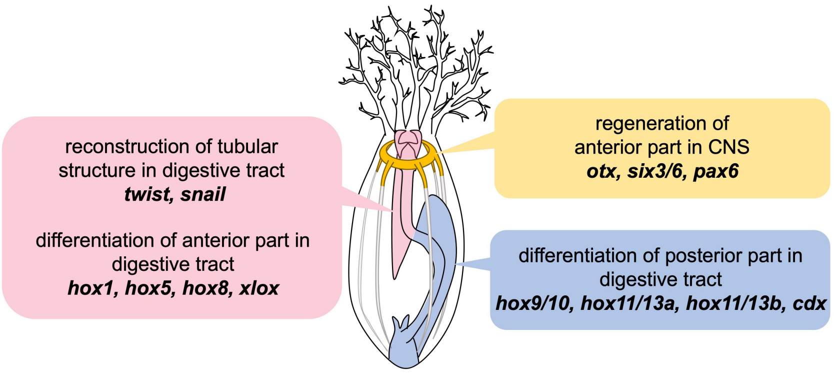

FIGURE 6
Schematic illustration of results in this study. Presented genes are suggested to be involved in the individual steps during regeneration after anterior evisceration in *E. quinquesemita*.

factors such as *pax*, *six* and *hox* genes are regulated downstream of the signals such as the bmp and wnt pathways (e.g., Grogg et al., 2005; Patel et al., 2022), so similar epistatic relationships are also suggested in the regeneration in echinoderms.

Conclusions

Based on our results, it is suggested that the regeneration after anterior evisceration in *E. quinquesemita* employs three conserved mechanisms: the digestive tract formation via EMT and MET under the regulation of *twist* and *snail*, spatial differentiation of the digestive tract along the A-P axis by Hox and Parahox genes, and reconstruction of the nerve ring by recruiting the conserved neuronal patterning genes (Figure 6). Understanding how these mechanisms relate to the upstream regulators will be important in order to clarify the whole regulatory system in the regeneration after evisceration in holothurians. Since the loss of the digestive tract or central nervous system is lethal in many vertebrates, understanding the rules that regulate the regeneration which reconstructs multiple organs in holothurians should offer new insights into deuterostome regeneration and regenerative medicine for indispensable organs.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here in the Sequence Read Archive of the DNA Data Bank of Japan (DDBJ) under the accession number DRA017482.

Ethics statement

The animal study was approved by Life Science Research Ethics and Safety, The University of Tokyo. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

AO: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Visualization, Writing – original draft, Writing – review & editing. SU: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. HK: Conceptualization, Investigation, Resources, Writing – review & editing. YH: Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. TM: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Aquaculture of emerging species in North-Eastern Atlantic and Mediterranean Sea: a systematic review on sea cucumber farming and potential development

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Sea cucumbers are considered a traditional food delicacy in Asian countries. In last decades high market demand, uncontrolled exploitation and inadequate management have led to stock overexploitation, driving commercial interest in new target species and new areas. In recent years, Mediterranean and North-East Atlantic sea cucumbers have become fishing targets, and, due to overfishing, a significant decline of wild populations have been observed, with detrimental effects on benthic communities and ecosystems. Indeed, in addition to being an important economic resource, sea cucumbers play a key ecological role in benthic dynamics where they are involved as ecosystem engineers in the processing of organic matter in the detrital food web pathway. In this context, aquaculture can play a crucial role in supporting the conservation of natural stocks by reducing harvesting pressure on wild populations and enabling restoration programmes for depleted stocks. Commercial sea cucumber farming is already an established reality in the Indo-Pacific region, where it has become one of the most profitable sectors of aquaculture production. In Europe, on the other hand, sea cucumber aquaculture is still an emerging sector, whose development is hampered by poor and fragmented information on the farming requirements of native species. In this context, this systematic review was carried out with the aim of providing a comprehensive summary of the state of the art of farming practices of the European sea cucumbers considered as a target of commercial fisheries. A total of 34 original articles were included in this review, after title, abstract and full-text screening. The results highlighted a sharp increase in the number of studies over the last six years. Portugal, Turkey and Italy are the countries where most research has been carried out. Among the selected species, *Holothuria tubulosa* was the most studied, while only one article was found on *Parastichopus regalis*. The main aims of this review were i) to gather all available literature on the farming practices of North-East Atlantic and Mediterranean sea cucumber species, ii) to outline the most common farming practices, iii) to identify gaps and future directions in this emerging aquaculture sector.

KEYWORDS

holothurian, echinoderm, Trepang, IMTA, co-culture

1 Introduction

Holothurians, or sea cucumbers, have been a popular and traditional food in Asian countries since ancient times and, today, represent an economically important market due to their high market value (Conand, 2018; Purcell et al., 2023). Sea cucumbers are processed and preserved as a dried product, commonly called "Trepang" or "Bêche-de-Mer" in the global market. The edible part of the animals, the body wall, is an appreciated food, characterised by a high protein content and a low concentration of fats, a large proportion of which are ω3-fatty acids, molecules of great value for their beneficial effects on human health (Aydin et al., 2011; Roggatz et al., 2018). Sea cucumbers are also used in traditional Chinese medicine for their bioactive compounds with anti-tumoral, antioxidant and anti-inflammatory properties, and are also applied in the cosmetic industry (Santos et al., 2016). In the Indo-Pacific region, the high harvesting pressure on sea cucumbers has led to the overexploitation of many natural stocks, even towards lower-value species (Conand, 2018). Similarly, due to the decline in tropical species abundance, Mediterranean and Atlantic sea cucumbers have recently been subject to strong fishing pressure, with some European countries starting to export their wild catches to the main Asian markets (Taiwan, Hong-Kong or Singapore; Conand, 2018). Several species inhabit European seas, but only eight are currently considered of commercial interest (Purcell et al., 2023; Rakaj and Fianchini, 2024). *Holothuria poli* (Delle Chiaje, 1823), *H. tubulosa* (Gmelin, 1791), *H. sanctori* (Delle Chiaje, 1823), *H. forskali* (Delle Chiaje, 1823), *H. arguinensis* (Koehler & Vaney, 1906) and *H. mammata* (Grube, 1840) belonging to the family Holothuriidae, *Parastichopus regalis* (Cuvier, 1817) and *P. tremulus* (Gunnerus, 1767) belonging to the family Stichopodidae, are the species that have recently experienced a significant increase in catches to meet the demands of international markets. *P. regalis*, is the only species that is consumed in Europe and precisely in coastal localities of Catalonia and Valencia regions, where the muscular bands are extracted and commercialized as a culinary delicacy known as "espardeña". In the European Atlantic *H. arguinensis, H. forskali*, and *H. mammata* are collected, while in the Mediterranean Sea the most harvested species are *H. poli, H. tubulosa* and *H. mammata*, with Turkey being the main exporter (González-Wangüemert et al., 2018; Dereli and Aydın, 2021; Aydin et al., 2023; Rakaj and Fianchini, 2024).

The increase in harvesting pressure has led to a rapid decline in natural stocks of marketable species, resulting in reduction of abundance, size and genetic diversity, with detrimental effects on benthic communities and ecosystems (González-Wangüemert et al., 2014, 2015; Maggi and González-Wangüemert, 2015; Ventura et al., 2023). Indeed, in addition to being an important economic resource, sea cucumbers play a key ecological role as ecosystem engineers, and their decline may affect the entire ecosystem. In fact, sea cucumbers, being deposit-feeders, ingest large amount of sediment contributing to nutrient cycling and energy fluxes in benthic ecosystems, regulating dissolved oxygen and nutrient concentration and promoting nitrification and denitrification processes (Purcell et al., 2013; Boncagni et al., 2019).

To counteract stock depletion and protect natural populations from the spread of illegal, unreported and unregulated (IUU) fishing, some countries have introduced restrictions to regulate harvesting and catches. In Galicia, the harvest of *H. forskali* has been regulated since 2016 (González-Wangüemert et al., 2018). In Italy, the increasing number of massive and uncontrolled catches, especially in the southern regions, led the Italian Ministry of Agriculture, Food and Forestry (MIPAAF) to impose a ban on "fishing, holding on board, transhipping, or disembarking of sea cucumbers". The ban has been extended annually pending the establishment of a scientific framework to support the adoption of structural measures in the management of sea cucumber stocks (Ministerial Decree n.156 of 02/27/2018).

In Asia, particularly in China and Japan, farming practices of the most popular species have been developed since the 1950s to make up for the lack of wild sea cucumbers and, in particular, aquaculture activities are focused on *Apostichopus japonicus* (Selenka 1867), the most prized temperate species (Brown and Eddy, 2015). In China, the cultivation of this species allows the production of juveniles (6 billion per year) to be reared at sea, in cages and in ponds, producing more than a quarter of the global wild catch in dry weight (Purcell et al., 2012).

As in the Indo-Pacific, aquaculture could be a potential solution for North-Eastern Atlantic and Mediterranean sea cucumbers. The farming system could sustain market demand by counteracting harvesting pressure on wild stocks, and the development of full-cycle production may support restocking programmes in depleted areas. Finally, the diffusion of sea cucumber aquaculture practices may contribute to the diversification of aquaculture products and the development of Multitrophic Integrated Aquaculture models (IMTA) which are essential to cope with principles of sustainable development. In this context, a systematic review of the available literature on the status of sea cucumber aquaculture in Europe was carried out. Specifically, among the species found in the North East Atlantic and the Mediterranean Sea, those currently subject to fishing activities were selected (Purcell et al., 2023) and all literature found was reviewed with the aim of providing a comprehensive summary of the most common farming practices and identifying gaps and future directions in this emerging area of aquaculture.

2 Materials and methods

Scientific literature was searched in Web of Science (https://www.webofscience.com) and Scopus (https://www.scopus.com) from 2000 to 21 August 2023, using the following search string:

"*Holothuria arguinensis*" OR "*Holothuria forskali**" OR "*Holothuria mammata*" OR "*Holothuria poli**" OR "*Holothuria sanctori*" OR "*Holothuria tubulosa*" OR "*Parastichopus regalis*" OR "**Stichopus tremulus*" AND "*cultur*" OR "IMTA" OR "farm*" OR "rear*" OR "diet*" OR "feed*" OR "food*" OR "bioremediat*" OR "bioturbat*" OR "nutriti*" OR "growth" OR "biomass" OR "*product*".

Only original articles (i.e. experimental field studies, observational studies) published in English and in full text, providing information on rearing practices were considered eligible. In particular, studies on culture conditions (feed, temperature, stocking density, salinity, sediment size) and reproduction (spawning induction, larval development, juvenile culture) were included in the present review.

Exclusion criteria were: (i) studies on the ecology and biology of selected species; (ii) studies on the contribution of holothurians to pollution control without information on their fitness or growth; (iii) studies on the quality of species of commercial interest; (iv) studies on bioactive compounds of pharmaceutical or biotechnological applications.

Titles and abstracts of articles identified by the search strategy were reviewed independently by two authors, and full-text articles potentially meeting the inclusion criteria were retrieved. From the full texts, the same two authors independently established whether the studies met the inclusion criteria. Studies that did not fulfil all the inclusion criteria were excluded, and the reasons for exclusion were reported. Any disagreements were resolved by consensus. If the two review authors disagreed, a third review author was involved to resolve the disagreement. The review authors were not blinded to authors, journals, results, etc.

3 Results

The search yielded a total of 417 results, 253 in Web of Science and 164 in Scopus. 79 items were removed (68 in Web of Science and 11 in Scopus), resulting in 338 articles. Duplicates (125) were removed and the remaining 213 articles were screened for inclusion or exclusion (Figure 1). After title and abstract screening, 161 articles were excluded and 52 articles were selected for full-text screening. After final screening, 34 articles were retained for review (Supplementary Table S1).

The distribution over the years showed that production was low from 2000 to 2015 with only 5 articles published, 1 in 2005 and 2013 and 3 in 2015 (Figure 2). In contrast, from 2017 to 2023 (August), 29 studies were conducted on North-Eastern Atlantic and Mediterranean sea cucumber aquaculture, with a peak in 2018 with 6 articles published.

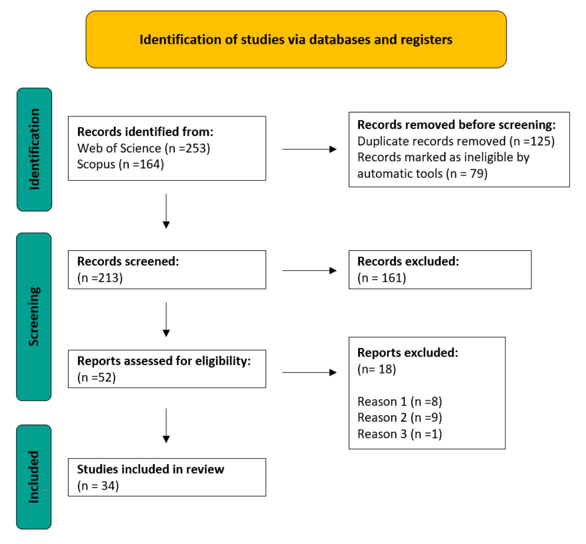

FIGURE 1
Search strategy following the PRISMA flow diagram. Reason 1: Articles on ecology and biology of selected species; Reason 2: Articles on the contribution of holothurians to pollution abatement without information on their well-being or ability to grow in those conditions; Reason 3: Articles on molecules of pharmaceutical or biotechnological interest.

Among the 34 articles included in the review, the highest number of studies was found in Portugal (11), followed by Turkey (6), Italy (5), Spain, France, Sweden, Norway and Malta (2), Egypt, Greece and United Kingdom (1) (Figure 3). Among the selected species *Holothuria tubulosa* was the most investigated with 11 papers, followed by *H. arguinensis* and *H. poli* with 8 articles each, while only 1 paper dealt with on *Parastichopus regalis* (Figure 4). Most of the included papers were related to culture conditions to improve fitness and promote fattening of adult or juvenile sea cucumbers.

Feed was the most studied issue, with 10 experiments conducted under integrated multitrophic aquaculture (IMTA) conditions, in mariculture (5) or in-land systems (5). Fish were the most commonly associated species in IMTA studies, with only two experiments combining sea cucumbers with other invertebrates. Temperature and stocking density were the most commonly investigated variables. Only 9 studies were conducted on artificial reproduction and larval rearing, focusing on spawning induction methods, larval feeding regimes and larval development (Figure 5).

Among the outcomes used to evaluate the response of sea cucumbers to culture conditions, the most investigated were survival (survival, survival rate or mortality rate) and growth (specific growth rate, growth rate, weight gain, relative weight gain) (Figure 6).

4 Discussion

Given the recent increase in sea cucumber catches and trade in North-Eastern Atlantic and Mediterranean countries, the development of aquaculture practices represents a promising solution to protect natural populations without losing the economic

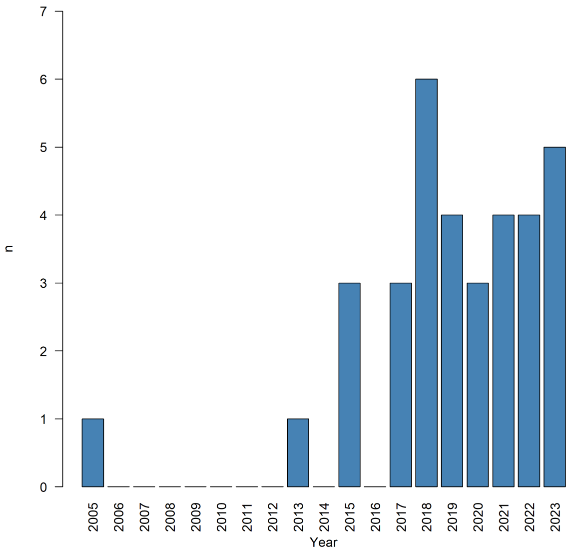

FIGURE 2
Distribution of publications across the years.

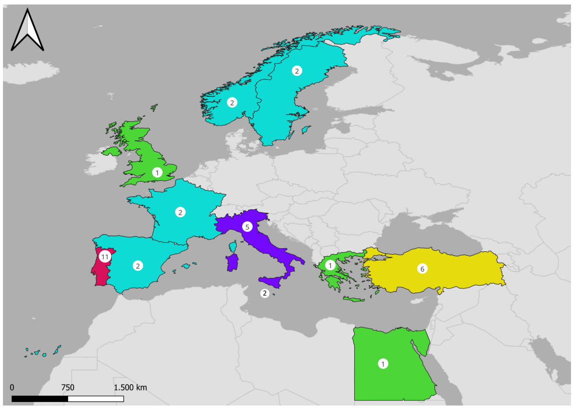

FIGURE 3
Geographical distribution of the studies included in the review.

business generated by sea cucumber sales. The findings of this systematic review showed a sharp increase in scientific production related to sea cucumber farming in recent years (from 2017), probably in response to the natural stock depletion observed for commercial holothurians. The countries most involved in scientific research were Portugal and Turkey, which have recently reported the first signs of stock depletion in their seas (González-Wangüemert et al., 2014, 2015; Maggi and González-Wangüemert, 2015). Similarly, the most investigated species were *Holothuria tubulosa*, *H. poli* and *H. arguinensis*, Atlantic species also reported in the western Mediterranean Sea (Rodrigues, 2012; González-Wangüemert et al., 2014; Mezali and Thandar, 2014). Surprisingly, only one study was found for *Parastichopus regalis*, the most valuable European species (Roggatz et al., 2018). This is probably because this species occurs at great depths (50 - 1200 m), making it more difficult to study than shallower water species (Ramón et al., 2022). The results showed that a much high percentage of papers focused on the study of environmental variables and feeding, as optimal culture conditions are fundamental to ensure animal fitness and promote growth of sea cucumbers at all life stage. Most studies were conducted on adult specimens, whit less attention paid to larval and juvenile stages and, in particular, studies on integrated multitrophic aquaculture (IMTA) have only been carried out on sub-adult and adult specimens. Knowledge of the biology and ecology of European sea cucumber species is generally poor and almost absent for the early stages of the life cycle. Indeed, to date, there are no full-cycle farming systems able to produce juveniles and the few facilities working on the reproduction process under controlled conditions are still at the experimental stage. It is therefore not surprising that most experiments have been conducted on large specimens, which are easy to find in the wild and can be handled under controlled conditions. The large number of outcomes used to assess the response to experimental conditions also underlines the lack of defined protocols and consolidated information. Although several authors analysed the survival and growth ability of sea cucumbers, the data are not always comparable. For example, to express sea cucumber growth, some authors use total wet weight, others eviscerated weight or dry weight, while in some cases length measurements are also included. However, the ability of holothurians to change in size and weight significantly depending on the amount of water contained in their bodies makes growth determinations critical, which is probably the reason for such different measurement protocols. The lack of knowledge on the biology and ecology of marketable North-Eastern Atlantic and Mediterranean sea cucumbers is one of the main bottlenecks for the development of their culture. In particular, little information is available on their natural food sources and even less is known about effective diets that could promote the growth of sea cucumbers in culture condition at each stage of their life cycle. As the most investigated topics were reproductive techniques for hatchery production and culture conditions to promote sea cucumber growth, these two categories were used to organise the information of the reviewed articles.

