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Morphological and genetic variation in Southern Ocean Echinoderms with main focus on brittle stars (Ophiuroidea)

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SUMMARY

AIMS AND STRUCTURE OF THE THESIS

In recent years, changes to traditional taxonomic methods to incorporate new technologies and techniques have greatly improved the quality of species hypotheses, but more work can be done to improve the speed of new species discovery and documentation. The mitochondrial COI DNA barcode has been successfully used to identify species with high accuracy since the early 2000s and is now currently used in conjunction with morphological examinations to detect and delimit new species.

The first aim of this thesis was the definition of a general standard sampling protocol to be applied in remote and extreme areas such as the Southern Ocean to pinpoint new species and the establishment of a procedural approach for samples treatment to obtain and record all the metadata and information associated with the collected organisms that will undergo molecular identification based on DNA barcoding.

Distributional information's regarding the Ross Sea quadrant are still scarce and sparse, despite the continued research that has been held in the area since the 50's of the past century. To fill this gap, the second objective of the thesis concerns the production of distributional data and species lists of taxa belonging to the phylum Echinodermata from the Terra Nova Bay area (Ross Sea, Antarctica) for which there is still no information in the scientific literature, i.e. the classes Holothuroidea and Crinoidea.

To assess this objective, a "*Reverse taxonomy*" approach was applied to echinoderms samples stored in the permanent biological collections of the Italian National Antarctic Museum (MNA, Section of Genoa). According to this approach, analysis of morphological features (e.g. skeletal elements) was performed only after having obtained molecular data and a robust phylogeny, to assess the congruence between morphological and molecular data. In order to do that, all the available organisms belonging to the phylum Echinodermata suitable for molecular investigation were gathered and analyzed. A collection of cytochrome c oxidase subunit I (COI) sequences amplified from specimen of the MNA collections was produced and used to define species boundaries. DNA

sequences were then deposited in permanent databases (BOLD and GenBank).

The application of this technique allowed us to increase the available information for the benthic community of the Terra Nova Nay, as well as to re-evaluate the previous knowledge and to correct gross classification mistakes present in literature due to not appropriate approaches in species determination that led to a perpetration of wrong evaluation of biodiversity in the area for more than 50 years. Due to the diversity of the different classes included in the phylum Echinodermata and the specific analyses necessary to assess the correct identification of each taxon, these will be treated and detailed in separate chapters.

The Thesis starts with an Introduction, where the most general topics covered by the entire thesis are presented. Each chapter, with the exception of the first one, which has a different structure from the others, has its own specific "Introduction" to the concepts discussed in that study, followed by the sections "Materials and Methods", "Results" and "Discussion". Chapters 1 to 4 have already been published as book chapters (Chap. 1 in *Marine Genomics - Methods in Molecular Biology*), occurrence dataset (Chap. 2, in the *Global Biodiversity Information Facility*, https://www.gbif.org/) or regular articles in ISI Journals (Chap. 3 and Chap. 4 in *Diversity*).

Considering the specificity of the different chapters, notwithstanding the fact that they pertain to the same, general topic of research, no general Discussion for the entire thesis was produced. Final remarks are instead reported at the end of the whole thesis ("Thesis Conclusions").

INTRODUCTION

Antarctic marine biodiversity can be considered as the result of a combination of very peculiar environmental conditions [1]. The origin of these environmental conditions date back to the latest part of the Cenozoic era, during which the numerous glacial-interglacial cycles, together with the isolation of the continent from all the other land masses, influenced the Antarctic ecosystems at a degree that has no parallel on Earth [2]. More than 8,000 species have been described for the Southern Ocean [3] and new molecular techniques approaches are continuously identifying new and cryptic species that remained undetected to this day, progressively increasing the knowledge on the true diversity of this region [4]. However, the limited accessibility of the continent led to uneven and sporadic sampling efforts, resulting in areas still not properly investigated and meaning that the true diversity of Antarctic marine communities might still be largely unknown [4]. Despite numerous oceanographic campaigns conducted in recent decades, the lack of comprehensive information on species distribution and recurrence in the Southern Ocean remains a significant barrier to a full comprehension of the biological richness of this ecosystem.

The Southern Ocean is confronted with several drivers that contribute to the loss of biodiversity in this fragile marine ecosystem [4]. Climate change stands as a primary, negative driver, with rising temperatures leading to an increase of sea ice melting and altering ocean currents, which, in turn, disrupts the delicate balance of the whole ecosystem. Ocean acidification, resulting from increased carbon dioxide absorption, poses a significant threat to marine organisms, especially those with calcium carbonate shells or skeletons. Overfishing and illegal fishing practices exert intense pressure on commercially valuable species, such as the Antarctic toothfish [5], impacting the trophic structure and causing cascading effects throughout the food web. Additionally, the introduction of invasive species due to human activities disrupts native species' habitats and ecological interactions [6, 7]. Pollution, including plastic waste and chemical contaminants, further degrade the environment and negatively impact marine life [8, 9].

All these drivers collectively contribute to the ongoing decline of biodiversity in the Southern Ocean, emphasizing the urgent need for effective conservation measures and international collaboration to understand and mitigate their detrimental effects. These key drivers are expected to have substantial influences on future biological communities [4] and pose significant challenges to the adaptability and survival of Antarctic marine species. Understanding the ecological responses and vulnerabilities of these communities to environmental change is crucial for effective conservation and management strategies in the region.

Addressing the knowledge and data gaps in the Southern Ocean requires increased investment in scientific research, enhanced international cooperation, and the development of robust monitoring programs. It is essential to prioritize systematic and interdisciplinary research efforts that encompass a range of taxa, habitats, and environmental variables. Only by filling these knowledge gaps, we can improve our understanding of the Southern Ocean ecosystem, enhance conservation strategies, and effectively manage this unique and valuable marine environment.