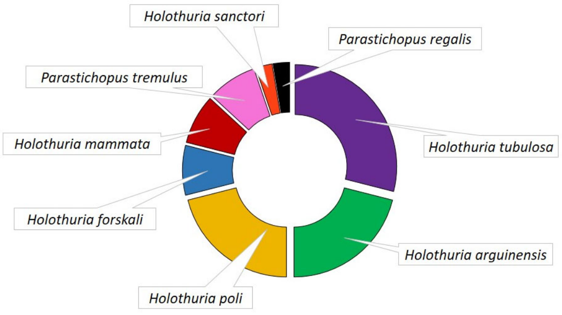

FIGURE 4
Frequency of the species used in the selected studies.

4.1 Hatchery productions

4.1.1 Spawning induction

The development of an effective protocol for full-cycle production is fundamental to counteract fishing pressure on natural sea cucumber stocks, as it would reduce the dependence of culture practices and market demand on wild specimens. Two studies have been conducted on the use of asexual reproduction with encouraging results the (Toscano and Cirino, 2018; Moussa et al., 2019), while other experiments have been carried out aiming at the production of larvae and juveniles in hatcheries. Protocols for artificial reproduction are available for all North-Eastern Atlantic and Mediterranean sea cucumbers of commercial interest, with the exception of *P. regalis*. Induction of gamete emission is a key challenge to optimise spawning and fertilisation rates in invertebrate hatcheries, so it is important to know the appropriate spawning stimulation method for each species. For *H. arguinensis* and *H. mammata*, spawning was successfully induced by thermal stimulation (gradual increase in water temperature) (Domínguez-Godino et al., 2015; Domínguez-Godino and González-Wangüemert, 2018a). Differently, for *H. poli* (Rakaj et al., 2019), *H. forskali* (Laguerre et al., 2020) and *P. tremulus* (Schagerström et al., 2022), spawning was obtained with thermal shock (rapid increase in water temperature). A combination of induction methods was used to induce gamete release in *H. tubulosa* (thermal stimulation and thermal shock, Rakaj et al.,

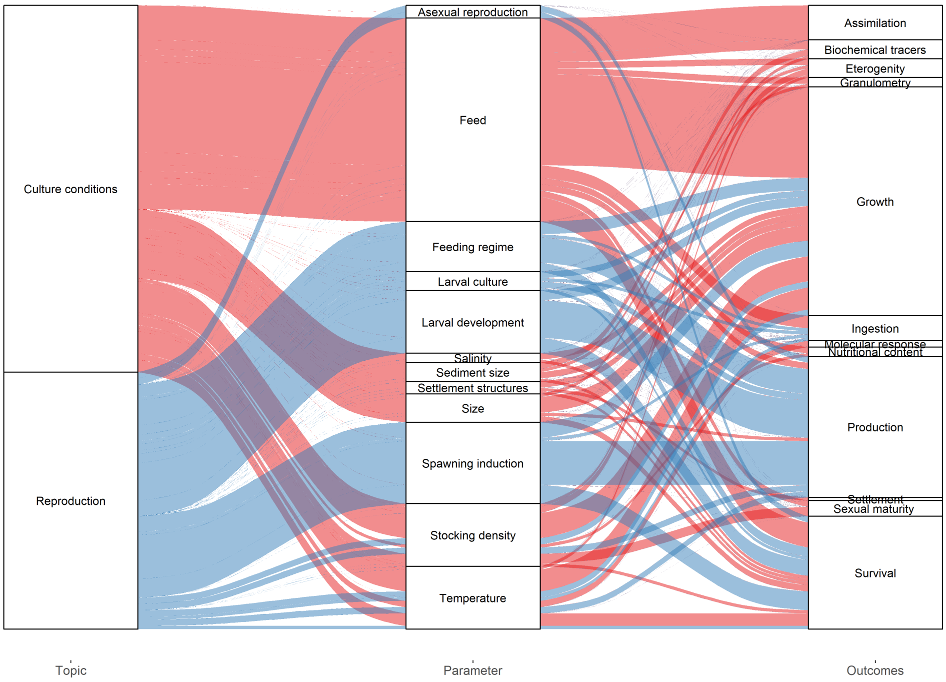

FIGURE 5
Alluvial diagram showing the distribution of Topic, Parameter and Outcomes investigated in the included studies.

2018) and *H. sanctori* (mechanical and algal stimulation, Magdy et al., 2021). Like other echinoderms, sea cucumbers showed sex order, with male specimens spawning first and presumably stimulating female gamete release, with the exception of *H. forskali*, which showed synchronous spawning (Laguerre et al., 2020).

4.1.2 Larval rearing

The larval cycle was similar in all species, with three planktonic and feeding stages (early, mid- and late auricularia) followed by two metamorphic non-feeding stages (doliolaria and pentactula respectively). However, some species-specific differences in the duration of each larval stages were reported. In *H. poli* and *H. arguinensis* the juvenile stage was reached after 15 and 18 days respectively, whereas *H. tubulosa*, *H. forskali*, *H. sanctori* and *P. tremulus* needed more than 20 days to reach the late auricularia stage.

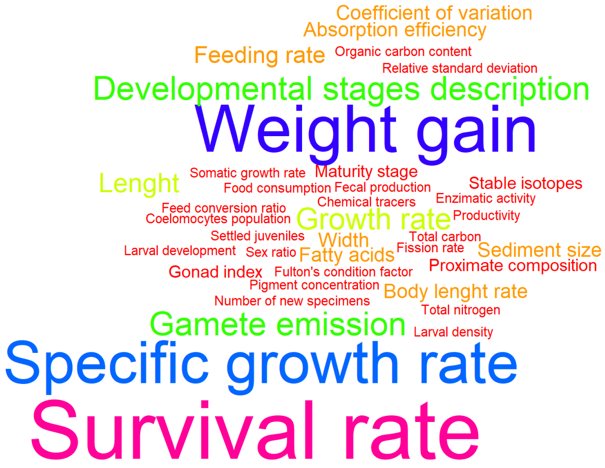

FIGURE 6
Word cloud analysis using outcomes used in the selected studies. The size of the words is proportional to the observed frequency. Same colours indicate same frequency.

Larval development seems to be influenced by culture conditions. Temperature and rearing time are inversely proportional, with temperate species characterised by a faster life cycle than species typical of cold waters (Schagerström et al., 2022). Stocking density affected larval survival, with low survival rates at high density (>3 larvae/ml; Domínguez-Godino et al., 2015; Rakaj et al., 2018; Domínguez-Godino and González-Wangüemert, 2018a; Magdy et al., 2021). In all selected studies, microalgae were used as feed for larval culture stages. *Tetraselmis chuii*, *Chaetoceros calcitrans*, *Isochrysis galbana*, *T. suecica*, *Rhodomonas baltica*, *Skeletonema marinoi*, *Phaeodactylum tricornutum*, *Dunaliella tertiolecta* and *C. mulleri*, were considered suitable to sustain larval development. However, the mixture of microalgae promoted higher larval survival rates than treatments with single algae (Domínguez-Godino and González-Wangüemert, 2019b). In terms of nutritional content, diets rich in lipids resulted more suitable for larval development since the lipids assimilated from the food during the first larval stages are stored in specific cells, called hyaline spheres, and become important energy resources for the subsequent non-feeding larval phases (Ramofafia et al., 2003; Sousa et al., 2023). Larval development was also influenced by the feeding regime with overfeeding conditions promoting morphological malformations, while underfeeding regime resulted in high mortality rates (Rakaj et al., 2018; Schagerström et al., 2022).

Also in this case, optimal conditions were species-specific with low algal concentration suitable for *H. tubulosa* and *P. tremulus*, while *H. arguinensis*, *H. mammata*, *H. poli* and *H. forskali* preferred high algal concentration (Domínguez-Godino et al., 2015; Rakaj et al., 2018, 2019; Laguerre et al., 2020; Venâncio et al., 2021; Schagerström et al., 2022).

The differences observed in larval development could be the result of specie-specific adaptive strategies, since the length of the planktonic phase influences the mortality rate and hence their range of colonisation (Domínguez-Godino and González-Wangüemert, 2018a). In addition, the duration of larval development may be related to the different reproductive effort. Indeed, it was observed that *H. poli*, which is characterised by faster larval development, produces large eggs (around 240 μm of diameter), suggesting the ability of this species to store high amounts of nutrient and take advantage of high feeding regimes (Rakaj et al., 2019). Similarly, *H. tubulosa* produced smaller eggs, preferring lower feeding conditions and showing slower larval development (Rakaj et al., 2018).

Finally, as already observed in other marine invertebrates characterized by planktonic larval stages, high mortality occurs during settlement. Settlement is induced by chemical cues provided, for example, by benthic diatoms (Schagerström et al., 2022). In addition, survival rates during this phase appear to be influenced more by the available surface area of the settlement structure than by its shape (Sousa et al., 2021).

4.2 Juvenile and adult culture requirements

Temperature is one of the most important physical variables affecting metabolism, growth and physiological performance of sea cucumbers (Yang et al., 2005; Dong et al., 2006). Sea cucumbers can respond to extreme temperatures by entering in a hypometabolic state called aestivation (high temperature) or hibernation (low temperature), in which specimens become inactive, stop feeding and start intestine digestion and metabolic rate depression (Zhao et al., 2022). Temperate species, such as *H. tubulosa* and *H. arguinensis*, can grow at temperature range of 20-25°C while lower temperatures (12-18°C) induce weight loss and low enzymatic and feeding activity (Günay et al., 2015, 2020; Domínguez-Godino and González-Wangüemert, 2019a; Madruga et al., 2023). High temperatures (23-28°C) are above the tolerance range of *P. regalis*, a species that lives in high depth where water temperatures are generally much lower. However, the ability of this species to survive at 18°C opens up the possibility of testing its cultivation also in shallow waters, throughout winter months (Galimany et al., 2018). Temperature also affects the ability of sea cucumbers to tolerate changes in salinity. *H. tubulosa* can grow between 30 and 45 psu in summer (25°C), reducing its tolerance to high salinity at winter temperatures (15°C).Similarly, *H. poli* can only tolerate salinity increases with warm water. However, both species are not able to survive under low salinity conditions (< 25 psu) (Tolon, 2017).

The stocking density is another important variable to take into account in sea cucumber culture practices in order to avoid intraspecific competition for space and food. Low stocking density is adequate for *H. tubulosa* (250 g/m^2) and *H. arguinensis* (520 g/m^2), while *H. mammata* prefers medium density (720 g/m^2, Tolon et al., 2017b; Domínguez-Godino and González-Wangüemert, 2018b). In general, growth performance decreases with increasing stocking density but, while medium densities (600 – 700 g/m^2) can be maintained for short periods, high densities must be avoided as negative growth was observed for all species studied (Tolon et al., 2017b; Domínguez-Godino and González-Wangüemert, 2018b). However, the critical biomass values vary between species, so species-specific tests are needed to define the appropriate rearing protocol.

Finally, as since sea cucumbers have the ability to select organic particles of different size (Ramón et al., 2019), it is important to understand size class preference for by North-Eastern Atlantic and Mediterranean sea cucumbers in order to facilitate sediment ingestion, and consequently food intake. Large sediment particles (7 mm) are not easily handled by juvenile specimens, while too small particles (0.05 – 0.2 mm), although preferred by sea cucumbers, may not be able to reach the bottom due to the aeration and water flow typical of tank systems (Hudson et al., 2005; Tolon et al., 2015). The best sediment size range seems to be between 1 and 3 mm, but as for other environmental variables, particle size class must be selected based on the cultured species and culture system.

In summary, food quality and availability are among the main factors influencing the fitness and growth of sea cucumbers under controlled conditions. Holothurians include both suspension feeders, able to collect phytoplankton and organic particles from the water column, or, like all European species of commercial interest, deposit feeders, able to ingest large amounts of sediment from the upper layers of the seabed or tank bottom. They are able to extract organic matter from sediment, which is composed of bacteria, microalgae and detritus, thus contributing to the recycling of sediment organic matter and improving sediment oxygenation. In line with these considerations, firsts experiments on the maintenance of sea cucumbers in tank conditions were carried out by feeding adults of *H. arguinensis* with natural sediment (Domínguez-Godino and González-Wangüemert, 2019a). The positive feeding rates (FRs) and absorption efficiency (AE), despite some variability associated with temperature changes, confirmed the suitability of this method and highlighted the ability of *H. arguinensis* to actively select organic-rich sediments and increase AE to compensate for the reduction in FR. Based on these encouraging results, further studies were conducted to stimulate sea cucumber growth using natural sediments enriched with macroalgae or seagrasses. Enriched substrates promoted higher growth rates compared with natural treatment, in both adult and juvenile specimens, but with similar survival rates (Rodrigues et al., 2023). In addition, while growth rates were high regardless of the ratio of sand to macrophyte, FR and AE increased with sediment enrichment (Sunde and Christophersen, 2023). Sediments enriched with seagrasses (*Cymodocea nodosa* and *Zostera noltii*) showed different performance, probably due to the composition of the seagrass litter (Domínguez-Godino et al., 2020). As deposit-feeding sea cucumbers have low cellulase activity, the high cellulose content typical of some seagrasses might have affected their growth. Sediment enrichment seems to favour the feeding activity providing easily access to food and reducing searching time and energy expenditure. In addition, a lower heterogeneity in growth performance was observed in juveniles when fed with enriched substrates, suggesting that enriched sediments might contrast the typical non-homogeneous growth patterns of sea cucumbers, an issue that could seriously affect their culture, as cohorts tend to reach commercial sizes at different times (Rodrigues et al., 2023).

4.3 Integrated multitrophic aquaculture

As sea cucumbers are considered suitable extractive species to be included in integrated multitrophic aquaculture (IMTA) systems, the effect of sediments enriched with waste deriving from the farming system was also tested. Most studies associated sea cucumbers with fish *(Sparus aurata, Dicentrarchus labrax, Seriola dumerili, Salmo salar)*, while only two studies used other invertebrates (the sea urchin *Paracentrotus lividus* and the mussel *Mytilus galloprovincialis*) as primary species. The choice to combine sea cucumbers mainly with fish is probably due to the fact that fish farming is the most common aquaculture activity in Europe. In addition, fish farming may impact the surrounding environment due to organic enrichment caused by the accumulation of faeces and uneaten feed (Janssen et al., 2017), conditions that could be favourable for sea cucumber growth. Although fish farming is the most widespread in Europe, mussel farming produces more than 1/3 of aquaculture products, making it one of the most important farming practices also in terms of extension (Avdelas et al., 2021). The high extension makes mussel farms particularly suitable for the development of co-culture with sea cucumbers, as the growth of holothurians is strongly affected by high stocking densities under culture conditions (Grosso et al., 2023). Finally, the development of a co-culture between sea cucumbers and *P. lividus*, the most prized sea urchin in the Mediterranean Sea, could be a valuable solution to reduce the impact of fishing pressures on both organisms, since *P. lividus*, like sea cucumbers, is now overexploited in several Mediterranean areas (Grosso et al., 2021; Elmasry et al., 2023). According with the literature, the ability of sea cucumbers to survive and grow under IMTA conditions is species-specific. *H. tubulosa* showed high survival rates and positive growth with fish, mussels and sea urchins (Tolon et al., 2017a; Grosso et al., 2021, 2023), but also showed signs of disease in highly eutrophic conditions, such as those that may occur near fish cages (Sadoul et al., 2022). Similarly, *P. tremulus* growth rates were enhanced by the addition of salmon sludge to natural sediments, suggesting the suitability of this species to reduce the impact of salmon farming, which is widespread in northern European countries (Sunde and Christophersen, 2023). Fish waste was also found to be palatable to *H. forskali* but further studies on growth performance are needed (MacDonald et al., 2013). In contrast, survival and growth of *H. poli* were generally affected by IMTA conditions, regardless of the primary species associated (Chatzivasileiou et al., 2022; Cutajar et al., 2022a, 2022b; Grosso et al., 2023), and similarly, *H. arguinensis* showed a reduction in weight, low FRs and negative AE when fed sediment enriched with fish (seabream) waste (Domínguez-Godino and González-Wangüemert, 2019a). The differences observed between species could be caused by several factors, including a different ecological compatibility due to a specific feeding strategy in the wild. For example, *H. tubulosa* actively selects organic matter from the upper sediment layer, while *H. poli*, which lives inside the sediment, consumes deeper sediments, which may be characterized by unfavourable physicochemical conditions when affected by waste enrichment, especially if the enrichment is due to fish waste accumulation (Grosso et al., 2023). The responses of sea cucumber to IMTA conditions is also influenced by the feed administered to the associated primary species (Grosso et al., 2021). Finally, stocking density in IMTA systems may also play a fundamental role in driving growth patterns of sea cucumbers. Exceeding the optimal stocking density may improve organic matter bioremediation, but compromise the growth yield of sea cucumbers (Slater and Carton, 2007; Domínguez-Godino and González-Wangüemert, 2018b). However, the survival of sea cucumbers in co-culture with fish or other invertebrates confirmed their compatibility with many of the rearing conditions expected in IMTA systems, and suggest a high potential of these species for the development of sustainable aquaculture project. Finally, as highlighted by Grosso et al. (2021), the use of sea cucumbers in an IMTA would also bring significant economic benefits due to their high market value, especially when combined with other prized species (*P. lividus*). However, in order to encourage the development of these farming practices, a more comprehensive economic evaluation of the inclusion of sea cucumbers in large-scale IMTA systems would be appropriate.

5 Conclusion

The number of papers on North-Eastern Atlantic and Mediterranean holothurian aquaculture has increased considerably in recent years, probably due to the growing interest in these organisms for commercial purposes. Most of the studies concern *Holothuria tubulosa*, *H. poli* and *H. arguinensis* and *H. mammata*, which are the most exported species, while little knowledge is available for the other marketable European sea cucumbers. Experiments revealed species-specific responses to the main environmental parameters (temperature and salinity) and stocking density. Hatchery production protocols are successfully described for all European species except *Parastichopus regalis*. The settlement stage is considered to be the main bottleneck, while there is still little and fragmentary information on the optimal maintenance conditions for larvae, both in terms of stocking density and food source and feeding regime. The most commonly used method to efficiently feed sea cucumbers, juveniles or adults, is the enrichment of natural sediments by the addition of macroalgae. Several algal species have been tested, but no studies have been conducted to compare the nutritional composition of the algae with the growth performance of sea cucumbers. However, enzymatic activity analysis suggests that protein-rich diets may be most suitable for these organisms. Finally, it has been confirmed that Mediterranean holothurians can be successfully included in multi-trophic aquaculture systems, achieving good survival and growth performance both by in combination with fish and other marine invertebrates. Again, interspecific differences were observed, hence further research is needed. In particular, more information on stocking densities and tolerable organic enrichment is essential to develop and consolidate this farming practice. Finally, there is important to stress the lack of information on the culture of *P. regalis*, a species that may soon be overexploited, due to its high value.

Author contributions

LC: Investigation, Writing – original draft, Writing – review & editing, Data curation, Conceptualization. GS: Writing – review & editing, Validation, Investigation, Data curation. GC: Writing – review & editing, Visualization, Validation, Formal Analysis. AR: Writing – review & editing, Supervision, Data curation. SV: Conceptualization, Funding acquisition, Supervision, Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2024.1381836/full#supplementary-material

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Current knowledge of Holothuriida (Holothuroidea: Echinodermata) from Brazil

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Sea cucumbers are the second most diverse class among echinoderms. They are characterized by an elongated body and dermal ossicles in the body wall. Among the included groups in that class, the order Holothuriida encompasses many species of commercial interest. Faced with the threats of biodiversity loss resulting from the climate crisis and the advancement of cities, this study provides an overview of recent research involving the Holothuriida of the Brazilian Coast. Searches were carried out in the main periodical databases, using appropriate keywords, targeting publications on the subject in the period between 2000 and 2023. In total, 69 publications were found, most of them corresponding to scientific articles in international journals. Most of the studies come from the Northeast region in Brazil (56.52%). Regarding lines of research, those involving taxonomy and molecular analysis were the most prevalent. Over these 20 years, seven species constituted new occurrences. Of note are the studies focused on *Holothuria* (*Halodeima*) *grisea*, which makes up 39.13% of papers. Despite progress, little is known about morphological variations, genetic distance between populations, population densities, and on reproductive biology of species. Brazil has a vast, heterogeneous coastal area, with unique characteristics to which the species are adapted. The findings reinforce the importance to expand studies aiming on knowing the biodiversity, reproductive biology, and population densities of species. Such data are essential for the creation of public policies aimed at the conservation of species of Holothuriida and their habitats.

KEYWORDS
Sea cucumber, Holothuroidea, Holothuriidae, *Holothuria*, Brazilian coast, *Holothuria grisea*

1 Introduction

Class Holothuroidea is represented by the taxonomic orders Apodida, Elasipodida, Holothuriida, Dendrochirotida, Persiculida, Molpadida, and Synallactida (Miller et al., 2017), that together include 29 families, 246 genera and about 1700 species (WoRMS, 2023). About 50 species of this class are known for the Brazilian coast; of these, 14 belong to the order Holothuriida, including species of Mesothuriidae (Ancona Lopez, 1958, 1962; Brito, 1960, 1962; Tommasi, 1969, 1971, 1972; Mondin, 1973; Tommasi and Aron, 1988; Freire and Grohmann, 1989; Moura et al., 2010, 2016; Martins et al., 2012, 2017; Prata et al., 2014a; Prata et al., 2014b, Prata and Christoffersen, 2017). Holothuriida is one of the most diverse orders of the class Holothuroidea. Its main synapomorphy is the presence of the gonad on only one side of the dorsal mesentery; nowadays it comprises the families Holothuriidae and Mesothuriidae (Miller et al., 2017).

Species of Holothuriida usually display medium to large size, peltate tentacles, and body classified according to arrangement of the ambulacral podia as bivium (dorsal) and trivium (ventral) (Pawson et al., 2010). Its diversity is high in shallow tropical environments (O'Loughlin et al., 2007), living in coral reefs and on soft or hard substrates, usually hidden in crevices, under rocks, on the sediment or gravel, and buried in the sediment (Prata et al., 2014b). Sea cucumbers is an important component of the marine fauna and can comprise up to 95% of the benthic community in some areas (Heezen and Hollister, 1971). Several species ingest and rework large amounts of sediment, being bioturbators of soft-bottom habitats (Miller and Pawson, 1990).

Some species are important fishery resources, considered as healthy food in several Asian countries (Conand, 1989, 2004). With the great demand for this delicacy, the price per kilo became exorbitant (Purcell et al., 2012) and fishing has expanded to other locations where its consumption is not common, such as the Western Atlantic (Conand, 2018). Because of disordered fishing globally, now about 16 species integrate the red list of threatened species of the Union for Conservation of Nature (IUCN) (Purcell et al., 2023). In Brazil, sea cucumber fishing is not regulated. Their capture and/or sale is considered illegal in accordance with federal laws 9,605/1998 and 11,959/2009. These regulate environmental crimes and fishing activities in the country, respectively.

However, fishing of *Holothuria* (*Halodeima*) *grisea* has already been reported for some states, as São Paulo and Ceará (Hadel et al., 1999; Lima et al., 2001; Souza Junior et al., 2017). Fishing appears to be more pronounced in the State of Ceará, where captures are carried out in an artisanal way through the manual picking of sea cucumbers in tide pools (Souza Junior et al., 2017; Ponte and Feitosa, 2019). According to Ponte and Feitosa (2019) the intense fishing pressure on reefs located at Xavier beach (Ceará State) caused a local collapse of the population of sea cucumbers. This led to the development of fishing on submerged reefs, where snorkeling is required. These authors report that sea cucumber fishing in these locations represents an important source of income for fishermen, and it is necessary that government agencies regulate this activity in order to avoid overexploitation of this resource.

Before the 2000s, few studies had been carried out covering the Holothuroidea fauna from Brazil. Examples are Verrill (1868); Rathbun (1879); Ludwig (1881; 1882); Sluiter (1910); Oliveira (1949); Ancona Lopez and Sawaya (1955); Ancona Lopez (1958; 1962); Tommasi (1957; 1969; 1971; 1972; 1974; 1999); Brito (1960; 1962); Deichmann (1963); Lima-Verde (1969); Freitas et al. (1973), Mondin (1973); Tommasi and Aron (1988); and Freire and Grohmann (1989). In general, these studies present species lists and taxonomic inventories from a determined locality, with little information on ecology and biology of the species.

Studies on Holothuroidea from the Brazilian coast is still insufficient to estimate its current diversity and, especially, their population stocks. Considering the ecological importance of the Holothuriida species and that they are more common in tropical waters, as well as that this group is the target of predatory fishing, this work presents a review of the current knowledge of the order from the Brazilian coast, from the 2000s to the present day.

2 Materials and methods

The bibliographic research on Holothuriida from Brazil was conducted between April and October 2023. Studies were selected according to type: scientific articles, books, book chapters, monographs, dissertations, and thesis. The preparation of the research was done locating scientific documents in bibliographic bases. Search was made in Scopus, Web of Science, Scielo, Google Scholar, and Biblioteca Digital Brasileira de Teses e Dissertações (BDTD [Brazilian Digital Library of Theses and Dissertations]), using the keywords: Holothuroidea, Aspidochirotida, Holothuriida, Actinopyga, *Holothuria*, *Mesothuria*, and *Zygothuria*, always in association with the word 'Brazil' or 'Brasil'. Those keywords could be present in the title, in the keywords and/or in the abstract of the documents, in order to increase search results. Regarding time, the searches were limited to studies published between 2000 and 2023, published in any language.

The processes of analysis of quantitative data followed Gil (2002), through the steps: establishment of categories, tabulation, and statistical analysis of data. Categories followed these descriptors: a) type of production; b) temporal distribution; c) geographical distribution; d) institutions originating publications; e) link with postgraduate programs; g) thematic focus. A list of species from Brazil is provided, indicating the recent papers found with species descriptions (from 2000 to 2023). Tabulation, analysis, and graphs of data were done in the software Excel, with each information listed below the respective descriptors, following the established categorization. The complete data can be accessed in the Supplementary Material.

3 Results

In this research 69 academic studies were found, among monographs, dissertations, thesis, book chapters and scientific papers published in periodicals.

3.1 List of the species

The current work reports 14 species of the order Holothuriida from the Brazilian coast, including oceanic islands (Table 1). Most species came from shallow waters, with six from the deep sea (Moura, 2016), of which *Holothuria* (*Cystipus*) *pseudofossor*

TABLE 1 Synthesis of species belonging to Holothuriida with records for the Brazilian coast, including oceanic islands.

Order	Family	Genera	Species	Recent bibliography
Holothuriida	Holothuriidae	*Actinopyga* Bronn, 1860	*Actinopyga agassizii* (Selenka, 1867)	Prata et al. (2014b), Martins et al. (2017)
		Holothuria Linnaeus, 1767	*Holothuria* (*Cystipus*) *pseudofossor* Deichmann, 1930	Prata et al. (2014b), Moura (2016); Martins et al. (2017)
			Holothuria (*Semperothuria*) *surinamensis* Ludwig, 1875	Prata and Christoffersen (2012), Prata et al. (2014b), Martins et al. (2017)
			Holothuria (*Holothuria*) *dakarensis* Panning, 1939	Prata et al. (2014a, 2014b)
			Holothuria (*Theelothuria*) *princeps* Selenka, 1867	Martins et al. (2012), Prata et al. (2014b)
			Holothuria (*Platyperona*) *parvula* (Selenka, 1867)	Prata et al. (2014b)
			Holothuria (*Halodeima*) *grisea* Selenka, 1867	Prata et al. (2014b), Martins et al. (2017)
			Holothuria (*Vaneyothuria*) *lentiginosa brasiliensis* Tommasi & Oliveira, 1976	Moura (2016)
			Holothuria (*Halodeima*) *manningi* Pawson, 1978	Martins et al. (2017)
			Holothuria (*Thymiosycia*) *arenicola* Semper, 1868	Prata et al. (2014b), Martins et al. (2017)
	Mesothuriidae	*Mesothuria* Ludwig, 1894	*Mesothuria gargantua* Deichmann, 1930	Moura (2016)
			Mesothuria maroccana Perrier R., 1898	Moura (2016)
			Mesothuria verrilli (Théel, 1886)	Campos et al. (2010); Moura (2016)
		Zygothuria R. Perrier, 1898	*Zygothuria* sp.	Moura (2016)

The bibliography indicated corresponds to taxonomic studies between 2000 and 2023 that provide descriptions of the species.

Deichmann, 1930 was also found in shallow waters (Prata et al., 2014b; Martins et al., 2017).

Except for *Holothuria* (*Halodeima*) *grisea* Selenka, 1867 and *Holothuria* (*Thymiosycia*) *arenicola* Semper, 1868, the species reported from Brazil have limited distributions along the coast. There are only specific taxonomic studies carried out for them, and few specimens were deposited in scientific collections or observed with Remotely Operated Vehicles (ROVs).

3.2 Types of production

The most frequent academic papers analyzed were scientific articles, representing 75.36% of the total (n = 52), followed by master dissertations (17.39%, n = 12), doctoral thesis (2.90%, n = 2), and book chapters (4.35%, n = 3). Most of the analyzed articles were published in international journals and in English. 28 (40.57%) were studies published in Brazilian journals and 27 (39.13%) in Portuguese. Only one study was published in French.

3.3 Temporal distribution

The focus of this research were the works produced in the present Millenium. The findings show that the quantity of studies increased between 2012 and 2019, reaching a peak in 2013 and 2014 with 7 studies published (Figure 1). From the early 2000s to 2010, the number of studies was low, reaching a maximum of two papers per year. A decrease in production was also observed between 2020 and 2021, increasing again in 2022.

This increase observed between 2012 and 2019 is related to the development of the 'Programa de Reestruturação e Expansão das Universidades Federais' (REUNI [Program to Support Restructuring and Expansion Plans of Federal Universities]). The REUNI started in 2007 and resulted in a considerable expansion of public universities and in opportunities for higher education throughout the country, with a view to its internalizing (Bizerril, 2020). The reduction in studies between 2020 and 2021 is related to the public emergency caused by COVID-19, a time when people had to confine themselves to their homes.

3.4 Geographical distribution of publications

The analyzed papers were performed in 11 federal units in Brazil. Three regions were represented, with 56.52% of the total located in Northeast Brazil (n = 39), followed by the Southeast Region (36.23%, n = 25), and then the Southern Region (7.25%, n = 5) (Figure 2). No related studies were observed for the Northern Region.

Northeast Brazil evidences a promising scientific production, particularly concerning Science Teaching in Biological Sciences. Promising scientific production concentrates most of the work on the Holothuriida. Lack of studies along the Amazon littoral, bathing the Sates of Amapá and Pará, point to a conspicuous a knowledge gap.

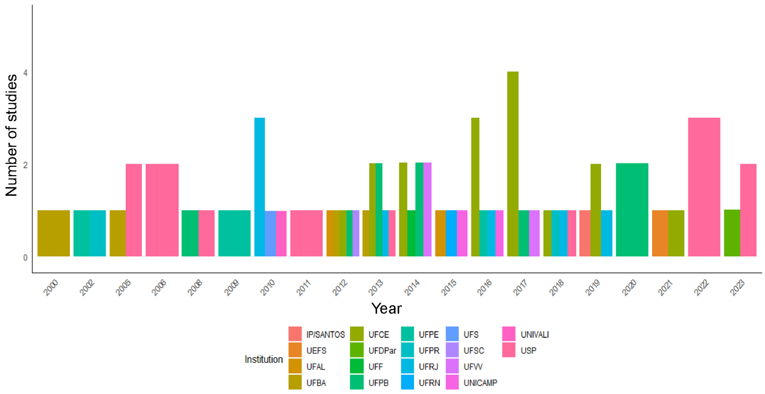

FIGURE 1
Number of studies by year and institution. UFCE, Federal University of Ceará; USP, University of São Paulo; UFPB, Federal University of Paraíba; UFRJ, Federal University of Rio de Janeiro; UFBA, Federal University of Bahia; UFPE, Federal University of Pernambuco; UFAL, Federal University of Alagoas; UFVV, Federal University of Vila Velha; UNICAMP, University of Campinas; UFPR, Federal University of Paraná; UFRN, Federal University of Rio Grande do Norte; UFS, Federal University of Sergipe; UEFS, State University of Feira de Santana; UFDPar, Federal University of Delta do Parnaíba; UFF, Federal University Fluminense; UFSC, Federal University of Santa Catarina; UNIVALI, University of Vale do Itajaí; IP/Santos, Advanced Marine Fish Research Center of Santos/São Paulo.

3.5 Origin of publications

Most publications came from Public Institutions of Higher Education (97.10%) or government agencies (1.45%), and only one from private institution (1,45%). Some papers share authors belonging to different universities in a same paper. In these cases, however, we considered the origin of the paper as that of the first author. The most productive institutions were Federal University of Ceará, with 26.08% (n = 18) of the scientific productions, followed by University of São Paulo, with 17.39% (n = 12), Federal University of Paraíba, with 13.04% (n = 9), and Federal University of Rio de Janeiro, with 10.14% (n = 7) (Figure 1). Other institutions published between three to one study. Of these publications, 46.37% came from postgraduate programs.

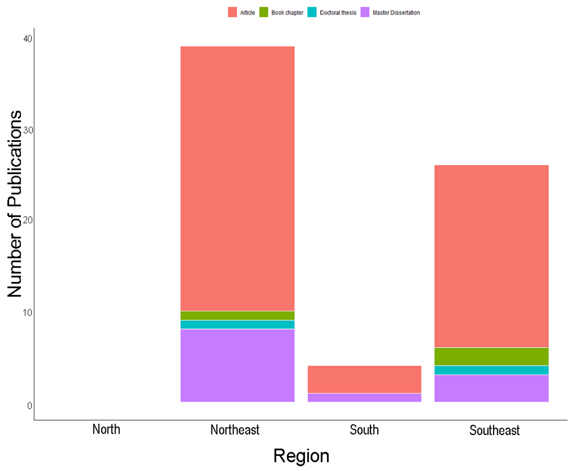

FIGURE 2
Number of papers by type and for region.

3.6 Thematic focus of publications

Most of studies analyzed correspond to Taxonomic papers (40%), molecular studies (29%), and studies in ecology (17.40%). Other studies considered reproductive biology (6%), fishery (6%), and reviews (1.6%) (Figure 3).

In the taxonomic category, works are more numerous, and can be divided into lists of echinoderm species for a given area; lists of species/taxonomic studies of Holothuroidea for a given locality or region; and new occurrence studies. Four studies focus exclusively on the Order Holothuriida. Most studies focusing on the Class Holothuroidea involve descriptions with detailed images and add data such as re-descriptions of species, ecological notes, and geographical and bathymetric distribution; some also include the morphometry of the dermal ossicles.

Among the 14 species belonging to the order Holothuriida along the Brazilian coast, the most common in the studies were *H.* (*H.*) *grisea* and *Holothuria* (*Thymiosycia*) *arenicola*. The studies cover the Northeast, Southeast and South regions. However, there is a lack of information about the fauna of Holothuriida from the North region, as well as for the States of Maranhão, Piauí, Sergipe (Northeast region), Espírito Santo (Southeast region), Paraná and Rio Grande do Sul (South region). In relation to deep-sea species, only four studies were found. All of them reporting species from Campos Basin, Southwest Atlantic (Campos et al., 2010; Moura et al., 2010).

Ecological studies show the spatio-temporal distribution of holothurian species, most focused on the species *Holothuria* (*Halodeima*) *grisea*; other studies cover behavioral aspects, diet, sediment and habitat analysis, and survival of species exposed to different variables. Two studies mentioned behavioral aspects of *H.* (*H.*) *grisea*. One of them evaluated the selectivity and food preference of juveniles of *H.* (*H.*) *grisea* subjected to different diets and the feeding behavior of the animals, in relation to two different photoperiods. The species had a nocturnal feeding habit

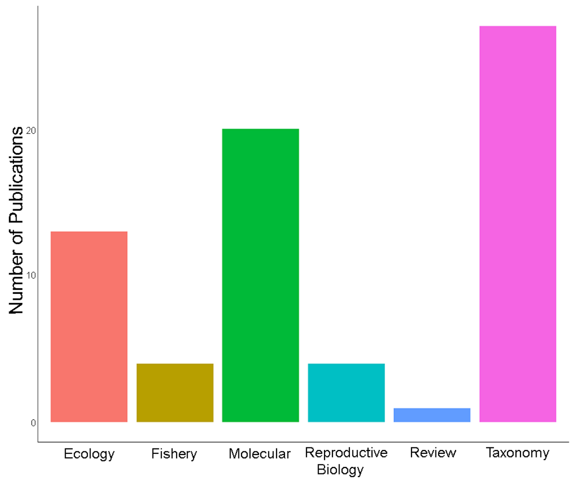

FIGURE 3
Thematic focus of publications.

and did not demonstrate selectivity or preference for the tested diets. The second study adds that knowing the behavior of holothurians can provide information about the basic learning mechanisms in Echinodermata.

Regarding spatial distribution patterns and populations, only three studies were found. Furthermore, the works deal with only one species, *H.* (*H.*) *grisea*, a species that has been studied by different lines of research. According with those studies, *H.* (*H.*) *grisea* was found more densely in the subtidal stratum, occurring in smaller quantities in autumn. Studies addressed the recycling of organic matter, correlating the survival of *H.* (*H.*) *grisea* with organic matter at different population densities, showing that the higher the density, the lower the chances of juvenile survival in controlled environments.

Another study related the content of organic matter to the presence of *H.* (*H.*) *grisea* in the environment and showed that there was a greater amount of calcium carbonate in samples collected in places where the species is found. There was one work reporting the parasite eulimid *Melanella eburnean* in *H.* (*H.*) *grisea*. Eulimids are gastropods that parasitize the external body wall of some species of Holothuroidea. Some studies have also analyzed the stomach contents in *H.* (*H.*) *grisea* to indicate if their diet is selective or not. These studies show that, in general, the diet of *H.* (*H.*) *grisea* is not selective. There was even a study on the nutritional value and yield of *H.* (*H.*) *grisea*.

Studies in the molecular category were diverse (n = 20). About 50% refer to studies on immune system cells, molecular markers, and analysis of body wall compounds. Some studies cover the microbiome of the body wall or the digestive tract; others focus on genetic diversity, primer design, and use of microchips, among other aspects. Some works characterized and described the effects of lectin in *H.* (*H.*) *grisea* (HGL). Among these studies, there was also a description of the HGL-2 (Melo et al., 2014). The lectin has been isolated and tested for its anti-inflammatory and hemolytic activities. Other compounds tested was fucosylated chondroitin sulfates type II, which were studied for their anticoagulant action and calcium affinities.