Consistent and standardized monitoring efforts are essential to track changes in biodiversity, assess the effectiveness of conservation measures, and inform decision-making processes [8, 9]. In addition to the spatial and temporal gaps in data, there are also limitations in the accessibility and sharing of existing information. Research efforts in the Southern Ocean often involve international collaborations (e.g. [12]), but data sharing and coordination among different research groups can be challenging due to logistical constraints, data ownership issues, and the lack of centralized databases. Improved data sharing mechanisms and the establishment of collaborative platforms are crucial for addressing these data gaps and promoting comprehensive understanding of Southern Ocean biodiversity.

The Southern Ocean presents a challenging environment for data collection, leading to significant information gaps. The harsh conditions, including extreme weather, sea ice, and remote locations, make it difficult to conduct comprehensive sampling and data collection efforts. Researchers face logistical

challenges, high costs, and limited access to certain areas, resulting in uneven spatial coverage and patchy data. These difficulties hamper efforts to gather sufficient material for analysis and contribute to the limited knowledge of the region. Furthermore, the Southern Ocean is characterized by a high level of biodiversity, including numerous cryptic and undescribed species. Furthermore, the lack of experienced morphological taxonomists poses a significant hurdle in species identification and classification.

Morphological identification is a fundamental aspect of biodiversity research, but it requires specialized expertise and extensive training. The shortage of skilled taxonomists with knowledge of Southern Ocean organisms slows down the identification process and creates bottlenecks in data analysis [13]. The scarcity of taxonomic expertise limits our understanding of species diversity and hinders the assessment of species' ecological roles and conservation status. Another contributing factor to the information gaps in the Southern Ocean is the time-consuming nature of morphological identification. The intricate nature of Southern Ocean species, their diverse life stages, and subtle morphological variations make accurate identification a laborious and time-intensive task. This prolonged identification process further delays the availability of data for analysis and hinders the progress of research and conservation efforts.

Overall, the combination of difficulties in material collection, a shortage of experienced morphological taxonomists, and long identification times contributes to the limited knowledge and data gaps in the Southern Ocean. These challenges impede our understanding of species distribution, abundance, and ecological interactions in this unique marine ecosystem. Addressing these issues requires increased investment in scientific research, training programs to build taxonomic expertise, and the development of efficient and standardized identification protocols. By overcoming these obstacles, we can enhance our knowledge of the Southern Ocean's biodiversity and support effective conservation and management strategies.

Molecular techniques are increasingly being utilized to uncover hidden diversity [14], but there is still much to learn about the true extent of species richness in

this ecosystem. The identification and classification of new species require taxonomic expertise and comprehensive sampling efforts across different habitats and depths. DNA barcoding has emerged as a valuable identification method for echinoderms (e.g. [13 to 17]), offering a powerful tool to discriminate between species based on their genetic sequences. Echinoderms exhibit morphological variations that can make traditional taxonomic identification challenging. However, DNA barcoding overcomes these problems by using a standardized DNA sequence, generally the mitochondrial cytochrome c oxidase 1 gene (COI), which is useful to make comparisons at the species level by distinguishing individuals that might belong to the same species from those that do not. This gene, in fact, has shown a higher degree of sequence variation between species compared to within-species variation, making it an effective tool in species discrimination. By comparing the DNA sequence of an unknown echinoderm specimen with a reference database, such as the Barcode of Life Data System (BOLD, https://www.boldsystems.org/), researchers can identify the species with greater accuracy, assuming the BOLD data set is highly complete and contains a high coverage of the world's diversity.

The application of DNA barcoding in echinoderm research has provided numerous benefits. It enables the rapid and accurate identification of species, particularly in cases where morphological identification is challenging or uncertain. It allows for the accurate identification of damaged specimens, juveniles, and larvae, which can be challenging using traditional taxonomy alone ([12], [16] to [20]). This is particularly important in the context of large-scale surveys to characterize echinoderm fauna in specific regions or within museum collections. DNA barcoding not only aids in species identification but also contributes to the understanding of species diversity, distribution patterns, and ecological interactions. These potentialities are particularly useful in monitoring biological communities, especially considering the increasing threat of biological invasions due to climate change [23].

It is worth noting that the process of DNA barcoding for echinoderms can involve different steps and approaches depending on the specific case. Some studies may utilize fully automated processes that involve third-party sequencing service

providers, while others may opt for customized and manually performed procedures in their own laboratories. The amplification and sequencing steps of DNA barcoding are highly variable and depend on the available resources and expertise of the researchers involved.

DNA barcoding has proven to be a valuable method for the identification of echinoderm species. It offers a standardized and reliable approach to overcome challenges in traditional morphological identification, especially for species with subtle morphological differences. The application of DNA barcoding in echinoderm research enhances our understanding of their biodiversity, distribution, and ecological roles. As technology continues to advance, DNA barcoding will likely play an increasingly important role in the study and conservation of echinoderms and other marine organisms. Considering these circumstances, traditional methods relying on morphological identification have failed to provide an appropriate solution to these issues (e.g., [21]). Neither molecular nor morphological methods alone are sufficient for accurate taxonomy [24]. One solution proposed to overcome this problem relies on the use of the "*reverse taxonomy*" [25], where morphological analyses are conducted for a re-examination of our molecular results based on DNA barcoding screening. This method gained more attention in the last decade due to the increase in speed in the processing and identification of organisms, its high reproducibility, short period of processing time and the fact that it partially allows us to overcome the problem of lack of specific experience of taxonomy.

A partial explanation for this deficiency in Antarctic bio-monitoring research must be identified in the logistic constraints, which are often driven by financial shortcomings and particularly relevant when sampling takes place in remote areas. In fact, polar areas are characterized by uncomfortable environmental settings, with temperatures exceeding the freezing point exclusively during the summer months, sea ice cover changing abruptly in a short period of time and harsh weather conditions, inevitably obstructing sampling activities. On top of this there is also the higher cost of maintaining personnel in these remote areas. Thus, when operating in Antarctica, the fulfilment of one of the most important

requirements of a sound monitoring program, i.e., a high sampling frequency, is generally difficult to be achieved [26].