Researches on the ecotoxicological aspects of the species of the order Holothuriida have also been conducted, particularly with *H.* (*H.*) *grisea*. These studies demonstrated its potential environmental bioindicator and capacity to maintain water in tissues during hyperosmotic conditions. They also observed that when exposed to air *H.* (*H.*) *grisea* could reduce the osmotic permeability of their body wall, preventing loss of water into the air or water entry/salt exit during rain.

Molecular studies also addressed characteristics of the microbiota of the digestive tract in comparison to the sediment and diet. An analysis showed different microbiota in larger and smaller individuals. Furthermore, it was observed in Holothuriida that bacteria selectively enriched in their guts were spread on ambient sediments via feces, maintaining the ecological role of the host and promoting the mineralization of organic matter. Studies characterizing microsatellite loci, drawing of primers, and structural studies of genetics were also carried out with *H.* (*H.*) *grisea*, further improving knowledge about the species. Another study with *H.* (*H.*) *grisea* described the morphology and function of cells in the organic matrix of the ossicles, involved in the calcification and developing appropriate protocols for maintaining cells in culture. Studies on the healing process in *H.* (*H.*) *grisea* showed a rapid wound closure action through the synthesis of new collagen fibers. Those fibroblasts and two spherulocyte populations are involved in this process.

Regarding fisheries, the studies reported and characterized such activities for certain beaches on the coast of Ceará (Northeast region), showing the process from fishing to selling. The results indicated an annual catch of 12,341 kg per year in some localities, which are totally commercialized, since sea cucumber is not consumed in Brazil (Souza Junior et al., 2017). According to the authors, Brazilian environmental agencies need to act regarding *H.* (*H.*) *grisea* fishing, since there is no information on population stocks, nor supervision or management in Brazil. Another issue highlighted in these studies was the need to improve the infrastructure of sea cucumber processing sites and to encourage the adoption of hygienic-sanitary practices by fishermen.

The studies that deal with reproductive biology address the gonodal cycle and larval development of *Holothuria* (*Halodeima*) *grisea*. Four studies involved those aspects, detailing the gonodal cycle of this species in two localities of Northeast and Southern Brazil, respectively. According to the findings, the gonads of *H.* (*H.*) *grisea* increase between October and February in the Southern coast, while in the northeast coast, gonad growth occurs from December to February. There are mature individuals along the year and the sexual activity increased in summer. The maturation period and variations in body components do not present notable differences between the sexes.

Fewer studies dealing with review (n = 1) were found. The review presented the historical development of echinoderm studies and current research in Brazil and cited some species of the order Holothuriida. New methods and protocols were addressed in four studies. One of the proposals suggests the use of a proteolytic enzyme to dissolve holothurian tissues, resulting in ossicles with a more preserved surface. This suggestion has not been widely used, because the method of dissolving the tissue with commercial bleach solution is more accessible. Another study proposes the identification of holothurian taxa from disarticulated ossicles.

In the molecular area, a study suggests an integrative approach to study coelomocytes. For this approach, the authors used live and stained cells, scanning electron microscopy and morphometric analyses. They were able to separate seven cell types from the immune system of *Holothuria*. Considering sea cucumber fishing and the need to understand the populations of *H.* (*H.*) *grisea* in Brazil, a master dissertation proposes the application of microchips to these animals. The microchip was implanted in *H.* (*H.*) *grisea* using eugenol as an anesthetic. The author concluded that would be a way to monitor individuals and an efficient approach for short-term research.

In general, the majority of studies with Holothuriida as their main object deal with the species *Holothuria* (*Holodeima*) *grisea*, from ecological to molecular aspects, totaling around 39.13% of the documents. Studies considering the family Holothuriida, in general, represent approximately 20.28%, in which are addressed the analysis of enzymatic compounds, tissue regeneration mechanisms, characterization of coelomocytes, among others. Those who address the Class Holothuroidea (17.39%) include taxonomic studies, such as species inventories, ecological aspects, and molecular studies. Studies that focus on echinoderms and mention species of Holothuriida represent 23.20% and constitute taxonomic studies or reviews.

4 Discussion

The revision of Brazilian Holothuriida reports 14 species, most from shallow waters (e.g.: *Holothuria* (*Theelothuria*) *princeps*, *Holothuria* (*Holothuria*) *dakarensis*, *Holothuria* (*Platyperona*) *parvula*, *Holothuria* (*Cystipus*) *pseudofossor*). Except for *Holothuria* (*Thymiosycia*) *arenicola* and *Holothuria* (*Halodeima*) *grisea*, the species reported from Brazil have restricted distributions, as a result of taxonomic studies performed, mainly, with material held in collections. These materials may include specimens collected decades ago (e.g. Prata et al. (2014b), includes specimens collected in 1981, but which were analyzed and identified only recently). The populations of several species of marine invertebrates have been decreasing with the exposure to various environmental impacts (Sigwart et al., 2023). If we consider the changes that coastal environments have undergone in recent decades, such as the expansion of cities, waste disposal, loss of habitat, and climate change, it is not possible to say whether these species still occur in those locations sampled decades ago. Therefore, inventorying biodiversity remains an urgent task.

Along the period from 2000 and 2023, the publication of studies on Holothuriida began to increase in 2012, with a small drop in 2020, during the Covid-19 pandemic. From 2022, the number of studies increased again. With the emergence of effective vaccines against the virus, life gradually returned to normal, which consequently made it possible for research to resume. The increase in the number of publications from 2012 matches with the academic training of the first researchers to obtain master and doctorate levels after the REUNI Program. Furthermore, research mostly comes from public institutions. This shows the importance of public universities and academic programs for advancing science in Brazil.

The analyses indicates that most research is published in international journals and in English, in order to ensure a broad audience. In general, the studies are the result of master and doctoral researchers. This indicates that researchers tend to continue their research activities after completing graduate studies. On the other hand, it is noticed that researchers focused on different research lines rarely work together (see Supplementary Material). The studies came from 11 of the 27 federative units of Brazil, being concentrated in the Northeast and Southeast regions. This finding is particularly interesting, given that before the 2000s research was more linked to institutions in the Southeast and South regions of the country. These data also show that little is still known about the echinoderm fauna of the Northern region. This represents a problem, since the reefs at the mouth of the Amazon River, despite having recently been discovered (Moura et al., 2016), already faces enormous dangers of exploitation by oil extraction (Francini-Filho et al., 2018).

The survey showed studies focused on aspects of taxonomy, molecular analysis, ecology, fishery, and reproductive biology. In less quantity on reviews and some studies propose new methodologies or protocols. Rarely two or more themes are combined, about 7.24% (n=5). Studies are usually descriptive; they describe characteristics and do not involve experimental testing or extracting components. Experimental studies, about 36.23%, are related to ecological and molecular research. Molecular studies have focused on testing or isolating compounds from the genus *Holothuria*, mainly *H.* (*H.*) *grisea* (Moura et al., 2013, 2015) and in analyzing its microbiome (Yamazaki et al., 2019). These subjects have special importance for ecology, aquaculture, and pharmacology.

For most species, information is limited to taxonomic and distribution aspects. Highlighted are a large gap in knowledge about their areas of occurrence, population size, and biology, among others. Being a continental and unequal country, efforts are necessary to expand the geographic and bathymetric distribution of species. Taxonomic and systematic research is still extremely important to know biodiversity (Prata et al., 2014a; Moura et al., 2016; Martins et al., 2017). Brazilian researchers need to be encouraged to expand their research to understand ecological and reproductive biology aspects of Holothuriida (Bueno et al., 2015). Without these data, it is not possible to propose effective measures for the conservation of species and of their habitats.

Regarding fishery, the consumption of sea cucumbers is not part of the Brazilian diet, despite small records of consumption and fishing of *H.* (*H.*) *grisea* in some locations still in the 90s (Hadel et al., 1999). Seizures of the Federal police and recent studies show that fishing for these animals has increased in Brazil. Souza Junior et al. (2017) and Ponte and Feitosa (2019) show unregulated fishing of *Holothuria* (*Halodeima*) *grisea* in coastal areas of the state of Ceará. These areas are far from large urban centers and fishing is not monitored. The fishing occurs on a small scale and in an artisanal way. Ponte and Feitosa (2019) described the fishing cycle in two locations on that coast. They estimated that more than 12,3 tons of *H.* (*H.*) *grisea* per year in 2015-2016, corresponding to 380 thousand individuals. This fishing occurs at spring tides through active search and snorkeling. These authors emphasize that there are other communities along the coast of Ceará that also capture these animals for commercialization. The fishing supplies the international illegal market, with China being the largest buyer (Ponte and Feitosa, 2019; Valenti et al., 2021).

Purcell (2010) finds that the price per kilo varies from species to species, with those better known as *Apostichopus japonicus* being more expensive compared to other sea cucumbers that are less known and small, such as *H.* (*H.*) *grisea*. Furthermore, poorly processed animals, from places where there is no sanitary control, also have a value below the market. Souza Junior et al. (2017) and Pontes and Feitosa (2019) observed that fishing carried out on the coast of Ceará is carried out improvised by fishermen, which reduces the added value. Freire et al. (2021) built a database on fishing in Brazil and mentions sea cucumbers for the first time. These authors reinforce the need to evaluate the impact of fishing and environmental factors on population stocks.

Holothuria (*Halodeima*) *grisea* is distributed across practically the entire Brazilian coast, including ocean islands (Tommasi, 1969; Prata et al., 2014b; Martins et al., 2017). According to the International Union for Conservation of Nature (IUCN) red list and list of Brazilian threatened species, *H.* (*H.*) *grisea* is considered of less concern. However, the near absence of studies on the species and its populations may be masking the impact it has been suffering over the last decades. A decline in population has already been observed in some locations (Souza Junior et al., 2017). Controlling sea cucumber fishing is a challenge, first because these animals are not recognized as fishing resources in Brazil, and there is still no legislation to regulate this fishing (Brasil,1998; 2009); secondly because there is not enough knowledge to estimate population stocks, as well as their reproduction. Furthermore, if fishing is regulated, another demand that arises is the breeding and maintenance of these animals outside of their natural environment.

Population and genetic data are particularly important, as there are increasingly more studies emphasizing the economic and pharmaceutical potential of sea cucumbers, which could be explored through their cultivation under controlled conditions (Valenti et al., 2021). However, growing sea cucumbers is still a challenge and has not been enough to control fishing in most consumer countries. Valenti et al. (2021) points out that there is research underway to cultivate *H.* (*H.*) *grisea* to obtain commercial quantities of heparin in Brazil.

Other studies emphasized the bioturbation role played by sea cucumbers and recommended considering it in coastal environment monitoring programs. However, there is still a lot to know about the relationship between the substrate and sea cucumbers. *H.* (*H.*) *grisea* is sensitive to heavy metals such as lead and tin (Pereira et al., 2014). Studies on osmosis and ionic gradients in the body wall of *H.* (*H.*) *grisea* showed that this species has a greater capacity to maintain water in tissues during hyperosmotic conditions. These studies recommend considering this species in coastal environment monitoring programs.

In relation to studies involving a species as a focus, only papers on *Holothuria* (*Halodeima*) *grisea* were found. Despite that, there is scarce information about its population density, preferences, areas where the largest populations are concentrated, and even about its reproduction and life cycle along the coast. The Brazilian coast is extensive and heterogeneous, presenting diverse environmental characteristics (Ventura et al., 2013), to which species of Holothuriida can adapt. Furthermore, many of these areas can be subject to environmental impacts, whether due to the increase of cities or to climatic crisis. Given the little knowledge about the species, studies combining one or more aspects are of special interest for conservation. Studies with an emphasis on the analysis of compounds for pharmaceutical purposes also need to find a sustainable solution for obtaining these compounds just in case a large-scale quantity is needed. Solutions for fishing species of commercial interest must be found in order to avoid their extinction in the environment (Purcell et al., 2023).

Although it is difficult for Brazilian authorities to control sea cucumber fishing in the country, fishing cannot be regulated without first having an accurate assessment of stocks and knowing the distribution of populations along the coast. Environmental managers need advice to build an action plan for commercial species. The first step is to improve knowledge about the species along the coast with the development of genetic, ecological, reproductive biology and cultivation studies, among others. Brazil has specialized professionals, in different areas, that could cover the gaps of knowledge, able to form a research group on sea cucumbers and articulate management strategies with public authorities in order to prevent overexploitation of this resource and to recover populations. Creating a research group on sea cucumbers with these researchers to propose an action plan that directs the necessary research and coordinating management strategies with public authorities could be a decisive step towards avoiding over-exploitation of this resource and recovering the populations.

This review provides a better understanding on actual knowledge of the Holothuriida for Brazilian waters. Researchers of Holothuriida cover few areas of knowledge, being concentrated in taxonomic and molecular studies. The data collected emphasizes that investigations about Holothuroidea in Brazil were carried out by few researchers, which could explain the gaps of knowledge. Certainly, the interaction between these researchers should be encouraged, to fill the gaps, and to provide measures for the conservation of the Holothuriida and to promote their sustainable development.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

JP: Data curation, Formal analysis, Writing – original draft. MC: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2024.1333253/full#supplementary-material

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Dispersal patterns of sandfish (*Holothuria scabra*) larvae in Manus Province, Papua New Guinea

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In many tropical nations, coastal communities seek to manage their sea cucumber fisheries by establishing locally managed marine areas on their traditional fishing grounds. These managed areas can protect spawning stocks, however the extent to which they help to replenish nearby sea cucumber fisheries is debated, as nothing is known about the scales at which sea cucumber larvae disperse. In this study, we used genetic parentage analyses and statistical modelling to provide the first empirical measure of larval dispersal patterns for a sea cucumber species. We analysed tissue samples from 765 adult and 827 juvenile sandfish (*Holothuria scabra*) collected from five traditional fishing grounds of the Titan tribe, from Manus Province, Papua New Guinea. All adults were sampled from the Pere fishing grounds (the larval source area), with juveniles sampled from the Pere, Mbunai, Tawi, Timonai and Mbuke fishing grounds. Parentage analysis identified 15 juveniles that were the offspring of parents sampled from Pere fishing grounds, with six of these juveniles located in the same fishing grounds as their parents. The best-fit larval dispersal kernel predicted that the average *H. scabra* larvae travelled 15 km from its spawning location, with 50% of larvae settling within 6.7 km of their parents and 95% of larvae settling within 59 km of their parents. These results suggest that the Titan tribes' desire to protect their *H. scabra* spawning stocks by establishing a network of locally managed marine areas across 65 km of continuous coastline is a culturally appropriate strategy, which has merit from both an ecological and fisheries standpoint.

KEYWORDS

customary marine tenure, connectivity, locally managed marine areas, fisheries management, genetic parentage analyses, larval dispersal

Introduction

Understanding the larval dispersal patterns of exploited marine species is important to fisheries managers as it informs the spatial scales at which these harvested populations should be managed (e.g. Sale et al., 2005; Pelc et al., 2010; Jones, 2015). From an ecological perspective, when mean larval dispersal distances are short (i.e. tens of kilometers, e.g. Almany et al., 2007) and a large proportion of larvae are locally retained, local marine reserves can be self-sustaining, and nearby fisheries can receive larval subsidy. From the perspective of economic and social theory, local larval dispersal makes small-scale management more viable (Almany et al., 2007; Hamilton et al., 2021), and creates the conditions for self-sustaining territorial user-rights fisheries – in particular, it means that the community who manage their fisheries are the primary beneficiary of sustainable behavior (Costello et al., 2015). Such management becomes much more challenging where mean larvae dispersal distances are large (i.e. in the hundreds of kilometres, e.g. Nanninga et al., 2015; Herrera et al., 2016; Williamson et al., 2016). Where larval dispersal scales substantially exceed management scales, connectivity creates economic and social externalities.

Our understanding of the scales and patterns of population connectivity in marine species has increased markedly in recent decades. Empirical field studies based on new methodologies such as genetic parentage analysis have allowed direct observations of larval dispersal events (Jones et al., 2005). These studies have assisted in the validation and calibration of increasingly sophisticated biophysical dispersal simulations (Bode et al., 2019). However, most experimental studies on larvae dispersal have focused on small fish species that are not harvested (e.g., Planes et al., 2009; Pusack et al., 2014; Shulzitski et al., 2016). Literature pertaining to large commercial species (i.e., reef fish) is gradually increasingly (e.g., Harrison et al., 2012; Almany et al., 2013; Hamilton et al., 2021). Yet despite the global interest in managing sea cucumber fisheries, we know of only one published study that has attempted to quantify sea cucumber larval dispersal (Brown et al., 2022). The study by Brown et al. (2022) involved conducting a high-resolution genomic audit of sandfish (*Holothuria scabra*) populations at six sites in Fiji that were separated by a maximum of 300 km. Brown et al. (2022) could not infer larval dispersal patterns of sandfish in Fiji, as they did not identify any parent-offspring relationships, however they did detect some full sibling and half sibling relationships, and concluded that sandfish in Fiji are made up of three genetically distinct populations.

Sea cucumbers, or bêche-de-mer, as they are known in their dried and processed form, have been a luxury sea food in Asia for centuries (Toral-Granda et al., 2008). Many commercially valuable sea cucumbers occur in shallow tropical waters, where they are typically exploited by small-scale fisheries that operate in low income and low governance contexts (Barclay et al., 2019). Consequently, many sea cucumber fisheries are severely overfished (Toral-Granda et al., 2008; Friedman et al., 2011), with patterns of boom and bust inherent in sea cucumber fisheries globally. In the Pacific Islands region both bottom-up and top-down management efforts have been utilized to manage these high value fisheries. Coastal communities in Fiji, Papua New Guinea and Solomon Islands have established locally managed marine areas (LMMAs) on their customarily owned reefs to allow populations of sea cucumbers to recover (e.g. PNG National Fisheries Authority, 2007; Hamilton and Lokani, 2011; Hamilton et al., 2015; Lalavanua et al., 2017). This type of community-based fisheries management is made possible by the existence of customary marine tenure systems that are common throughout the Pacific, often involving communities closing an area to some or all types of fishing for a certain period (Ruddle, 1996; Johannes, 2002; Almany et al., 2015). National fishery agencies also routinely place moratoriums on these fisheries once they are considered over-exploited at a regional scale (e.g. Christensen, 2011; Hair et al., 2019; Brown et al., 2022).

For the past two decades, the Titan communities, located along the south coast of Manus Island in Papua New Guinea, have established LMMAs within their traditional fishing grounds as a fisheries management tool for a variety of harvested species (e.g. Hamilton et al., 2005). To evaluate the effectiveness of these LMMAs, Titan fishers and staff from James Cook University and The Nature Conservancy investigated the dispersal of squaretail grouper (*Plectropomus areolatus*) larvae that were spawned at a fish spawning aggregation that is protected by one of these LMMAs (Almany et al., 2010, Almany et al., 2013, Almany et al., 2015). This work showed that *P. areolatus* had a mean larval dispersal distance of 14 km, with 20% of larvae retained within the fishing grounds of where they spawned (Almany et al., 2013).

When the findings on *P. areolatus* connectivity were shared with Titan fishers in 2011, communities realized that while they would obtain some direct larval subsidies from protecting fish spawning aggregations located within their fishing grounds, the scale of larval connectivity meant that this fishery would be best managed at a larger spatial scale. This information provided Titan leaders with the ecological rationale for establishing the Mwanus Endras Asi Resource Development Network (MEARDN) tribal network. MEARDN encompasses a sea area of approximately 24,000 km^2 and 10,000 people; the Titan communities that make up MEARDN share a common language, religion, and ethnic identity. Most MEARDN inhabitants live in communities that are located along a continuous 65 km stretch of the south coast of Manus Island, with a smaller number of people residing on outer islands that lie up to 100 km south or east of Manus Island (Almany et al., 2015). MEARDN was established in 2013, with the goal of improving the livelihoods of the Titan people, by maintaining healthy marine ecosystems and strengthening the governance and financial capacity of the Titan people.

One of the most valuable marine resources that is found within MEARDN is *H. scabra*. Often called “sandfish”, this is the most valuable species in the Papua New Guinea sea cucumber fishery (Purcell et al., 2014; Hair et al., 2019). Sandfish have been commercially exported from Papua New Guinea since the 1870s (few local people traditionally consumed sea cucumber), but the trade accelerated dramatically in the 1980s, by which time Papua New Guinea had become the third largest supplier of bêche-de-mer to Hong Kong (Kinch et al., 2008). Historically sandfish were very abundant in nearshore seagrass habitats in Papua New Guinea, with reported densities in the 1980s-1990s often exceeding 1000 per ha (i.e. Lokani, 2001; Hamilton and Lokani, 2011). Because of its shallow distribution children, women, men, and the elderly participate in the sandfish fishery (Barclay et al., 2019). Its shallow distribution and high value have resulted in sandfish populations being depleted throughout Papua New Guinea (e.g. Dalzell, 1990; Hamilton and Lokani, 2011; Hair et al., 2019) and much of its range (Hamel et al., 2022). Given this vulnerability to overexploitation, *H. scabra* was listed as Endangered on the IUCN Red List in 2010 (Hamel et al., 2013). At the time of this study, 2014, a Papua New Guinea wide moratorium on sea cucumber fisheries had been in place since 2009, following low stock assessments for all commercially valuable sea cucumber species (Hair et al., 2016).

Sexes are separate in *H. scabra* and this species is a broadcast spawner. Their larvae are planktonic, settling in shallow seagrass habitats, approximately 14 days post-spawning (see review in Hamel et al., 2022). Adults inhabit seagrass areas and deeper sand and silt habitats. The species has a narrow habitat range, and is only found in shallow, low-energy environments that have muddy or sand substrates (see review in Hamel et al., 2022). Uthicke and Benzie (2001) found restricted gene flow between *H. scabra* populations along the north-east coast of Australia, suggesting a low dispersal ability in this species. Because of their broadcast spawning behavior many closely related congeneric species of sea cucumbers can hybridize, particularly in areas where both populations are abundant (e.g. Gkafas et al., 2023). In Papua New Guinea the golden sandfish (*H. lessoni*) occurs in similar habits to *H. scabra*, although in Manus this species occurs in very low abundances (authors, personal observations). The two species are readily distinguishable based on markedly different color patterns and presence/absence of wrinkles in the body wall (Uthicke et al., 2005). *H. scabra* and *H. lesoni* have been shown to hybridize, although the genetic integrity of either holothurian species remains intact through an unknown postzygotic mechanism that may be hybrid sterility (Uthicke et al., 2005).

During a MEARDN tribal meeting in 2013, Titan leaders expressed interest in establishing a business arm within their tribal network, as a means of enhancing the financial security of their people. Titan leaders were particularly interested in sustainably harvesting, processing, and exporting *H. scabra* directly to Hong Kong once the national moratorium on sea cucumber harvesting was lifted. By running all aspects of the bêche-de-mer business themselves, MEARDN and its fishers hoped to capture a greater share of the profits in this fishery. To ensure the sustainability of their *H. scabra* fisheries, Titan leaders also discussed the merits of establishing a network of nearshore LMMAs within MEARDN, to protect local spawning stocks of *H. scabra* once the fishery was opened. Since nothing was known about the larval dispersal patterns of *H. scabra*, TNC staff who were present at the 2013 tribal meeting were asked if they would conduct research on their larval dispersal patterns within MEARDN. This request formed the premise for the research that is reported on here.

Methods

Environmental setting

The Titan communities of Pere, Mbunai, Locha, Tawi, Timonai and M'buke participated in this study These communities are all situated near the mainland of Manus Island, and their traditional fishing grounds are shown in Figure 1. At the time of this study (2014) the national moratorium on sea cucumber harvest had been in place for five years, and Titan fishers reported that their sandfish populations were showing signs of recovery (Feary et al., 2014). In water surveys that we conducted in 2014 revealed that the average densities of sandfish in shallow seagrass habitats in Titan fishing grounds ranged from 192 to 422 individuals per hectare, with *H. scabra* populations dominated by juvenile size classes (Feary et al., 2014). These 2014 densities were on par with areas that have undertaken experimental, small-scale fishing of sandfish (i.e., Warrior Reefs within Torres Strait, Australia, Murphy et al., 2012).

Collection of tissue samples

Although juvenile *H. scabra* in Papua New Guinea have been categorized as being ≤ 21cm total length (TL) (Lokani, 1990), maturity can occur from 17cm TL to 21cm TL (Hamel et al., 2022). To be conservative, for our connectivity study we considered adults ≥ 22cm TL and juveniles to be ≤ 15 cm TL. Between 19th May to the 22nd June 2014 we worked with over one hundred Titan fishers to sample 6,465 *H. scabra* from 57 sites located in the Pere, Mbunai, Locha, Tawi, Timonai and Mbuke fishing grounds (Table 1). Juveniles were sampled from all six communities' fishing grounds, whereas adults were only sampled from the Pere fishing grounds (i.e. the 'natal site': see Feary et al., 2014 for further details). Once collected, each *H. scabra* was allowed to rest for 5 mins, as individuals are prone to constrict when moved. Following this, each individual was measured (mouth to anus), a small sample of body wall tissue from the dorsal side of the animal collected and this sample immediately preserved in 98% ethanol. Between individual sample collection, all sampling instruments were placed in 98% ethanol, to reduce contamination. All individual sandfish were then replaced in the habitat they were collected. A subsample of 765 adults and 827 juveniles were analysed for parent – offspring relationships (Table 1).

Estimating the amount of *H. scabra* habitat within the Pere fishing grounds

To estimate the amount of *H. scabra* habitat within the Pere fishing grounds, we asked knowledgeable sea cucumber fishers from Pere to demarcate sandfish habitats on satellite images of their fishing grounds (Feary et al., 2014), a process known as participatory mapping. Local fishers identified three readily distinguishable *H. scabra* habitats in the Pere fishing grounds, that being dense and sparse seagrass habitat that occurred in

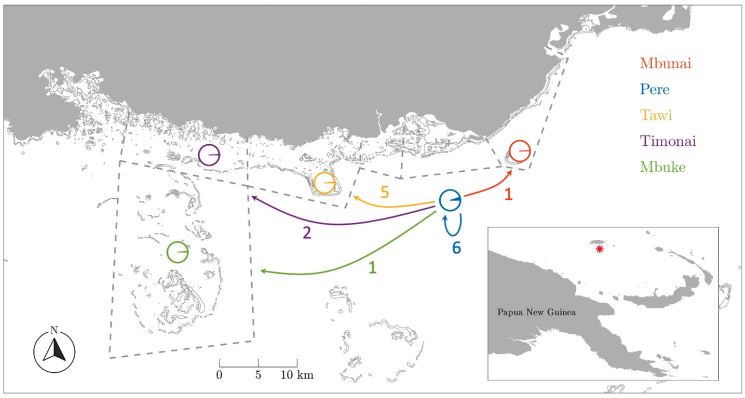

FIGURE 1
Dispersal map by fishing grounds. Dashed lines delineate customary marine tenure boundaries between communities. Circles indicate the proportion of juveniles sampled in each fishing ground that were assigned to parents from among the Pere samples. Arrows indicate the number of juveniles assigned to parents from Pere. Note the high number (6) and proportion (5.1%) of self-recruits from Pere.

shallow (1-2 m) water depths, and sand/silt habitat that occurred in water depths of 5-10 m. *H. scabra* habitats that were identified through participatory mapping were digitized in GIS, enabling us to calculate the total area of each *H. scabra* habitat in the Pere fishing grounds.

Underwater visual census surveys

For the purposes of dispersal kernel estimation, we needed to estimate the adult abundance of *H. scabra* in the Pere fishing grounds (the adult source area). This abundance estimate can be used to determine what proportion of the larvae were genetically marked. Between the 25th to 30th of May 2014, 180 100m^2 (50m * 2m) underwater visual census (UVC) surveys were undertaken within the Pere fishing grounds (Supplementary Table S1). We surveyed dense seagrass, sparse seagrass, and sand/silt *H. scabra* habitats in the Pere fishing grounds. Shallow seagrass habitats were surveyed on snorkel while deeper sand/silt habitat was surveyed using SCUBA (Supplementary Table S1). All UVC surveys were conducted at night using underwater torches to coincide with the highest density of *H. scabra*, since this species will burrow during the day and emerge during the night (Hamel et al., 2022). On each UVC survey all *H. scabra* encountered were counted, with each individual's length visually estimated and placed within 50 mm size classes (total length, TL). To determine the density of sandfish in juvenile versus adult phases, all individuals were split into ≤ 21cm TL (juvenile) and ≥ 22cm TL (mature [adult] phase) (following Lokani, 1990).

Microsatellite development and genotyping

Microsatellites were identified from a *de-novo* assembly of Illumina 2 x 150 bp paired-end sequencing from genomic DNA of a single *H. scabra*. The genomic library was prepared and sequenced on an Illumnia MiSeq platform according to the manufacturer's instructions, using a Nextera DNA sample prep kit (Illumina). Reads were first trimmed of low-quality bases in TRIMMOMATIC v0.36 before forward and reverse reads were merged in Pear v0.9.10 (Zhang et al., 2014). Candidate tri- and tetra-nucleotide microsatellite repeats were detected using MSATCOMMANDER v 1.0.8 (Faircloth, 2008). A total of 6,473 suitable loci were identified, of which 48 were selected for multiplex PCR amplification. Primers were designed in msatcommander with optimum lengths of 26 bp, optimum annealing temperature of 60°C, and a 3' GC clamp for higher specificity in multiplex PCR. Forward primers were labelled with 5'-modifications (6-FAM, VIC,

TABLE 1 **Total number of adult and juvenile *H. scabra* collected from each fishing ground and the subsample of these that were genetically analysed.**

Fishing ground	Adults collected	Juveniles collected	Adults analysed	Juveniles analysed
Pere	2094	1294	765	117
Mbunai	0	936	0	162
Locha	0	139	0	0
Tawi	0	1004	0	291
Timonai	0	612	0	201
Mbuke	0	386	0	56
Total	**2094**	**4371**	**765**	**827**

NED and PET) for fragment analysis on an ABI 3370xl DNA Analyzer (Applied Biosystems) with the GeneScan 500 LIZ internal size standard (Applied Biosystems).

All loci were first amplified in simplex PCRs on three individuals and later combined in multiplex PCRs in eight individuals to assess the quality of amplification and adjust primer concentrations. A total of 26 loci were selected for amplification in four multiplex reactions. All four multiplex reactions were performed in a total volume of 10 μL containing 5 μL of Qiagen Multiplex Master Mix (Qiagen, Germany), 3 μL of distilled water, 1 μL of primer premix, and 1 μL template DNA. Multiplex PCRs were performed on Veriti thermocyclers following a 'touch-down' sequence to increase the binding specificity of primers and reduce artifacts: 15 min initial denaturation at 95°C, 5 cycles of 30 s at 95°C, 90 s at 62°C, and 60 s at 72°C, then 5 cycles of 30 s at 95°C, 90 s at 60°C, and 60 s at 72°C, then 20 cycles of 30 s at 95°C, 90 s at 58°C, and 60 s at 72°C, followed by 30 min at 60°C. All adult and juvenile samples were genotyped at all 26 loci following DNA extraction using a Nucleospin tissue extraction kit (Macherey-Nagel). Individual genotypes were scored in GENEMAPPER v4.0, and unique alleles were distinguished using marker specific binsets adapted from MSATALLELE (Alberto, 2009).

Parentage analysis and accuracy tests

All collected juveniles were screened against the total pool of adults to reveal parent–offspring relationships using a maximum-likelihood approach implemented in FAMOZ (Marshall et al., 1998; Gerber et al., 2003). The program computes log of the odds ratio (LOD) scores for assigning individuals to candidate parents based on the observed allelic frequencies at each locus. Minimum LOD score thresholds for accepting assignments to single parents and parent pairs were determined from simulation adapted from Harrison et al. (2014). Briefly, we generated five simulated datasets of individual genotypes of known and unknown decent, simulating incomplete sampling of the adult populations. The proportion of known parents in the sample was fixed at 20% and genotyping error was introduced at a rate of 1% for each locus. Simulated datasets were analysed using the pairwise likelihood score method implemented in FAMOZ and the results analysed to identify a LOD threshold resulting in the highest overall accuracy of assignments. Accuracy was measured as the proportion of correct assignments to single parents or parent pairs and the number of correct exclusions over all possible assignments (Harrison et al., 2013).

Fitting dispersal kernels to genetic parentage data

We fit several isotropic dispersal kernels to the parentage data, using the maximum likelihood methods described in Bode et al. (2018), implemented in Matlab (see *Data availability statement*). We considered four kernel shapes from the family of generalized normal distributions: two were thin-tailed distributions (the Gaussian and the Ribbens), and two were heavy-tailed (the Laplacian and the Cauchy). We represented the sampling locations of individuals as single points in each of the fishing grounds, creating distance and parentage matrices (the final row accounting for unassigned juveniles). We did not consider the Locha fishing grounds in this analysis as none of the juveniles from Locha were genetically analysed.

The best-fit kernel functions and parameter values were estimated using both those juveniles which could be assigned to parents, and also unassigned juveniles, since these also offer information about the scale of dispersal.

Results

Microsatellite genotyping and locus characteristics

In total, 765 adults from Pere and 827 juveniles from five fishing grounds (Pere, Mbunai; Tawi; Timonai; Mbuke) were successfully genotyped at 26 microsatellites for parentage analysis. Of the 26 loci amplified, seven were excluded from further analyses due to null alleles or poor amplification. All remaining loci were highly polymorphic (Supplementary Table S3) ranging from 7 to 51 alleles with a mean of 24.5 (SE: 2.8) alleles per locus and average heterozygosity of 0.730 (SE: 0.04). The high level of polymorphism provided a strong exclusion power (Supplementary Table S3) with an overall accuracy of 98.6% across simulated datasets with LOD scores above 6, providing very strong confidence in assigned parent-offspring pairs. The probability of assigning a juvenile to a parent that was not its true parent, knowing that the true parent was not sampled was 1.3% (false positive – type I error). Conversely, the probability of a true parent–offspring pair not being identified knowing that the true parent was sampled was 0.1% (false negative – type II error). Fifteen juveniles were genetically assigned to a single sampled parents in our samples (mean LOD score: 8.6). All fifteen individuals were assigned to adults from Pere (Table 2, Figure 1).

Larval dispersal within the MEARDN customary fishing grounds

The participatory mapping of *H. scabra* habitats (Supplementary Figure S1) and UVC data (Supplementary Tables S1 and S2) indicated that the 765 surveyed adults represented 3.1% of the total adult population in Pere, across all three habitat types. Using this proportion, and the number of assignments across the five fishing grounds along the coastline, we found that the best fit was provided by the Cauchy dispersal kernel function:

$$\rho(d_{ij}, k) = \frac{k}{2}\exp[-(d_{ij} \;\; e^{k})^{\frac{1}{2}}],$$

with $k = -0.99$. The fit implies that the mean dispersal distance in the system was 15 km, and that half of the larvae are settling

TABLE 2 Number and proportion of juvenile *H. scabra* in each fishing ground that were assigned back to a genetically analysed adult in Pere.

Fishing ground	Juveniles analysed	Assignments	Not assigned	Proportion assignments
Pere	117	6	111	5.13%
Mbunai	162	1	161	0.62%
Tawi	291	5	286	1.72%
Timonai	201	2	199	1%
Mbuke	56	1	55	1.79%
Total	**827**	**15**	**812**	

within 6.7 km of their natal population. The kernel predicts that the overwhelming majority of settlement (95%) happens within 59 km of the larval source. The Cauchy kernel is particularly steep at the origin (Figure 2), suggesting that the strength of dispersal drops off very rapidly with distance.

Confidence bounds around this fit were generated using bootstrap resampling at the scale of the 5 sampling locations, which showed that 95% of the fits had values in the range $k \in [-5.0, -0.38]$ (Figure 2). This choice of bootstrap resampling addresses the uncertainty produced by the choice of sample locations. Along with the relatively small number of assignments, this choice produces wide confidence bounds, since the dataset changes substantially between resamples.

Discussion

In this study, we combined genetic parentage analysis and modelling to provide the first description of larval dispersal patterns in a commercially valuable sea cucumber. We identified 15 juvenile *H. scabra* in the Mbuke, Mbunai, Pere, Tawi and Timonai fishing grounds that were the offspring of parents sampled from the Pere fishing grounds. A fitted dispersal kernel based on the sampling location of adult and juvenile *H. scabra* predicted that the mean distance of larval dispersal is 15 km. These findings imply high levels of self-recruitment within the Pere fishing grounds, as well as larval connectivity between the five MEARDN fishing grounds investigated in this study – particularly between adjacent communities (Figure 2).

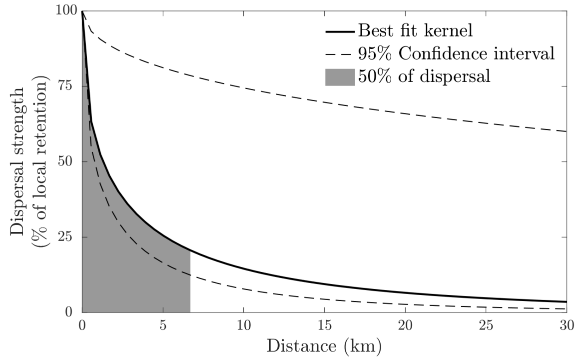

FIGURE 2
Best-fit larval dispersal kernel (solid black line) – a Cauchy kernel – estimated from the parentage data. Dashed lines enclose ± 1 SD of the fits to the bootstrap resampled data. Dispersal strength is given relative to the strength of local retention. The best-fit kernel estimates that 50% of larvae settle within 6.7 km of the natal reef. The estimated mean dispersal distance is 15 km.

We found that 50% of larvae settle within 6.7 km of their parents and 95% of larvae settle within 59 km of their parents, indicating that the studied *H. scabra* population is reasonably demographically closed (Sale et al., 2005). This implies that the spatial scale of MEARDN, which extends across 65 km of the Manus coastline, is likely adequate for sustainably managing the stock. Establishing a network of nearshore LMMAs that protect adult spawning stock of *H. scabra* would be a culturally appropriate strategy for MEARDN that has merit from both an ecological and fisheries standpoint. Although demographically closed populations can be managed at restricted spatial scales that match the size of tribal networks such as MEARDN, they are more vulnerable to recruitment overfishing (Begg et al., 1999; Walters and Martell, 2004) and localized extinction at that scale. Once depleted, demographically closed populations are unlikely to be rescued by the arrival of larvae from distant intact populations (van der Meer et al., 2015; Bonin et al., 2016). Indeed, some sea cucumber fisheries that have been overfished have failed to recover, despite many decades of complete closure (Friedman et al., 2011). The limited dispersal observed in this study confers with findings from Brown et al. (2022), who found three genetically distinct populations of *H. scabra* across sites that were separated by a maximum of 300 km in Fiji.