Marine protected Areas (MPAs) are an important management tool that can be used to protect, maintain, and restore biodiversity and ecosystem services. Different MPAs have been established in the Southern Ocean, in both the continental and sub-Antarctic regions [27]. The MPA established in 2016 in the Ross Sea still represents the largest single-body marine reserve to date (the network of North-East Atlantic Marine Protected Areas is bigger in extension, with 1.5 million km^2, but comprises 583 different MPAs reunited under the OSPAR Commission (Oslo and Paris Conventions). The Ross Sea region Marine Protected Area (RSr MPA) includes, besides the area bounded by the General Protection Zone, a "Krill Research Zone" and a "Special Research zone" adjoining the former [28]. The region has been protected by both its remoteness and harsh weather conditions includes four ecoregions (of those evidenced by Douglass *et al.*, 2014 [29]) and several environmental types, ranging from the Ross and Oates continental shelves to the Pacific abyssal plain, from the Scott Seamounts to the Balleny Islands, making it particularly relevant for the conservation of Antarctic communities.

Terra Nova Bay (TNB) is a ~70 kilometers long inlet, lying between Cape Washington and the Drygalski Ice Tongue along the coast of Victoria Land, in eastern Antarctica (https://data.aad.gov.au/aadc/gaz/scar/). Since its discovery, which took place during the British National Antarctic Expedition (1901-1904), this area has been extensively studied only after the establishment of the Italian research base "Terra Nova Station" (later called "Mario Zucchelli Station", MZS), in 1985 and the first Italian oceanographic expedition that was conducted from 1987 to 1988 [30]. The Italian research base MZS is located approximately at the center of the bay and provides facilities and support for 85 people on average (between research and logistic personnel) operating only during the Austral summer from mid-October to the beginning of February.

The Italian National Antarctic Museum (MNA) was established in 1996 with the specific intent to preserve, study and make available to the scientific community all the material collected in Antarctica by the Italian National Antarctic Program (PNRA) scientific activities [31]. This institution has an interuniversity organization, with different universities hosting specific typologies of samples, with the section hosted by the University of Genoa specialized in preserving biological samples [31]. Since its establishment, the section of Genoa of the MNA acquired more than 15,000 biological samples and has continuously contributed to the major repositories of species occurrences such as the Global Biodiversity Information Facility (GBIF), through a variety of publications ([30] to [40]).

The main objective of this thesis is to provide the first information regarding species community composition of echinoderms from the Terra Nova Bay area. Within these Antarctic and sub-Antarctic waters, echinoderms are one of the most represented benthic macrofaunal phyla in terms of biomass and diversity, encompassing more than 779 accepted species [43]–[45]. They play an important role in structuring marine assemblages [46]. In the Southern Ocean this Phylum counts 235 species of Asteroidea (sea stars) [47], 43 Crinoidea (feather stars) [48], 82 Echinoidea (sea urchins) [49], [50], 219 Ophiuroidea (brittle stars) [51] and 200 Holothuroidea (sea cucumbers) [52]. This Phylum shows a high flexibility in trophic strategies, spanning from deposit and suspension feeder species (highly sensitive to spatial variations in primary production and detritus) to predators like sea stars [53]–[58]. Echinoderms also show a wide distribution, occupying different habitats. This feature together with the large size, facilitates the efficiency of sampling regardless the used sampling tool [59]. Despite the role that is attributed to Echinoderms in Antarctic waters, the information about the real number of species occurring in this Phylum is still limited and available only for selected taxa.

Most of the information on the benthic communities of the Ross Sea derives from limited coastal areas, in particular for the areas adjacent to the research bases of Italy (Mario Zucchelli - Terra Nova Bay), United States (McMurdo) and New Zealand (Scott Base) (e.g., [60]–[62]). Currently a complete checklist for this

phylum in the Terra Nova Bay area is not yet available. Chiantore *et al.* (2006) [63] provided the first list of species for echinoids, ophiuroids and asteroids and described their bathymetric distribution. Cecchetto *et al.* (2017) [34] added 3 new Ophiuroidea records for the Terra Nova Bay area bringing to 18 the number of known species for this class of echinoderms.

The aim of this thesis work is to provide the complete DNA-based alpha taxonomy for the phylum Echinodermata in the Terra Nova Bay area (Ross Sea, Antarctica) and, where necessary, to apply the «*reverse taxonomy*» method. With this approach, the molecular data obtained from DNA Barcoding are used as a first 'filter' to recognize robust clades and delimit the species and, secondly, a morphological analysis is applied in order to highlight 'unpublished' morphological characters able to speed up future identifications even without the use of molecular techniques. Specifically, in this work the employment of molecular techniques integrated with a morphological approach allowed us to assess the missed information on Holothuroidea (sea cucumber) and Crinoidea (feather stars) from TNB. The use of this approach also allowed us to re-examine the scientific knowledge available for the area and to make corrections of errors in the scientific literature and online molecular open data libraries. The possibility of misleading identification carried out could have led to erroneous information flow into other science fields with inaccuracies that would persist in the scientific literature (chapter 3 and 4). A joint action of revision is thus fundamental for understanding the current level of diversity, speciation events of the past, and for implementing actions aimed at the conservation of these ecosystems and the species that occupy them.

The first chapter of this thesis will cover the development of guidelines for DNA barcoding approach from sampling to laboratory procedures in remote areas and hostile environment. DNA barcoding is a powerful and widespread method used to identify large numbers of species collected in the framework of sampling activities in the field [12]. With the exception of research projects that may count on large teams characterized by tasks' delegation and where many activities may run in parallel, in the majority of cases the barcoding effort is handled by a

limited number of persons. The guidelines here reported focus on this second case, with a special attention paid to field procedures, whose efficiency and smoothness are often overlooked. This chapter was published in Schiaparelli, S., Alvaro, M. C., Cecchetto, M., & Guzzi, A. (2022). *Barcoding of Antarctic Marine Invertebrates: From Field Sampling to Lab Procedures*. In Marine Genomics: Methods and Protocols (pp. 177-194). New York, NY: Springer US [64].