Aside from limited larval dispersal, there are several additional reasons why *H. scabra* stocks are well-suited to being managed through the establishment of a network of LMMAs in southern Manus. Firstly, adult *H. scabra* habitat is common near MEARDN communities. Establishing LMMAs for sandfish that were in line-of-sight of communities makes social enforcement more feasible (McClanahan et al., 2006), and was a topic of discussion and the 2013 MEARDN meeting. Secondly, in Papua New Guinea and across the Pacific, most LMMAs are usually< 0.5 km^2 (Mills et al., 2010; Weeks et al., 2010). Because sea cucumbers are relatively sedentary, small LMMAs are likely to be sufficient for protecting their spawning stocks. For example, some large-bodied species such as the elephant trunkfish (*Holothuria fuscopunctata*) move less than 10 m on average over annual timescales (Purcell et al., 2023). Movement studies on *H. scabra* indicate that *H. scabra* will cover a total distance of 2–8 m per day and have home ranges of<0.05 km^2 (Lee et al., 2018).

For LMMA networks to serve their fisheries management objectives individual reserves must be large enough to protect adult spawning stocks that export larval subsidies to fished areas and nearby LMMAs (Green et al., 2015). For sea cucumbers, the configuration (size, spacing and location) of LMMAs within a network should be informed by the home ranges of benthic life stages and the dispersal patterns of pelagic larvae (Green et al., 2015). We suggest that a LMMA network for *H. scabra* should be made up of LMMAs ≥ 40 ha (Purcell and Kirby, 2006) and separated by no more than 15 km (the approximate distance that the majority of larvae travel from their natal sites). It is noteworthy that a study by Hamel et al. (2019) challenges the notion of that *H. scabra* adults have restricted home ranges. Hamel et al. (2019) describes how juveniles and adult *H. scabra* in aquaculture enclosures in Madagascar were able to modify their buoyancy, leading them to tumble or float at speeds much faster than they can obtain through benthic crawling. Hamel et al. (2019) termed this behavior active buoyancy adjustment, and found that in experimental trials this behavior was triggered by high conspecific density, increasing water turbidity, and decreasing salinity, with active buoyancy adjustment only observed in enclosures that were located near land. If active buoyancy adjustment is common in wild populations of *H. scabra*, then LMMAs would need to be large to be effective once protection leads to high densities.

The findings of this study demonstrate that *H. scabra*, and possibly other species of the genus *Holothuria*, are well suited to parentage analysis. In this study, 19 *H. scabra* microsatellites were successfully genotyped. Each of these microsatellites had high levels of polymorphism, which provided strong exclusion power in the parentage analysis and high confidence in assigned parent-offspring pairs. The *H. scabra* dispersal kernel presented here is based on a low number of assignments, 15 out of out of 827 total juvenile samples. This 2% assignment rate is relatively low in dispersal analyses based on parentage assignments (Bode et al., 2018), most of which assign between 1% and 15% of juveniles. This low juvenile assignment is a result of only sampling a small proportion of a single source population, and is reflected in the large uncertainty around the best-fit larval dispersal kernel. UVC surveys indicate that there were 24,653 adult sandfish in the Pere fishing grounds when this fieldwork was conducted (Supplementary Table S2), of which only 3.1% (n = 765) were analysed for the purpose of this study.

In this study, the shallow distribution of abundant and sedentary *H. scabra*, combined with the assistance of knowledge local fishers, made it possible to sample 6,465 sandfish in a month. However, the human resources required to amplify all the samples were unavailable. – In particular, the costs of the technical specialists' time to extract and genotype the samples, the cost of the necessary extraction kits, and the time to process the output from the genotyping. In detail, to extract batches of samples (e.g., 2 x 96 samples) and prepare them for fragment analysis would take 1–3 days, while processing of genotyped samples would take 3–4 days. As a consequence, we were only able to process 36.5% (n = 765) of the adult and 18.9% (n = 827) of the juvenile samples. Advances in genetic technologies may allow genotyping of a very large number of tissue samples in a small number of sequencing runs, saving months of data processing and making large-scale parentage studies on sea cucumbers more feasible.

The limited larval dispersal of *H. scabra* that we observed in this study provides compelling evidence that coastal communities who establish LMMAs to protect spawning stocks of sea cucumbers can directly benefit from their efforts. These LMMAs can replenish stocks within the fishing grounds they were established in, complementing national management measures such as closed seasons and size limits. Our study has also shown that although *H. scabra* appear to have limited larval dispersal, the animals nevertheless move across spatial scales that are larger than most communities' traditional fishing grounds. Networks of LMMAs that span multiple fishing grounds – at the scale of MEARDN, for example – are needed to generate the greatest benefits for these fisheries. LMMAs that are protect prime adult habitat of *H. scabra*, are in line-of-sight of communities, are ≥ 40 ha, and are separated by other LMMAs in a network by a maximum of 15 km are likely to provide the greatest fisheries management benefits for sandfish.

Data availability statement

The code for fitting dispersal kernels to parentage data are available at https://github.com/MikeBode/Parentage_kernel_fitting/. The cleaned datasets used for the Manus example, including the code for creating Figure 2, can be found in this repository in the archive "Manus_Holothuria_scabra.zip". The GenBank accession number for the sequences reported in this paper are PP548095-PP548120. Additional markers are available upon request to the authors.

Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional requirements because Research clearance, which included ethics clearance, was provided by the Mwanus Endras Asi Resource Development Network and the Papua New Guinea National Fisheries Authority.

Author contributions

PW: Project administration, Data curation, Formal analysis, Software, Validation, Writing – review & editing. DF: Data curation, Writing – review & editing, Project administration, Investigation, Methodology. MB: Formal analysis, Methodology, Software, Writing – original draft. MM: Conceptualization, Data curation, Investigation, Project administration, Writing – review & editing. HH: Data curation, Writing – review & editing, Formal analysis, Methodology. MLB: Writing – review & editing, Project administration, Resources. CM: Writing – review & editing, Data curation, Investigation. MK: Data curation, Writing – review & editing, Investigation. RH: Investigation, Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2024.1380235/full#supplementary-material

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Development and application of molecular markers in fisheries, aquaculture, and industry of representative temperate and tropical sea cucumbers: a review

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Sea cucumber has emerged as a crucial economic species in aquaculture in China because of its remarkable nutritional and medicinal value. However, wild sea cucumber populations have experienced a decline due to overfishing and environmental factors, underscoring the urgent need for genetic resource conservation and biotechnology innovation within the sea cucumber aquaculture and breeding industry. The development of the sea cucumber industry is still impeded by challenges and difficulties. Nevertheless, significant progress has been made through the utilization of molecular markers, which have effectively addressed a number of fisheries and aquaculture issues. In recent years, diverse types of molecular markers including mitochondrial DNA, microsatellites, and SNP markers have been developed and extensively applied in various aspects of sea cucumber research. These markers play vital roles in genetic sex identification, germplasm resource evaluation, population structure assessment, as well as marker-assisted breeding in marine ranching and sea cucumber aquaculture and breeding industry. This review provides an overview of the fundamental principles, functions, and characteristics associated with various markers employed across various sea cucumber species while also discussing their applications within different aspects of the sea cucumber fisheries, aquaculture, and breeding industry.

KEYWORDS

sea cucumber, molecular marker, aquaculture, germplasm conservation, molecular breeding

1 Introduction

Sea cucumber is a marine invertebrate belonging to the class Holothuroidea with the phylum Echinodermata. In general, sea cucumbers appear in a cylindrical, highly contractile body with numerous papillae on the dorsal and tube feet on the ventral side. Sea cucumbers live in a wide variety of different habitats, including seaweed ecosystems and coral reefs, ranging from shallow water to depths of more than 3,000 meters (Sulardiono et al., 2022). They feed on microalgae, organic debris, and material in sediments (Madduppa et al., 2017). There are more than 1,700 species of sea cucumber in the world, of which about 50–60 are edible (Elvevoll et al., 2022). Total sea cucumber fisheries increased from 26,000 tonnes of live weight in 2007–2016 to 48,000 tonnes in 2018–2019, with a slight decline to 43,000 tonnes in 2020 (FAO). Most cultured sea cucumber species belong to the order Aspidochirotida, including two sister groups, Holothuriidae and Stichopodidae (Wen et al., 2010). In Asian countries, sea cucumber is a commercially important aquaculture species due to its high nutritional and medicinal value. The wild sea cucumber populations have declined because of overfishing and environmental factors (Peng et al., 2012).

To increase the yield of sea cucumber, the sea cucumber breeding industry is rising gradually. Since the 19th century, sea cucumber farming in most countries has experienced boom-and-bust cycles, with the exception of China, where the majority of sea cucumbers have come from the aquaculture industry since the late 1980s (Liu, 2016). Even though sea cucumber aquaculture has been developed in the past decades, a series of problems occurred that could affect the larva development and juvenile growth, mainly related to disease control, germplasm conservation, and large-scale production in sea cucumber industry (Du et al., 2012). Among these, the slow growth rate is one of the most important issues in the sea cucumber industry (Cui et al., 2021). To meet the increasing demand for sea cucumber in the market, sea cucumber aquaculture and management using molecular methods or based genomic methods are quite important.

The sea cucumber breeding industry requires the application of traditional and novel molecular methods to benefit production and ensure sustainable development. Genetic breeding has been widely used in the breeding of aquaculture species, which focuses on genetic variations among populations and individuals to improve different types of performance traits. Molecular markers are commonly used molecular methods for detecting biological diversity at the DNA level, which is a powerful informatic molecular tool in genetic breeding and population genetics. Commonly used molecular markers include mitochondrial DNA (mtDNA), restricted fragments length polymorphism (RFLP), random amplification polymorphic DNA (RAPD), microsatellite (SSR), and single nucleic polymorphism (SNP), which have been widely used for species identification, population genetics, genetic breeding, marker-assisted selection, stock enhancement, and aquaculture management (Maqsood, 2017).

At the end of the 20th century, with the emergence of restriction enzymes and polymerase chain reaction (PCR), many molecular marker technologies have been developed and widely used. RFLP marker is a highly polymorphic and co-dominant marker that can be used for species identification, which is rarely used at present due to its complex process (Wen et al., 2010). RAPD marker is a simple technique without molecular hybridization and radioautography, which can be used to evaluate the geographical origin of species and genetic diversity analysis (Yun et al., 2017). Microsatellite marker is abundant and widely distributed throughout the genome (Oliveira et al., 2006), which is a simple and fast marker that have high mutation rates and reliable results, which can identify sea cucumber species, which will benefit germplasm conservation and management (Zhan et al., 2007). Subsequently, the advent of single nucleic polymorphism (SNP) molecular marker technology made it possible to perform genetic analysis of the genome at a precise level (Oliveira and Azevedo, 2022). Microsatellite marker and SNP marker are ideal molecular markers because of their high polymorphism and co-dominant inheritance (Maqsood, 2017), which have been widely used in population structure analysis (Kanno et al., 2006), genetic linkage map construction (Yan et al., 2013), QTL mapping (Chen and Li, 2007), marker-assisted selection (MAS) (Wang et al., 2009). Mitochondrial marker is a simple, fast, reliable molecular marker that is inherited from maternal lineage, which have high mutation rates and can be used in species identification, population genetic structure analysis, and phylogenetic analysis (Sulardiono et al., 2022). The advantages and disadvantages of various types of molecular markers are summarized in Table 1. At present, molecular markers are quite useful in the sea cucumber aquaculture and breeding industry. For instance, using sex-specific tags and SNPs to identify the sex can develop and breed a single-sex population, which could improve breeding efficiency, and lay a molecular foundation for studying the sex determination mechanism of sea cucumber (Cui et al., 2021); The high-density genetic linkage map of sea cucumber was constructed based on SNPs, which laid a foundation for mapping and analyzing QTL for performance traits like growth rate (Wei et al., 2021). This review introduces and summarizes the development and application of different types of molecular markers and their based genomic methods in sea cucumbers (Figure 1, Table 2) and examines the present status and prospects in fisheries, aquaculture, and industry.

2 Development of various molecular markers in sea cucumber

2.1 Mitochondrial DNA markers

The DNA barcoding using mitochondrial COI (cytochrome C oxidase subunit I) and ITS (internal transcribed spacers) can be used for identifying species for *Stichopus* genus and analyzing genetic diversity and phylogenetic relationships (Madduppa et al.,

TABLE 1 Characteristics, advantages, and disadvantages of various types of molecular markers.

Molecular marker	Description	Advantage	Disadvantage	Application
RFLP	Changes in the length of DNA fragments produced by cutting relevant DNA molecules using specific endonuclease enzymes	High polymorphism; Co-dominant marker; High reproducibility	Require target DNA sequence information for the design of amplification primers; Complicated process	Species identification; Product mislabeling correction
RAPD	Genomic DNA is amplified by PCR using a series of single random primers (usually 10 nucleotides), and the polymorphism of PCR products is detected by electrophoresis	Random primers; Simple technique without molecular hybridization and radioautography; Require low amount DNA; Low cost	Poor experimental repeatability; unreliable results; Dominant marker	Genome mapping; Geographical origin and genetic diversity analysis
AFLP	A PCR-based molecular technique that uses selective amplification of a subset of digested DNA fragments	Large amount of markers with relatively little lab effort; No prior information about genome; Genome wide coverage; Small amount of DNA needed; Low cost; High experimental repeatability; reliable results	Dominant marker; Low reproducibility	Determine genetic differences among individuals, populations, and species; Investigate population structure and phylogenetic relationships based on genetic distances; Evaluate gene flow and dispersal, outcrossing, introgression, and hybridization
SSR	non-coding repetitive DNA regions composed of small motifs of 1 to 6 nucleotides repeated in tandem	Abundant, widely distributed throughout the genome; High mutation rate; Co-Dominant marker; High experimental repeatability; reliable results	Difficult to develop; High cost	Construction of genetic maps; Analyze genetic structure; Species identification; Genetic breeding and assessment of genetic variation
SNP	DNA sequence diversity caused by single nucleotide variation at the genomic level	Co-dominant markers; Abundance; Low genotyping cost; Typically biallelic; High-throughput genotyping; Lower mutation rate; Genotyping error rates	Difficult detection; Large workload	Construction of high-dense maps; Quantitative trait loci mapping; Assessment of genetic diversity; Parentage studies; Marker-assisted breeding
Mitochondrial DNA markers	Inherited from maternal lineage and efficiently identify and classify species based on a short DNA sequence in population and evolutionary biology	High mutation rate; Maternal inheritance; Simple, fast, and reliable	Limited information compared with nuclear genes	Species identification; Examine population genetic structure; Examine phylogenetic relationships

2017; Sulardiono et al., 2022). More specifically, the DNA barcode mitochondrial COI has been used for identifying sea cucumber species in two main families, including Stichopodidae (*Stichopus herrmanni*, *Stichopus ocellatus*, *Stichopus horrens, Stichopus monotuberculatus*) and Holothuridae (*Bohadschia bivittata*, *Actinopyga lecanora*, *and Holothuria leucospilota*), which will benefit germplasm conservation and management (Madduppa et al., 2017).

Using the mitochondrial DNA marker, three local sea cucumber species in Indonesia, Crengek Gamete, Pace gamete, and Kuning gamete, were close relatives to *Stichopus monotuberculatus* (Sulardiono et al., 2022). A next-generation

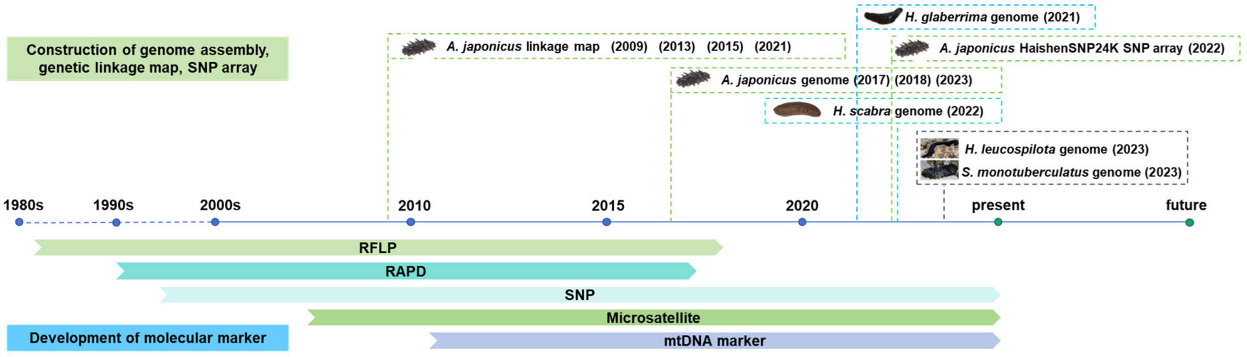

FIGURE 1
Time history of molecular markers and their based genomic methods applied in sea cucumbers.

TABLE 2 Development and application of various types of molecular markers in sea cucumbers.