The second chapter reports the dataset of Holothuroidea vouchers information's deposited in the biological collection of the Italian National Antarctic Museum (MNA) – Genoa section with standardized notation and format. This dataset adds vital occurrence and abundance data for holothuroids from eleven previously unexamined research cruises from the Ross Sea and Antarctic Peninsula sector of the Southern Ocean. All 328 occurrences reported here were classified to the possible lowest taxonomical level, specifically there are 23 taxa identified to species, 14 to genus and 7 to family rank. The data reported here represent an important source of information on the composition of benthic communities in the Southern Ocean. Furthermore, including samples of sea cucumbers from the Terra Nova Bay (Ross Sea), the data reported here represent the first species checklist for the area, providing an important baseline for future studies. Of all the specimens here represented, ~99% are currently stored in absolute ethanol or at -20°C, representing a potential resource for future genetic studies. Concerning this potential, approximately ~47% of the entire collection has been subject to DNA barcoding screening. The database presented in this chapter was published in Guzzi A, Alvaro M C, Cecchetto M, Grillo M, Cometti V, Noli N, Schiaparelli S (2023). *Distributional records of sea cucumbers (Echinodermata, Holothuroidea) based on the collection stored at the Italian National Antarctic Museum (MNA, section of Genoa)*. Version 1.3. Italian National Antarctic Museum (MNA, Section of Genoa). Occurrence dataset [39] .

The third chapter will discuss the application of the "*Reverse Taxonomy*" approach on the *Odontaster* genus. In this chapter, two species of sea stars, *Odontaster roseus* Janosik & Halanych, 2010 and *Odontaster pearsei* Janosik &

Halanych, 2010, are reported for the first time from the Terra Nova Bay area (TNB, Ross Sea, Antarctica) by using a combination of molecular (DNA barcoding) and morphological (coloration and skeletal features) analyses. Molecular results agree with external morphological characters of the two identified species, making occurrence in the area unequivocal. The two species were recently described from the Antarctic Peninsula, and went unnoticed for a long time in TNB, possibly having been confused with *O. meridionalis* (E. A. Smith, 1876), with which they share a bright yellow coloration. This latter species seems to be absent in the Ross Sea. Thus, the past literature referring to *O. meridionalis* in the Ross Sea should be treated with caution as these "yellow morphs" could be one of the two recently described species or even orange–yellow morphs of the red-colored congeneric *O. validus* Koehler, 1906. This work highlights the paucity of knowledge even in purportedly well-studied areas and in iconic Antarctic organisms. This chapter was published in Guzzi, A, Alvaro, MC, Danis, B, Moreau, C, & Schiaparelli, S (2022). *Not all that glitters is gold: barcoding effort reveals taxonomic incongruences in iconic Ross Sea sea stars*. Diversity, 14(6), 457 [22].

In the fourth chapter, 96 specimens, 74 belonging to echinoids and 22 to crinoids collected during seven PNRA expeditions and one AWI expedition undergo an investigation combining molecular (DNA barcoding) and morphological (skeletal features) analysis following the "*reverse taxonomy*" approach. With our analysis we were able to identify 13 species of sea urchins (of which 6 from the Terra Nova Bay area) and 4 of crinoids. Based on prior scientific literature, only 4 species of sea urchins were reported for the Terra Nova Bay (TNB) area. However, in our work we increase the number to 6 with the first records for *Abatus cordatus* (Verrill, 1876), *Abatus curvidens* Mortensen, 1936 and *Abatus ingens* Koehler, 1926. We also evidenced an erroneous taxonomical identification of *Abatus koehleri* (Thiéry, 1909) (previously *A. elongatus)* previously reported in the scientific publication for the area that led to mistakes in distributional record for the species. There is currently no faunistic inventory available for crinoid species of TNB, therefore, *Anthometrina adriani* (Bell, 1908), *Florometra*

mawsoni AH Clark, 1937, *Promachocrinus kerguelensis* Carpenter, 1879 and *Notocrinus virilis* Mortensen, 1917, identified in our work, represent all new records for the area. This MS was submitted to Diversity and is currently under review.

The fifth and last chapter of this thesis focused on the characterization of *Iophon* sponges found in association with Southern Oceans *Ophioplinthus* brittle stars by employing a morphological approach. A total of 96 *Iophon* specimens were analyzed in the current study, morphological analyses were conducted with the examination of different elements. In the specimens studied, the presence of *Iophon* has different degrees of development, indistinctly in the two species, but numerous specimens of *O. gelida* collected did not exhibit the association with a *Iophon* sponge, while all the *O. brevirima* specimens are in symbiosis with *Iophon* sponge. Therefore, we register the first record of *I. flabellodigitatum* on *O. brevirima* and the first record of *I. unicorne* on *O. gelida*. This MS is currently in preparation for submission.

CHAPTER 1
Barcoding of antarctic marine invertebrates: from field sampling to lab procedures

1 Introduction

Since its introduction in 2003 [14], DNA barcoding has been increasingly employed as a routine species identification tool in the framework of large-scale surveys to characterize the fauna of a given site or large museum collections [65]. This technique relies on the use of a short-standardized sequence of DNA, that is, the mitochondrial cytochrome c oxidase 1 gene (COI), which was found to be effective in discriminating animal species thanks to a between-species sequence variation which is generally higher than the within-species one [14]. When a sequence of an unknown specimen is obtained, this is compared with the pool of existing ones through the Barcode of Life Data System (BOLD) (http://www.barcodinglife.org) [66]. This is one of the major online repositories of COI sequences and provides services such as the registry of Barcode Index Numbers (BINs), which greatly speed up the process of putative species individuation by refining species hypotheses [67] and maintaining a high level of quality standards through the inclusion of additional information (e.g., sampling event information) and the collection of images of the barcoded specimens. The whole barcoding process can even be performed in the field, as long as all required lab equipment can be moved and made available to researchers [e.g., [68]]. In the majority of cases however, the molecular work takes place in a second phase, after sample collection in the field and shipping to the lab. The sampling and lab procedures below described can quicken specimen handling in the field, guaranteeing at the same time the achievement of the best results in a barcoding effort. Despite the availability of general sampling guidelines for the management of biological samples at sea in the framework of large sampling efforts, which may count on working teams with tasks' delegation [e.g., [69]], in the case of small scale, near-shore activities with limited personnel involved there are no available guidelines. Under these circumstances, field operations of a barcoding campaign have to be even more carefully planned and is mandatory

to count on clear protocols and have a smooth operational workflow. The following guidelines provide advice for this second case and are the results of several seasons of Antarctic fieldwork where sampling occurred from small vessels and one or two people only were in charge of all procedures. The description of DNA barcoding amplification and sequencing steps are omitted from this protocol as these may vary case by case, spanning from fully automated processes which use third sequencing service providers, to those made in own labs where all operations are customized and manually performed. In this context, we describe all operations until tissues' clipping for their later placement in TrakMate sample tube racks (with 96-wells, Fig. 4g) to be sent to BOLD. Details of front-end logistics of DNA barcoding can be found in [70].

2 Materials

2.1 Sample collection at sea

1. Nitrile gloves.

2. Digital camera.

3. Plastic label to record station data in the image of the catch.

2.2 Sorting in the field

1. Nitrile gloves.

2. Forceps.

3. Plastic jars and drums of different sizes.

4. Work bench or a box where to spread the catch for gross or refined sorting.

5. Preprinted labels to be placed in jars/containers to refer single specimens to the sampling stations.

6. Spare labels to be used in the field to mark extra samples/jars.

7. Digital camera.

2.3 Sorting in the lab

1. Nitrile gloves.

2. Forceps.

3. Trays.

4. Fridge at 4 °C.

5. Magnesium chloride (MgCl2).

6. Optical refractometer.

2.4 Vouchers labeling

1. Preprinted labels on archive-quality paper with field number codes.

2. Scissors to cut the labels.

3. PC with waterproof keyboard cover.

4. Field notebook.

2.5 Vouchers photographic documentation

1. A dSRL (digital Single Lens Reflex) camera equipped with a wireless light system (Fig. 3c).

2. Different photographic sets for organisms having different sizes.

3. Small aquaria and petri dishes for specimen photography.

4. Waterproof scale bars weighed down with small fishing sinkers.

5. Inverted forceps to keep in position the scale bars.

6. Small size fishing weights to keep the scale bars on the bottom of petri dishes.

7. Fridge for maintaining living specimens in a cool environment before photography and subsampling.

8. Supply of clean and cold seawater to fill the small aquaria and petri dishes.

9. PC to rename images according to stations and field codes.

2.6 Subsampling in the lab

1. Nitrile gloves.

2. Forceps.

3. Scalpel with disposable blades.

4. Dissecting scissors.

5. Bunsen burner.

6. Jars of different volume (from 100 to 1000 mL).

7. Supply of centrifuge tubes (15 and 50 mL).

8. Supply of cryovials.

9. Racks for centrifuge tubes and cryovials storage.

10. 96% ethanol, or RNA later, or solution of 20% DMSO (dimethyl sulfoxide) saturated with sodium chloride (NaCl).

11. Storage facilities for frozen samples.

3 Methods

3.1 Sample collection at sea

This part of the activities is the most variable one, relying on the logistic facilities available in the field. It is possible to imagine two typical situations, detailed below, ranging from sampling performed from a boat (Subheading 3.1.1) (Fig. 1a, e) to sampling performed by SCUBA divers (Subheading 3.1.2). Regardless the specific case, special attention has to be placed in finding the right containers (vials, jars, drums) where to place the sampled specimens that have to be kept alive in seawater in order to obtain a good photographic documentation of the vouchers. The containers need to have different sizes (Fig. 1e) in order to accommodate single specimens or species belonging to the same group, which will be preserved in this way to avoid further damage. All these containers (which may reach high numbers in the case of a successful sampling) must bear station data in order not to get mixed across stations (see Notes 1 and 2). Owing to time or facilities restrictions, even a gross sorting may not be feasible on board. In these cases, what is brought back in the lab is the whole sample ("bulk"), hence a variety of small containers of different size is not necessary. However, this latter procedure is never the best solution as barcoding usually targets representative and undamaged specimens in order to crosscheck the

coherence of molecular and morphological data or apply a "*reverse taxonomy*" approach [25]. In this latter situation the bulk sample may even not be kept in seawater, further compromising potential integrity of specimens which may die, intermix with the sediment, and so on, thus turning into bad quality and less representative specimens for the barcoding effort (see Note 3).

3.1.1 Sampling from a boat

Sampling from a small boat (i.e., not an oceanographic vessel) can be challenging due to the availability of only small operative spaces, the presence of safety hazards, such as winches in activity, slippery surfaces, and hanging weights. Thus, it has to be carefully planned with the personnel responsible for the safety on board where the sorting operation can be done and where to place and store drums, jars, etc., in order not to interfere with boat operations.

1. Prepare all the small containers to be dedicated to a given station inside a box (see Note 4) (Fig. 1e).
2. Remove container lids and fill the containers with seawater before reaching the sampling station.
3. When the catch is on board, place a label with the station number over the catch.
4. Take a picture of the whole catch with the label on it (Fig. 1b, c).
5. Take additional images of visible species emerging at the surface.