Types	Sea cucumber species	Location	Method	Population number	Description	Reference
16S rRNA gene region	*Stichopus hermanni*, *Stichopus chloronotus*, *Thelenota ananas*, *Thelenota anax*, *Bohadschia argus*, *Holothuria fuscopunctata*, *Holothuria leucospilota*, *Holothuria scabra*, *Actinopyga echinites*	Sanya, China	species-specific PCR method	15 individuals per sample	a simple, fast, and reliable protocol for the accurate identification of sea cucumber species	Wen et al., 2012a
mitochondrial COI	*Holothuria atra*	Hawaiian Islands, Line Islands, Marshal Islands, Bonin Islands, and Ryukyu Islands	Sanger sequencing	385	examining population genetic structure to aid coral reef management	Skillings et al., 2011
mitochondrial COI and 16S ribosomal RNA (16S), and nuclear histone (H3)	*Holothuria edulis*	Okinawa Main Island in Ryukyu Archipelago (Motobu, Oyama, Ryugu, Sunabe, Philippine Sea and Awase)	Sanger sequencing	438	Investigating the metapopulation structure to provide valuable data to help management in vulnerable locations of Okinawa	Soliman et al., 2016
COI DNA barcoding	4 species belonged to the Stichopodidae (*Stichopus herrmanni*, *Stichopus ocellatus*, *Stichopus horrens*, *Stichopus monotuberculatus*), and 3 species to the Holothuridae (*Bohadschia bivittata*, *Actinopyga lecanora* and *Holothuria leucospilota*)	Kepulauan Seribu reefs, Indonesia	phylogenetic analysis	96	species identification	Madduppa et al., 2017
COI DNA barcoding	23 sea cucumber species	Not applicable	Sanger sequencing and next-generation DNA sequencing	23	screening of commercially processed sea cucumber products for clearer labeling	Xing et al., 2021
COI and ITS loci DNA barcoding	Stichopus	Karimunjawa Island, Central Java province, Indonesia	Not applicable	100 individuals consisting of three sea cucumber types (30 Crengek gamete, 36 Pace gamete, and 34 Kuning gamete)	genetic diversity, species identification, and examine phylogenetic relationships of the Stichopus genus	Sulardiono et al., 2022
RAPD	*Apostichopus japonicus*	Dalian and Weihai, China	RAPD-PCR	26	geographical origin and genetic diversity analysis	Yun et al., 2017
RFLP	Aspidochirotida: Stichopodidae	Sanya, Dalian, and Guangzhou, China	PCR-RFLP and FINS	70	identify six species belonging to family Stichopodidae in commercial products	Wen et al., 2010
RFLPs	*Holothuria mammata* Grube, 1840 and *H. sanctori* Delle Chiaje, 1823	The Azores in the north-east Atlantic, nine volcanic oceanic islands and several islets	PCR-RFLPs	Not applicable	quick identification of two species of sea cucumber	Madeira et al., 2018
PCR-RFLP, 16Sar/ 16Sbr primers	16 commercial sea cucumber species	Sanya and Dalian, China	PCR	6 individuals for each species	identify 16 types of sea cucumber in raw materials and processed foods	Zeng et al., 2018
EST-SSRs	*Apostichopus japonicus*	Rongcheng, Shandong Province, China	EST database	48	population structure analysis, phylogenetic and comparative genomics studies and genetic linkage map construction	Peng et al., 2009

(Continued)

TABLE 2 Continued

Types	Sea cucumber species	Location	Method	Population number	Description	Reference
Microsatellite	*Stichopus japonicus*	Onagawa Bay in Miyagi Prefecture, Japan	Not applicable	Not applicable	tools for genetic analyses, investigate the genetic structure of *S. japonicus* and to detect reproductive isolation among color types through mating experiments	Kanno et al., 2005
Microsatellite	*Apostichopus Japonicus*	Aomori, Hokkaido, Miyagi, Oita, Japan; Qingdao, China	Not applicable	403	population identity of sympatric green and black, difference in the population structure, and further ecological and genetic research	Kanno et al., 2006
Microsatellite	*Apostichopus japonicus*	Not applicable	screening from SSR enriched library and EST database	45	population structure and the stock enhancement in fishery, the species/hybrid identification, and the linkage map construction	Zhan et al., 2007
Microsatellite	*Stichopus japonicus*	Penglai, China	expressed sequence tags	11	examining genetic population structure, parentage analysis and mapping studies	Chen and Li, 2007
Microsatellite	*Apostichopus japonicus*	Not applicable	Not applicable	Not applicable	an efficient procedure for isolating microsatellite DNA	Hu et al., 2007
Microsatellite	*Apostichopus japonicus*	Dalian, China	combining microsatellite DNA polymorphism and its relationship with body weight	208	molecular breeding, marker-assisted selection	Wang et al., 2009
Microsatellite	*Apostichopus japonicus*, *Parastichopus parvimensis*, and Pathallus mollis warty sea cucumber (*Parastichopus parvimensis*), and black sea cucumber (*Pathallus mollis*)	Qingdao, China; United States; Peru	Not applicable	60	future genetic breeding and the assessment of genetic variation within sea cucumbers	Liao et al., 2011
Microsatellite	*Apostichopus japonicus*	Jiaonan, Shandong, China.	expressed sequence tags	30	provide sufficient polymorphism for population genetic studies and genome mapping	Jiang et al., 2011
Microsatellite	*Stichopus chloronotus*	Okinawa Archipelago, Japan	Not applicable	60	detailing the genetic structure and gene flow	Taquet et al., 2011
Microsatellite	*Apostichopus japonicus*	Rongcheng, China	enrichment-colony hybridization protocol	48	population genetic studies and molecular marker-assisted breeding	Peng et al., 2012
Microsatellite	*Apostichopus japonicus*	Noto, Shinminato, and Uozu around Toyama Bay	PCR	236 wild individuals (146 red and 90 green variants)	clarify larval dispersal among populations by describing the levels of distinctiveness and gene flow among red and green variant populations, provide useful information for genetic conservation and fisheries management	Soliman et al., 2012
Microsatellite	*Stichopus japonicus*	Jeju island, Korea	multiplex assays with eight highly polymorphic	250	reveals the genetic composition and significant genetic differentiation between wild and hatchery-produced red sea cucumber samples in Korea	An et al., 2013

(Continued)

TABLE 2 Continued

Types	Sea cucumber species	Location	Method	Population number	Description	Reference
			microsatellite loci			
Microsatellite	*Apostichopus japonicus*	Liaoning, China	transcriptome sequencing	20	linkage mapping, comparative genome analysis and genetic breeding	Chen et al., 2013
Microsatellite	*Holothuria scabra*	Croker Island, Northern Territory, Australia	next-generation sequencing	50	population structure and mating systems, investigation of population structure, levels of gene flow and mating systems	Fitch et al., 2013
Microsatellite	*Holothuria leucospilota*	Hainan Island, China	amplified fragment length polymorphism	30	germplasm conservation, genetic diversity, population structure, and conservation strategy design	Dai et al., 2015
Microsatellite	*Holothuria scabra*	Hainan Island, China	amplified fragment length polymorphism	30	genetic diversity and its effective conservation strategy, population structure	Li et al., 2015a
Microsatellite	*Stichopus horrens*	Hainan Island, China	fast isolation by amplified fragment length	30	studying population structure and conservation strategy design, genetic diversity	Li et al., 2015b
Microsatellite	*Holothuria leucospilota*	Not applicable	amplified fragment length polymorphism	30	genetic diversity, and the design of conservation strategies, population structure studies and cultivation	Shangguan et al., 2015a
Microsatellite	*Stichopus horrens* (Selenka)	Sanya, Hainan, China	fast isolation method with amplified fragment length	35	genetic structure, population conservation, and breeding of wild *S. horrens*	Shangguan et al., 2015b
Microsatellite	*Holothuria mammata*	Atlantic and Mediterranean Sea	SSR enriched library on a Roche 454 platform	60	fisheries management including identification of stocks, assessment of genetic diversity, estimation of gene flow and monitoring the fishery effects on exploited populations	Henriques et al., 2016
Microsatellite	*Holothuria grisea*	Bitupitá beach, Barroquinha, Ceará, Brazil	Illumina paired-end reads of whole genome shotgun sequencing	30	proper conservation of the species and development of sustainable fishery and aquaculture	Pereira et al., 2018
Microsatellite	*Apostichopus japonicus* Selenka, 1867	Peter the Great Gulf, Japan	Not applicable	159	genetic variability and population structure	Yagodina et al., 2022
SNP	*Apostichopus japonicus*	Dalian, Yantai, Qingdao, and Wendeng, China	Not applicable	39	delineating population structure in the sea cucumber	Sun et al., 2010
SNP	*Stichopus monotuberculatus*	Xidao Island, Sanya, China	gene-based	80	future quantitative trait loci (QTL) analysis, and to facilitate marker-assisted selection (MAS)	Du et al., 2012
SNP	*Apostichopus japonicus*	Wendeng, China	mining EST	32	genetic diversity assessment, genome mapping, reproductive ecology analysis and SNP-based analysis in aquaculture practice	Yang et al., 2012

(Continued)

TABLE 2 Continued

Types	Sea cucumber species	Location	Method	Population number	Description	Reference
SNP	*Apostichopus japonicus*	Dalian, China	SNP markers associated with defense mechanism	50	useful for the construction of genetic linkage map and comparative genome analysis	Gao et al., 2013
SNP and SSRs	*Apostichopus japonicus*	Dalian, China; Vladivostok, Russia; Rajin, North Korea	transcriptome analysis of tube foot	45	provide abundant genetics and molecular ecology resources	Zhou et al., 2016
SNPs	*Apostichopus japonicus*	Changdao, Yantai, Jiaonan, Wendeng, Penglai, China; Oita, Aomori, Japan	EST sequences	40	guides selective breeding	Dong et al., 2016
SNPs and SSR	*Apostichopus japonicus*	Yantai, China	transcriptome sequencing	150	genes discovery and functional genomic studies of the sea cucumber	Zhou et al., 2013
SNPs	*Holothuria (Metriatyla) scabra*	Six locations throughout Fiji (Namuka district, Lakeba island, Tavuki district, Serua island, Raviravi district, Nacula island)	Illumina sequencing	211	investigate genetic structure, diversity, signatures of selection, relatedness and connectivity in wild populations	Brown et al., 2022
SNPs	*Holothuria leucospilota*	Islands from Northern Vietnam, Central Vietnam, and Southern Vietnam; Darwin, Northern Australia and Mooloolaba, Eastern Australia	restricted site associated DNA sequencing	180	investigate population genetics to provide basic genetic information, design restocking management plans, genetic conservation initiatives, and sea ranching programs	Chieu et al., 2023

sequencing-based DNA mini-barcoding using COI has been developed to screen various commercial sea cucumber products and correct mislabeled for clearer labeling guidelines (Xing et al., 2021). The complete mitochondrial genome and cox1 DNA barcoding will be helpful for the sea cucumber breeding industry and studies on population genetic diversity and molecular ecology resources (Fan et al., 2012). In addition, a species-specific PCR of 16S rRNA has been used to authenticate 11 sea cucumber species from processed products, providing a rapid and useful protocol for correct labeling of commercial sea cucumber products (Wen et al., 2012a). By calculating the genetic distance of COI gene among different sea cucumber, Lu et al. proved the feasibility of COI as a DNA barcode for species identification, and the probes designed based on COI gene were used to identify four species of sea cucumber (Lv et al., 2011). Hu et al. used COI gene and 16S rRNA gene as target genes to identify sea cucumber species based on DNA barcoding technology, and the results showed that COI gene or 16S RNA gene could identify most species of sea cucumber (Hu et al., 2019). According to the specific primers for cox1 gene of *Holothuria scabra*, eDNA technology was used to investigate *Holothuria* resources in Weizhou Island, and the results showed that the eDNA monitoring method was more suitable and reliable than traditional methods (E et al., 2023).

2.2 Traditional molecular markers

A PCR-RFLP method was developed to identify six species of sea cucumbers in commercial products, which can authenticate species and detect fraudulent labeling (Wen et al., 2010). Another application of the PCR-RFLP method was to identify 16 commercial sea cucumber species from food products to correct the mislabeling (Zeng et al., 2018). Moreover, a PCR-RFLP method using restriction nuclease Sau3AI on 16S rRNA fragments has been developed to rapidly and inexpensively identify holothurian species with no need for taxonomical or genetic expertise (Madeira et al., 2018). DNA polymorphisms identified by RAPD-PCR technology were used to evaluate the geographical origin of *A. japonicus* and genetic diversity analysis (Yun et al., 2017). EST databases of sea cucumber species have been built, including *Holothuria glaberrima* during intestinal regeneration (Rojas-Cartagena et al., 2007), multiple tissues (body wall, intestine, respiratory tree) in *Apostichopus japonicus* (Yang et al., 2009), which were submitted to NCBI Genbank. EST databases have provided a rich resource for the development of molecular markers of sea cucumber species, especially microsatellite markers. SSR can be identified from EST data using screening programs, for example, SSRHUNTER (Chen and Li, 2007; Zhan et al., 2007; Peng et al., 2009; Jiang et al., 2011).

In addition, SNP markers were characterized by mining EST data of *A. japonicus* and using a homogeneous Tm- (melting temperature) genotyping method (Yang et al., 2012; Dong et al., 2016).

2.3 Microsatellite markers

The majority of microsatellite markers in sea cucumber species were from an aquaculture species in the coastal area of the Northwest Pacific, *Stichopus (Apostichopus) japonicus*. The earliest 20 microsatellite markers of sea cucumber *Apostichopus japonicus* were developed by Kanno et al. in 2004, which could be excellent molecular markers to facilitate genetic structure analysis (Kanno et al., 2005). Eleven microsatellite markers were used to identify *A. japonicus* population from green and black color variants for further genetic population structure analysis and ecological research (Kanno et al., 2006). In sea cucumber *Apostichopus japonicus*, Hu et al. developed an efficient procedure for isolating a reasonable number of microsatellites for genetic analysis (Hu et al., 2007). Forty-five informative microsatellite markers (43 genomic SSRs and 2 EST-SSRs) were developed based on screening SSR-enriched library and EST library, which may contribute to population structure analysis, linkage map construction, stock enhancement, and species identification (Zhan et al., 2007). Eleven microsatellite markers derived from expressed sequence tags were developed to investigate population structure and conduct parentage analysis and QTL mapping (Chen and Li, 2007). Microsatellite markers associated with body weight were developed for not only molecular breeding but also marker-assisted selection (MAS) (Wang et al., 2009). Jiang et al. have developed 43 EST-SSR markers for future studies in population genetics and genomics (Jiang et al., 2011). Twenty novel microsatellite markers from *Apostichopus japonicus* were used for cross-species application in *Parastichopus parvimensis* from the United States and *Pathallus mollis* from Peru, which could facilitate genetic variation assessment and genetic resource management among Holothuroidea populations (Liao et al., 2011). A larger number of ~70 microsatellite markers were developed for the implementation of marker-assisted selection and population genetic studies (Peng et al., 2012). Furthermore, 45 gene-derived microsatellite markers were identified from transcriptome sequencing, which could be useful for comparative genomics studies, genetic linkage mapping, and molecular breeding like MAS (Chen et al., 2013). A total of 8 highly polymorphic microsatellite markers were used for genetic structure studies between wild and hatchery populations and implications in breeding programs and stocking strategies (An et al., 2013). Ten microsatellite markers have revealed the genetic variation and population structure of *A. japonicus* in Peter the Great Gulf, Japan. In studying microsatellite markers, the detected high heterogeneity among populations might be due to several factors: stochastic larval settlement at a location, and selection pressures on QTL (Yagodina et al., 2022).

Ten microsatellite markers were developed in a tropical sea cucumber species *Stichopus chloronotus* to investigate the genetic structure and gene flow among populations (Taquet et al., 2011). Eighteen microsatellite markers from another sea cucumber species *Holothuria scabra* were characterized, which were beneficial for genetic structure and gene flow studies for sea cucumber aquaculture and management (Fitch et al., 2013). A total of another 9 microsatellite markers of *H. scabra* were developed for conservation strategy and investigation of genetic diversity and population structure (Li et al., 2015a). Eight microsatellite markers were identified in a tropical species *Holothuria leucospilota*, which could be beneficial for genetic structure, population diversity, and germplasm conservation (Dai et al., 2015). Shangguan et al. identified 16 additional microsatellite markers from *H. leucospilota* that could be used for studies in genetic diversity and population structure and application in cultivation and conservation strategies (Shangguan et al., 2015a). Li et al. identified 9 microsatellite markers in another tropical sea cucumber *Stichopus horrens* (Selenka) that could be used as markers to study its conservation and breeding, population structure, phylogeny and evolutionary studies of Holothuroidea (Li et al., 2015b). 9 more microsatellite markers have been characterized in *S. horrens*, which could be helpful for conservation strategy and management, population structure and genetic diversity of *S. horrens* (Shangguan et al., 2015b). 9 novel microsatellite markers have been identified from *Holothuria mammata*, an exploited sea cucumber species in Mediterranean and North-Eastern Atlantic, which could be used for aquaculture and conservation management, gene flow, and genetic diversity assessment (Henriques et al., 2016). Furthermore, eight microsatellite markers in the West Atlantic sea cucumber species *Holothuria grisea* have been developed and used as markers for proper conservation and advancement in sustainable aquaculture of this species (Alves Pereira et al., 2018). Despite the popularity of SSR in diverse aspects of sea cucumber research, it is gradually replaced by genome-wide molecular markers such as SNPs.

2.4 Single nucleic polymorphism (SNP)

In *A. japonicus*, all identified 13 SNPs have two alleles and their heterozygosity could be used as molecular markers in analyzing population structure, population genetics, and QTL mapping (Sun et al., 2010). Fifteen more SNP markers were identified from ESTs and provided a complement to available molecular markers of *A. japonicus* (Yang et al., 2012). These SNPs may be implied in studies on genetic diversity, QTL mapping, and ecology analyses of *A. japonicus* (Yang et al., 2012). 26 SNP markers associated with defense mechanisms were developed by mutation scanning from unigene sequences in *A. japonicus* transcriptome, which may provide insights into genetic maps and comparative genomic studies (Gao et al., 2013). 51 gene-derived SNPs from ESTs using high-resolution melting can be used for MAS, genetic improvement, and aquaculture management (Dong et al., 2016). High throughput transcriptome analysis provided benefit to large-scale marker discovery of *A. japonicus*. 142,511 SNP markers and 6,417 microsatellite markers with high quality have been identified through transcriptome analysis, which can be used for gene discovery and functional genomic research (Zhou et al., 2014). A total of 219,860 SNP markers have been identified from tube foot transcriptome, providing plentiful resources in genetic and molecular ecology studies (Zhou et al., 2016).

3 Construction of genome assembly, genetic linkage map, and SNP array

Since 2017, the genome assemblies of sea cucumber species have been constructed using next-generation sequencing and third-generation sequencing, including one northern sea cucumber species *A. japonicus*, and three tropical sea cucumber species, *H. scabra*, *H. leucospilota*, *H. glaberrima* and *S. monotuberculatus* (Table 3). As the most common commercial sea cucumber species, the *A. japonicus* genome assembly have been constructed in 2017 (Zhang et al., 2017) and 2018 (Li et al., 2018) and updated in 2023 (Sun et al., 2023). The construction of genome assembly has been used to understand the molecular mechanisms on morphological evolution, visceral regeneration, saponin biosynthesis, aestivation regulation, providing a referable resource for understanding evolution and biodiversity of sea cucumber. In 2022, *De novo* genome assembly of *H. scabra* has been built using nanopore sequencing, which provides excellent genetic resource for studying genetic, phylogenetic, molecular biology, and breeding studies (Luo et al., 2022). In 2023, a draft genome of *S. monotuberculatus* was assembled using Nanopore sequencing, which provides insight into critical genes in fucosylated chondroitin

TABLE 3 Construction of genome assembly and molecular markers-based genomic methods in sea cucumbers.

Types	Sea cucumber species	Location	Method	Population number	Description	Reference
genome	*Apostichopus japonicus*	Laoshan, Qingdao, China	Illumina+PacBio (CLR)	NA	enables exploring key genetic mechanisms behind the unique biological characters, phylogenetic and comparative genomic analyses	Zhang et al., 2017
genome	*Apostichopus japonicus*	Shandong Province, China	Illumina+PacBio (CLR)	NA	reveals novel genomic features and molecular variations that contribute to the evolutionary adaptation, provides insights into saponin synthesis and aestivation regulation.	Li et al., 2018
genome	*Apostichopus japonicus*	Rushan, Shandong Province, China	Illumina+Pacbio (CCS)	NA	an excellent tool to better investigate the mechanisms that drive evolution and biodiversity	Sun et al., 2023
genome	*Holothuria glaberrima*	Puerto Rico, USA	Illumina	NA	a critical resource for future studies in animal regeneration	Medina-Feliciano et al., 2021
genome	*Holothuria scabra*	Guangxi Province, China	Nanopore	NA	provides a referable resource for research on genetic, phylogenetic, molecular biology, and breeding studies	Luo et al., 2022
genome	*Stichopus monotuberculatus*	Guangxi Province, China	Nanopore	NA	provides a genomic approach to investigate the structural diversity, as well as novel perspectives into evolutionary adaptation of critical genes in holothurian fucosylated chondroitin sulfates biosynthesis pathways	Zhong et al., 2023
genome	*Holothuria leucospilota*	Nanhai, China	Illumina+PacBio+BGI	NA	provides insights into molecular underpinnings of sacrificial organ expulsion in holothurian species	Chen et al., 2023
SNP array	*Apostichopus japonicus*	Liaoning and Shandong, China; Russia	a high-throughput 24 K SNP genotyping array	210	minmax concave penalty (MCP) regularization for sparse deep neural networks (DNN-MCP) high-throughput genotyping platform	Lv et al., 2022
Genetic linkage map	*Apostichopus japonicus*	Not applicable	AFLP and microsatellite markers	88	application of a marker-assisted selection breeding strategy, basis for mapping of the functional genes and quantitative trait loci, mapping QTL for growth heterosis and locating expressed genes in aquaculture.	Li et al., 2009
Genetic linkage map	*Apostichopus japonicus*	Shandong Province, China	microsatellites and SNPs	144	facilitate further sea cucumber genetic studies such as quantitative trait loci (QTL) mapping and comparative genomic analysis	Yan et al., 2013
Genetic map	*Apostichopus japonicus*	Penglai, China	genotyping-by-sequencing (GBS) methods, 2b-restriction site-associated DNA (2b-RAD) method	100	a powerful tool for research involving both fine-scale QTL mapping and marker assisted selection (MAS)	Tian et al., 2015

NA, not applicable.

sulfates biosynthetic pathway (Zhong et al., 2023). The high-resolution genome assembly of *H. leucospilota* was also built in 2023 using Illumina sequencing, Pacbio sequencing, BGI sequencing platform, which provides insights into molecular underpinnings of sacrificial organ expulsion in holothurian species (Chen et al., 2023).