Figure 1- Overview of field activities starting from sampling at sea to specimens' isolation in the lab. (a) The dredge sample has just been released in a stainless steel sorting tray. (b and c) images of catches from two different stations; note the plastic label on the top of both to uniquely identify them (in this case the roman number refers to the expedition followed by the sampling station name). Now the target will be the isolation of all the species form the catch. (d) The sorting has started and some specimens are removed by hand; note the thick gloves worn by the researchers doing the sorting and the two directions taken to cover all the catch. (e) A variety of jars and drums have already been filled with seawater while the dredge is working on the bottom; they are now ready to receive larger specimens from the catch. (f) Once in the lab the collected species are checked and further divided in trays (g–i). (g) Holothuroids and hydroids. (h) Pycnogonids. (i) Crinoids. (j) Four specimens of *Chorismus antarcticus* (Pfeffer, 1887) have been isolated from the station catch, one more should be added to reach the number of specimens that will have to be barcoded.

3.1.2 SCUBA sampling

This kind of sampling may produce single specimens collected by hand by the

divers or even a “bulk sampling,” for example, when a hard substrate is scraped. All underwater operations are limited by the severe environmental conditions (seawater temperature is generally -1.8 °C) and by the heavy equipment used by divers in polar areas. Here jars and containers, if several depths/stations are investigated during a single dive, have to be obligatorily marked in advance. If are not the researchers themselves to dive, it is necessary to coordinate the sampling activities with the divers appointed to this task, in order to let them to understand the sampling procedures and field codes in use. This kind of sampling has to be built on top of the existing security restrictions and procedures in use for diving in polar areas (see Note 5).

1. Prepare all the containers to be dedicated to a given station in advance, in the lab (see Note 6).

2. Mark with a permanent marker the external side of the containers with large, clearly visible numbers/codes.

3. Arrange the containers within the sampling bag in a logic way, for example, with their numbers ordered according to the expected underwater operations.

4. Arrange sampling bag (if each one is dedicated to a sampling station) in a way that enables an easy recovery from the water (see Note 7).

5. Once containers are taken out of the water, if mixed, divide them according to the station.

6. Verify that lids are closed not to lose the water inside; if needed, refill with seawater.

7. Organize the containers inside boxes for their transport in the lab.

3.2 Sorting in the field

Sorting in the field is usually limited to benthos samples brought on board by a dredge or a grab and require a “good eye” to spot as soon as possible those most fragile and delicate organisms that have to be immediately placed in seawater. According to the study targets, the number of requested specimens per species

may vary but, above all, it is not known the number of species that will be collected in a given station. It is therefore difficult to foresee in advance how many jars/containers have to be prepared and it is always a good idea to prepare an adequate number of spare containers (see Note 2). The place where the sorting will be performed has to be decided in advance with the boat crew, in order to avoid any potential risk to people and materials.

1. After having photographed the catch with the station label, spread it carefully on the sorting bench or box.

2. Proceed with the sorting by starting with a corner and then following a direction. Avoid random specimen picking (see Note 8) (Fig. 1d).

3. Divide specimens according to size, softness etc., or by taxon. The goal here is not to mix groups having different characteristics (see Note 9).

4. Add station labels in each container regardless its size (see Note 10).

5. Once sorting has been accomplished, the remaining organisms and sediment can be thrown at sea, possibly at the same sampling station.

6. According to the abundance of specimens in the sample or in the case of time restrictions, sorting may not be feasible and the whole catch has to be placed into a large drum/box and brought back into the lab for sorting operations.

3.3 Sorting in the lab

This is the part of the whole process where the choice of the final candidates as vouchers for barcoding is done or refined (Fig. 1f). Here the boxes/trays with already sorted taxa are resurveyed and labelled with field code numbers. If a "bulk sample" is present, it is sorted at this stage.

1. Organize the specimens in trays to cross check the quality of specimens and refine sorting wherever necessary (Fig. 1g, i).

2. Count specimens in order to verify if the requested numbers of specimens per taxon to be barcoded has been reached (see Note 11) (Fig. 1j).

3. Select and start with those specimens that require an immediate treatment

and have to be photographed first (see Note 12).

4. Try to keep a constant temperature at all stages in order not to further increase the thermic shock in the collected specimens and put in a fridge at 4 °C most sensitive taxa (see Note 13).

5. At this stage it may also be considered to relax some specimens by placing them into MgCl2 [71]. This step will save time at the photographic phase (see Note 14).

3.4 Vouchers labeling

This is the crucial part of the whole process as it is here that the link between each physical specimen, the field code and the station data is established. The field codes used to identify specimens have to be established and agreed in advance. They also have to be clearly explained in the field notes and in any data report that will be published after the expedition (see Note 15). If the sampling activities are performed with the collaboration of a museum, then all these steps would be quite straightforward as they fall within the museum's routines. In these cases, it is possible that preprinted museums labels and data recording sheets containing all the required fields to be filled in are made available by the museum itself (e.g., see Fig. 15.3 in ref. 6). It is a good advice to establish communication with a reference museum to get guidance about these procedures. In common practice it is much more common that working teams operating in the field will have to think and prepare their own materials and develop their own system of field codes. Here becomes mandatory to rely on an easy and robust system to associate preprinted labels to specimens and record the correspondent associated field data (see Notes 16 and 17). Official museum voucher numbers will substitute these field codes only in a later stage, that is, when the specimens will be sent to a museum for permanent storing after the barcoding effort and the field data will be entered into the museum's database.

1. Print the labels with the field codes on archive-quality paper (see Note 18) (Fig. 2a).

2. Cut the labels with the field numbers with scissors and place these in physical

contact with the single specimens (see Note 19).