Genetic linkage maps are constructed based on the gene linkage and genetic recombination rates to infer the relative positions of genes or genetic markers on chromosomes (Cui et al., 2021). Up to date, a total of four genetic linkage maps of *Apostichopus japonicus* based on molecular markers have been developed for QTL mapping and marker-assisted selection breeding since 2009 (Table 3). The first genetic linkage map was built on 484 polymorphic AFLP and microsatellite markers, which provides the application of molecular breeding and understanding the genetic basis of economic traits in sea cucumber industry (Li et al., 2009). In 2013, a medium-density genetic linkage map using co-dominant microsatellite and SNP markers was built to benefit QTL mapping, marker-assisted selection, and comparative genomic studies (Yan et al., 2013). A high-density and high-resolution genetic map of 7,839 markers based on 2b-restriction site-associated DNA (2b-RAD) was constructed in 2015, which facilitates fine-scale QTL mapping, precise marker-assisted selection, and chromosome assignment for whole genome assembly (Tian et al., 2015). A 6,144 SNP-based high-density genetic map using genotyping-by-sequencing was built, which provides an important genomic resource for marker-assisted selection with high performance traits like growth traits (Sun et al., 2021). With the development of whole genome sequencing and new molecular techniques, genome-wide molecular markers like SNPs in an SNP array chip have been used as an efficient genotyping tool for genetic research and genome breeding programs in aquaculture. In 2022, a high-throughput 24 K SNP array genotyping platform, HaishenSNP24K, was developed for genetic studies and application in selection and breeding programs with minmax concave penalty regularized deep neural networks (DNN-MCP) (Lv et al., 2022). The new SNP array has significantly improved the genomic assessment of sea cucumber populations for quantitative performance traits, including wet or dry weight, and survival time (Lv et al., 2022).

4 Application of molecular markers and based genomic methods in fisheries, aquaculture, and industry

The molecular marker technology facilitates germplasm resource evaluation, assessment of genetic diversity and population structure, sex identification for live animal, and selection of desirable traits, which has been extensively applied in various aspects of the sea cucumber aquaculture industry (Figure 2). These applications play a pivotal role in enhancing the efficiency of industrialized aquaculture and breeding programs for sea cucumbers, enabling precise breeding strategies and conservation of germplasm resources.

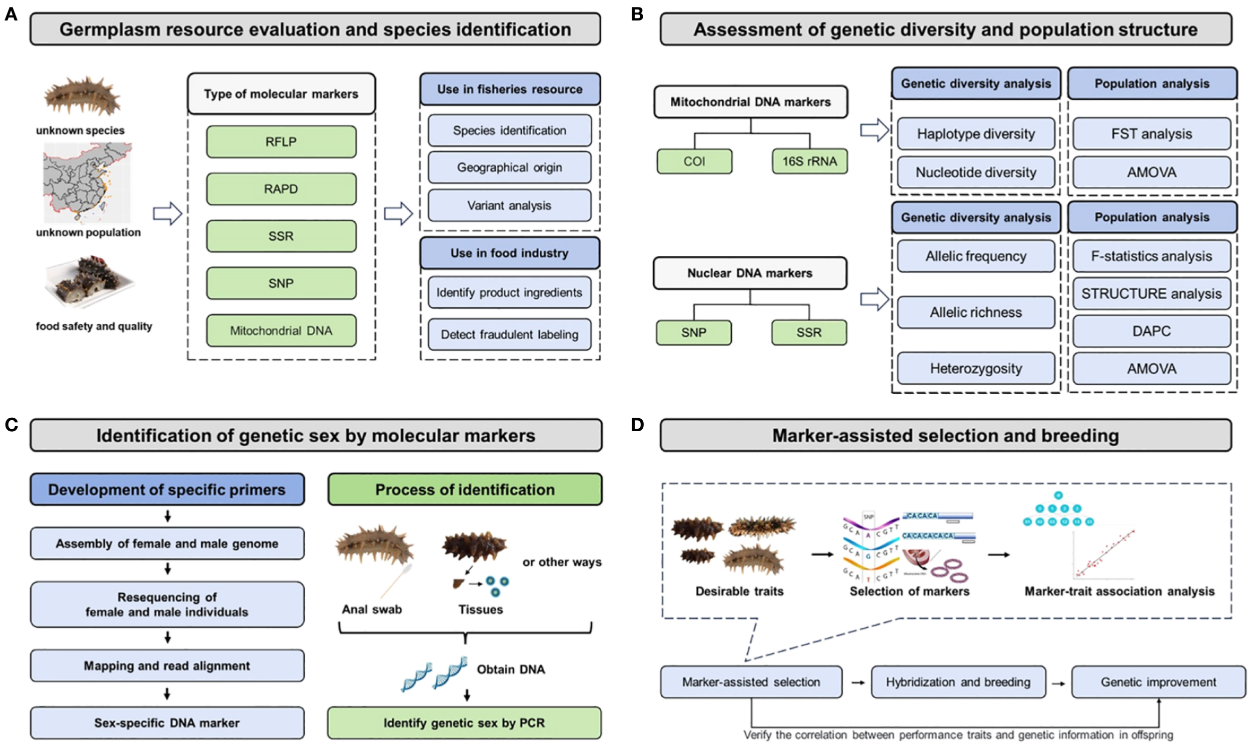

FIGURE 2
Flow chart of molecular markers or based genomic methods in sea cucumber fisheries, aquaculture, and industry. **(A)** Germplasm resource evaluation and species identification; **(B)** Assessment of genetic diversity and population structure; **(C)** Genetic sex identification in aquaculture; **(D)** Marker-assisted selection and breeding.

4.1 Germplasm resource evaluation and species identification

Germplasm resource evaluation mainly includes population resource information, ecological factors, habitat conditions, environmental variables, external disturbance factors, and strain identification. Germplasm resource identification has significant implications not only in the discovery and development of new germplasm but also in hybrid and cryptic species identification and the utilization of hybrid advantages (Ding and Chang, 2020). Specific sequence analysis plays an important role in natural hybridization and gene flow between populations. For example, skeletal characteristics could not distinguish *H. scabra*, colour morphs of *H. S.* var.*versicolor*, and intermediate phenotype hybrids in the species boundary study, as their color patterns, morphology, and small-scale distribution patterns were different (Uthicke et al., 2005). Both of population genetics and 16S mtDNA sequence analysis were used to identify new species *H. S.* var.*versicolor* and individuals with intermediate phenotypes represented F1 hybrids, demonstrating that the opportunity for introgression exists (Uthicke et al., 2005). The conventional method based on their morphology or external characteristics for species identification have gradually developed towards molecular markers with high accuracy and polymorphism, such as RFLP, RAPD, SSR, and SNP (Lv, 2012). In general, germplasm resource evaluation by molecular markers includes four important steps: sample collection, DNA extraction, PCR amplification using molecular markers, gel electrophoresis or DNA sequencing. For instance, PCR-RFLP was used to develop a method of DNA barcoding-based sea cucumber species identification, and mitochondrial COI gene was used as a DNA barcode to achieve germplasm identification of five sea cucumber species (Lv, 2012). Wen et al. successfully identified 19 commercial sea cucumber species using PCR-RFLP method (Wen et al., 2012b). With the completion of mitochondrial genome sequencing of *Apostichopus japonicus* and other sea cucumber species at the National Center for Biotechnology Information (NCBI), the basic characteristics of the mitochondrial genome of the taxonomic class Holothuroidea have been revealed, which could benefit germplasm resource evaluation using molecular methods (Shen et al., 2011). The use of all these molecular markers in fisheries resource evaluation include species identification, geographical origin, and variant analysis. In sea cucumber industry, molecular methods could identify product ingredients and detect fraudulent labeling for food safety and better quality.

4.2 Assessment of genetic diversity and population structure

Molecular marker technology has been widely applied in the assessment of population genetic structure and genetic diversity, providing guidance for the sea cucumber aquaculture industry and germplasm resource protection. Bottom-culture farming in marine ranching is an important means of germplasm resource protection, and the evaluation of genetic diversity and population structure of its natural geographical populations is crucial. In general, this includes four important steps: sample collection, DNA extraction, genotyping or sequencing, and data analysis. Numerous sea cucumber studies have utilized mitochondrial DNA markers (e.g. COI, 16S) and nuclear DNA markers (e.g. SNP, SSR) for this purpose. Mitochondrial DNA markers are used to conduct haplotype diversity (h) and nucleotide diversity (π) for genetic diversity analysis, as well as FST (fixation index) and AMOVA (Analysis of molecular variance) for population analysis. For instance, Yan (2006) used COI molecular marker methods to investigate the genetic diversity and population genetic structure of *A. japonicus* from five geographic subgroups in the Dalian Sea area and revealed that these five geographical subgroups had high genetic diversity. A mitochondrial COI gene analysis for the population genetic structure of *Holothuria atra* in the waters surrounding the Hawaiian Islands in order to aid coral reef management (Skillings et al., 2011). Additionally, nuclear DNA markers have proven to be an effective method for evaluating genetic diversity, including allelic frequency, allelic richness, and heterozygosity, as well as population analysis including F-statistics analysis (Fixation indices), STRUCTURE analysis, DAPC (discriminant analysis of principal components), AMOVA. For instance, Wang et al. conducted genetic diversity and population structure assessments of 18 geographical populations of *A. japonicus* from sea ranching in Shandong, Hebei, and Liaoning provinces using specific SNP loci, thereby enabling continuous genetic monitoring of the northern natural habitats of sea cucumbers in China (Wang et al., 2023). Whole-genome SNP data was used to identify three genetically distinct subgroups within populations of *Holothuria (Metriatyla) scabra* from Fiji, revealing a model of genetic structure isolated by distance (Brown et al., 2022). An investigation of population genetics between Australia and Vietnam using SNP markers in *H. Leucospilota* was conducted to compare gene flow and genetic similarities among populations and analyzing population genetic structure (Chieu et al., 2023). Now land et al. used population genetic analysis based on microsatellite sequences to assess the population genetic structure of *H. scabra* in Papua New Guinea and a broader region of northern Australia, in order to determine the genetic diversity within subpopulations and investigate the genetic impact of environmental changes on wild populations (Nowland et al., 2017). Eight polymorphic microsatellite loci were successfully developed in *H. leucospilota*, which provided crucial molecular markers for studying population genetic diversity and conservation strategies in sea cucumbers (Dai et al., 2015). Liao et al. utilized SSR fingerprinting technology to analyze the genetic diversity and construct fingerprinting profiles of sea cucumber populations from different regions in China, South Korea, and Russia (Liao et al., 2021). The constructed fingerprinting profiles successfully differentiated eight populations and provided technical support for the protection of sea cucumber germplasm resources and the identification of different geographical populations (Liao et al., 2021). Furthermore, genetic diversity and structure assessments of wild populations and hatchery-produced *H. scabra* in New Caledonia were achieved using nine polymorphic microsatellite markers, aiming to develop an appropriate breeding plan for restocking (Riquet et al., 2022).

4.3 Identification of genetic sex

Due to the lack of visible sexual dimorphism in the external appearance of most sea cucumber species, the development and application of a method based on molecular markers is useful to identify the sex of several sea cucumber species (Table 4). Generally, the development of a sex-specific marker is realized through the assembly of female and male genome sequences, resequencing of female and male individuals, mapping and read alignment, identification of sex-specific region, and design and verification of sex-specific marker. The verification of an accurate and stable sex-specific marker is usually completed by wild populations in multiple geographical regions. Tested samples can be collected by anal swab or tissue dissection, and then genomic DNA can be extracted by multiple protocols. The genetic sex of sea cucumber individual is identified by PCR amplification using sex-specific marker. The utilization of sex-specific molecular markers facilitates the determination of individual gender and enhances the efficiency of artificial breeding in large scale aquaculture. In *A. japonicus*, a sex-specific marker was developed utilizing 2b-RAD technology, which holds significant implications for genetic breeding (Wei et al., 2021). In addition, a loop-mediated isothermal amplification (LAMP) method was established based on male-specific sequences, which could rapidly identify the genetic sex of *A. japonicus* (Cong et al., 2024). Furthermore, a rapid and non-destructive method was devised for sex identification in aquaculture practices involving another tropical sea cucumber species (*Stichopus monotuberculatus*) (Wu et al., 2022). A non-invasive and rapid method based on anal swab sampling using a LAMP system (PCR and loop-mediated isothermal amplification) was employed for sex identification in tropical sea cucumber *Holothuria scabra* (E et al., 2023).

4.4 Marker-assisted selection and breeding

With the continuous development of breeding technologies, there is a gradual shift from traditional selective breeding and hybrid breeding techniques towards the integration of molecular marker-assisted breeding, whole-genome selection breeding, molecular design breeding, sex control, gene transfer, and gene editing (Ding et al., 2021). Molecular marker-assisted breeding is a technique in aquaculture breeding that involves directly selecting individuals with advantageous alleles or genotypes for specific traits using molecular markers closely associated with those traits, which applies the research findings of genomics and molecular biology to the selection and breeding of aquaculture species (Lu et al., 2019). It demonstrated that the construction and use of genetic linkage maps with a high number of molecular markers could connect desirable performance traits with molecular markers, which can benefit marker-assisted selection and breeding and accomplish genetic improvements for sea cucumber aquaculture. The accomplishments of marker-assisted selection and breeding can be evaluated through the correlation between performance traits and genetic information in offspring. In *A. japonicus*, the insertion/deletion (INDEL) marker of internal transcribed spacer 1 (ITS1) sequence divergence was successfully used to identify the genetic types of different body color varieties, and hybridization experiments showed that this marker could be used to distinguish and track the genetic composition of F1 hybrid larvae, which explored the relationship between body color variation and genetic background and its impact on larval development (Yoshida et al., 2012). The linkage map using AFLP markers serves as a foundation for constructing high-resolution genetic maps and identifying functional and quantitative trait loci (Li et al., 2009). Yan et al. constructed a common genetic map for sea cucumbers based on microsatellite and SNP markers, which was consistent with the haploid chromosome number of sea cucumbers (Yan et al., 2013). Liu et al. used a high-density genetic linkage map of sea cucumbers to preliminarily locate QTL regions associated with five traits: body length, body width, body weight, total number of spines, and survival rate (Liu et al., 2019). The "Sea Cucumber Superior No.1" was successfully bred using molecular marker-assisted breeding, which has strong resistance to *Vibrio splendidus* infection and fast growth rate (Ding and Chang, 2020).

TABLE 4 Development and application of sex-specific markers for genetic sex identification in sea cucumbers.

Types	Sea cucumber species	Location	Method	Population number	Description	Reference
sex-specific markers	*Apostichopus japonicus*	Liaoning, China.	2b-RAD	97	provide a solid basis to reveal the sex determination mechanism and the origin of sex-determining regions; improve the efficiency of genetic and sex control breeding in sea cucumbers	Wei et al., 2021
sex-specific markers	*Stichopus monotuberculatus*	Xidao Island, Sanya, China	genomic sequencing	80	an effective way to identify sex in sea cucumbers	Wu et al., 2022
PCR and LAMP system	*Holothuria scabra*	Zhanjiang, China	DNA sequencing	50	investigate the basis of sex determination in the Holothuriidae family	E et al., 2023
PCR and LAMP system	*Apostichopus japonicus*	Dalian, China	genomic sequencing	100	A rapid LAMP-based method of identifying the genetic sex without complex equipment.	Cong et al., 2024

The new germplasm was achieved through population selection, screening and verification of disease-resistant molecular markers that indicate disease-resistant functional genes (Ding and Chang, 2020). In *A. japonicus*, a new high-density genetic linkage map was constructed based on single nucleotide polymorphism (SNP) molecular markers, and quantitative trait loci (QTL) mapping analysis was performed to explore the genetic mechanism of sea cucumber growth traits, which could be helpful for future marker-assisted breeding (Cui et al., 2021).

Molecular marker technology has diverse applications in fisheries research and offers significant convenience and solutions to numerous challenges encountered in the aquaculture industry. In order to tackle the obstacles faced by the sea cucumber aquaculture and breeding industry, various molecular markers have been developed and implemented for multiple purposes, including germplasm resource evaluation, species identification, Assessment of genetic diversity and population structure, and marker-assisted selection and breeding. With innovations in genomic methods, molecular markers are expected to continue playing important roles in promoting the future development of sea cucumber fisheries, aquaculture, and industry.

Author contributions

XL: Investigation, Visualization, Data curation, Writing – original draft. XT: Investigation, Visualization, Data curation, Writing – original draft. MC: Supervision, Resources, Conceptualization, Writing – review & editing. GN: Supervision, Resources, Conceptualization, Writing – review & editing. YY: Visualization, Supervision, Resources, Conceptualization, Writing – review & editing, Writing – original draft.

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Conflict of interest

Publisher's note

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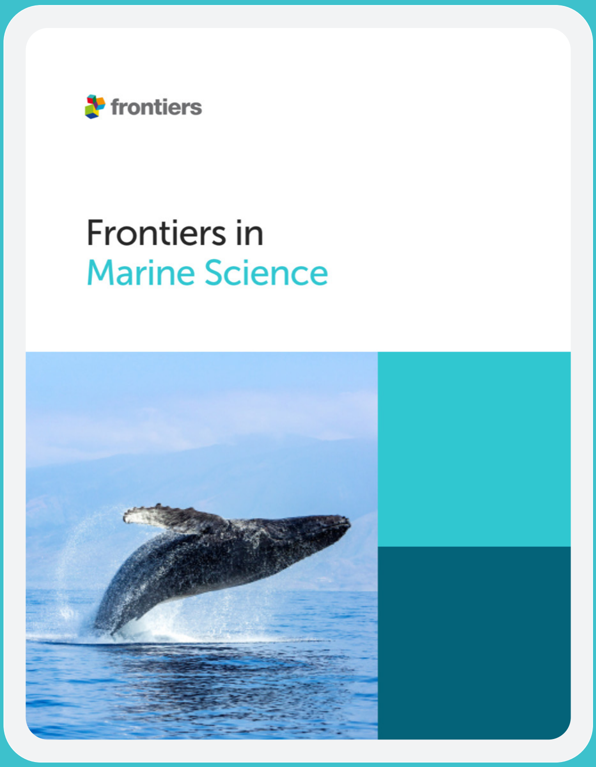
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