3. Write all necessary filed notes in the field book or in an Excel spreadsheet (see Note 20).

3.5 Vouchers photographic documentation

Take images of the live specimens already in the field has several advantages and hold a critical role in the sample's validation process. Specimens' colors will fade or will disappear once the organism will be fixed in ethanol, hence potential diagnostic characters will not be available anymore. Some specimens may show dramatically different color patterns but molecular data may later indicate that they belong to the same species [e.g., [72]]. If no color documentation is available this variability may fail to be described. Color images of good quality are also mandatorily required by BOLD (https://v3.boldsystems.org/index.php/resources/handbook? chapter ¼3_submissions.html&section ¼image_sub missions#photo_guide). Despite images can indeed be taken even after the fixation of the samples, the general appearance of these specimens will not be of great utility for comparisons as specimens will be contracted, fragmented or shrunk according to the preservation method adopted. It is therefore a very useful investment of time to get the best at this stage, that is, taking images of the alive specimens. This kind of photography has to be taken in seawater; hence a variety of containers ranging from small aquaria (Fig. 3a) to petri dishes (Fig. 3c) have to be prepared. Then, in order to avoid light reflection into the camera lens it is necessary to use flashes oriented at 45° vs the water surface (Fig. 3c). In this way, the light will reach the subject in the water but there will be no reflection in the lens. It is good practice to include a measurement scale in the image to provide a size reference (e.g., Fig. 2c, d, f; Fig. 3f, i, k, m), or a color scale to provide color reference. Background in a contrasting color is beneficial where possible (i.e., black). Some of these sets up are described in [6].

1. Place the petri dish with the specimen in the photographic set up or place the specimen in the aquarium (see Note 21).

2. Put a scale bar close to the specimen and the label with the field code number (see Note 22).

3. Adjust the position of the scale bar and that of the label in a way that both can be included together with the specimen in the same image.

4. Shoot the general image.

5. Take additional images to document specimen's morphological features by zooming where necessary. 6. Remove the specimen with the label and clean the aquarium by changing the water (see Note 23).

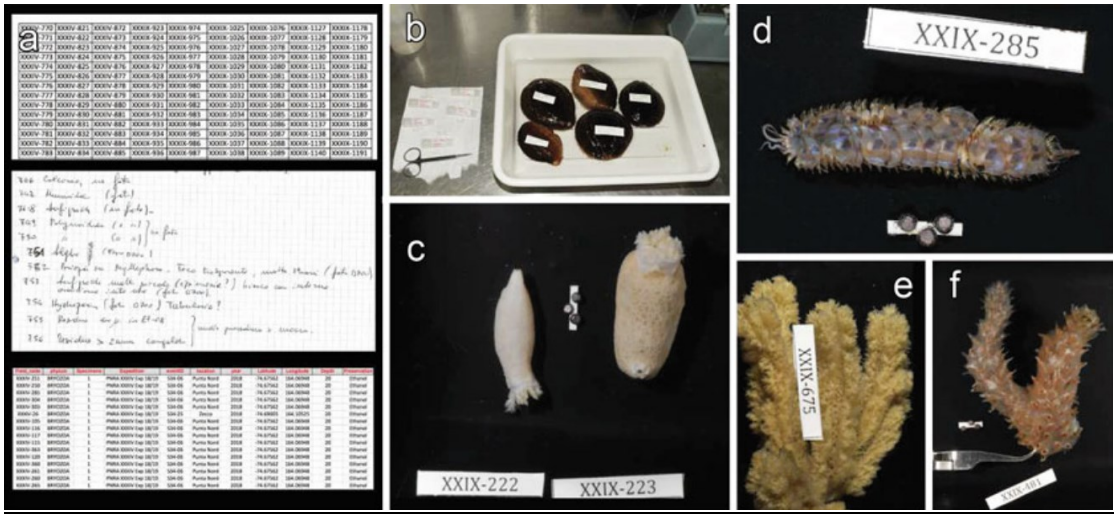


Figure 2 - Specimens labeling. (a, top) An image of a preprinted archive-quality museum paper where labels have been sized to fit into the smaller containers in use (i.e., cryovials of 2 mL); the roman number refers to the expedition, while the Arabic number will be associated to a physical specimen. (a, middle) An example of field notes: here only the consecutive numbers of the specimens (the right part of the previous code) were reported. (a, bottom) the final Excel spreadsheet with all information reported. (b) In this image, five holothuroids are prepared for the tissue clipping; each one has received a handwritten label and the cryovials for the tissue bits are ready on the left. In this example preprinted labels have not been used, but this approach is not feasible when there are large numbers of specimens to be processed. In this case, preprinted labels ready to be inserted in the cryovials and associated to the holothuroids (which will be frozen) would have saved much time. (c) Two different holothuroid species from the same station are photographed together to save time. The specimens are in seawater hence tentacles are partially expanded. Each specimen is marked by its label and in the middle of them there is the scale bar (1 cm) made with a piece of archive-quality paper kept on the bottom of the tray by small fishing weights. (d) A polynoid polychaete photographed with its label and the scale bar; note the natural appearance of the specimen and the possibility to appreciate its colors. (e) Here

the label has been placed directly over the specimen; a second image for reference will be taken after the label removal. (f) The pterobranch *Cephalodiscus*, which would float on the water surface, is kept in place by using inverted forceps. Note in images c–f the perfect black background is obtained by using black adhesive plastic layer placed on the bottom of the container; the absence of light reflections is achieved by using flashes oriented at 45° (see explanations of Fig. 3).

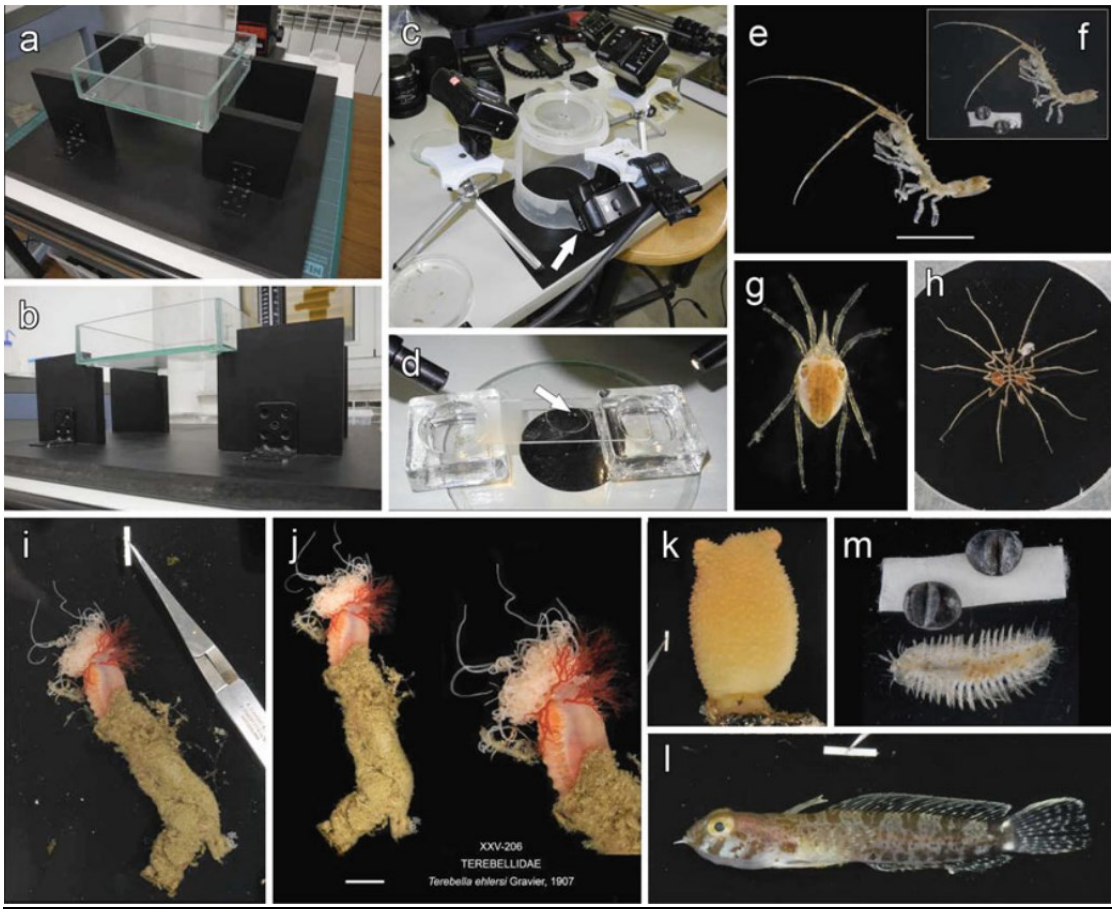


Figure 3 - Different photographic set ups that enable obtaining perfect images with a little effort helping in this way to deal with the high number of images of the specimens selected fort the barcoding that have to be taken. These images will then appear in BOLD "Specimen page" to document each sample morphological features. (a, b) A small glass rectangular aquarium (size 20 x 20 x 5 cm) is used for photographing larger specimens. The aquarium is suspended by small pillars to allow the positioning of extra-flashes (not figured here) also behind the subject; this extra flash enable capturing finer details of bristles and cirri which would disappear by using only frontal lighting. Pillars and the basement are painted in opaque black to avoid reflections (c) An "emergency" photographic set up was obtained by cutting a plastic laboratory pitcher to suspend petri dishes. Three wireless flashes are positioned around the petri dish and with a 45° inclination. The flash marked by the arrow will bring some light from behind the subject. Even

here a small black piece of adhesive plastic was attached to the table to provide the black background. (d) Similar set up can also be prepared "on the fly" to document tiny species. In this case a microscopic glass slide containing an alive marine mite (arrow) was suspended between two laboratory "salt cellars," the light being provided by optical fibers oriented at 45°. (e) An arcturid photographed with the set up shown in c. This image was corrected for sharpness and contrast in Photoshop; debris and dirt were digitally removed from the background and a scale bar (¼1 cm) added in the desired position. (f) This is the original image from which was produced; note the scale bar made with the archive paper that was used as reference to add the final scale bar in e. (g) The marine mite photographed with the set up shown in d. (h) A pycnogonid photographed with the set up shown in c. (i) A tererebellid photographed in a small plastic box with a black background. This is the original image where the scale bar (¼1 cm) is kept in place by inverted forceps and there is a lot of debris around the subject. (j) The same image of i optimized in Photoshop for publication. (k) A large ascidian, *Cnemidocarpa verrucosa* (Lesson, 1830) photographed inside a large box with a black background. (l) A tiny fish (scale bar ¼1cm) photographed with the scale bar kept in place by inverted forceps. (m) A tiny polynoid polychaete was photographed close to the scale bar; here two small fishing weights were used to keep the scale bar on the bottom of the container (scale bar ¼1cm).

3.6 Subsampling in the lab

Subsampling in the field may be required when specimens are large (e.g., in the case of sponges, cnidarians, fishes) and the fixation of the whole organism may thus represent an unfeasible solution. In these cases, therefore, a small bit of tissue is clipped (Fig. 4a, d) and preserved in ethanol (or other media ideal for preserving tissues destined to molecular analyses), while the remaining part of the body is generally frozen. Whenever subsampling has to be performed, this step should follow clear protocols and instructions, decided in advance, where it has to be specified whether whole or partial specimens are required and, when necessary, which parts have to be clipped (see Note 24).

1. Wear nitrile gloves.

2. Crosscheck labels to verify any possible mismatch before tissue clipping (see Note 25).

3. Prepare new blades or clean the tools (e.g., scissors) from previous use before

dissecting another specimen in order to avoid contamination (see Note 26).

4. Proceed with the subsampling.

5. Place the tissue bit for the subsampling in a cryovial containing absolute Ethanol, 20% DMSO or DNA later (see Note 27 and 28) (Fig. 4d).

6. Place the label relative to the subsampling in the cryovial.

7. Place the whole specimen in a centrifuge tube or a plastic container of adequate volume and containing ethanol (or frozen it in a plastic bag if too large for the containers).

8. Place the label relative to the specimen inside the container.

9. Store the cryovials and the other containers at the best temperature for the fixative in use.

10. Discard used blades in proper displacement containers.

11. Clean all the tools for the next use.

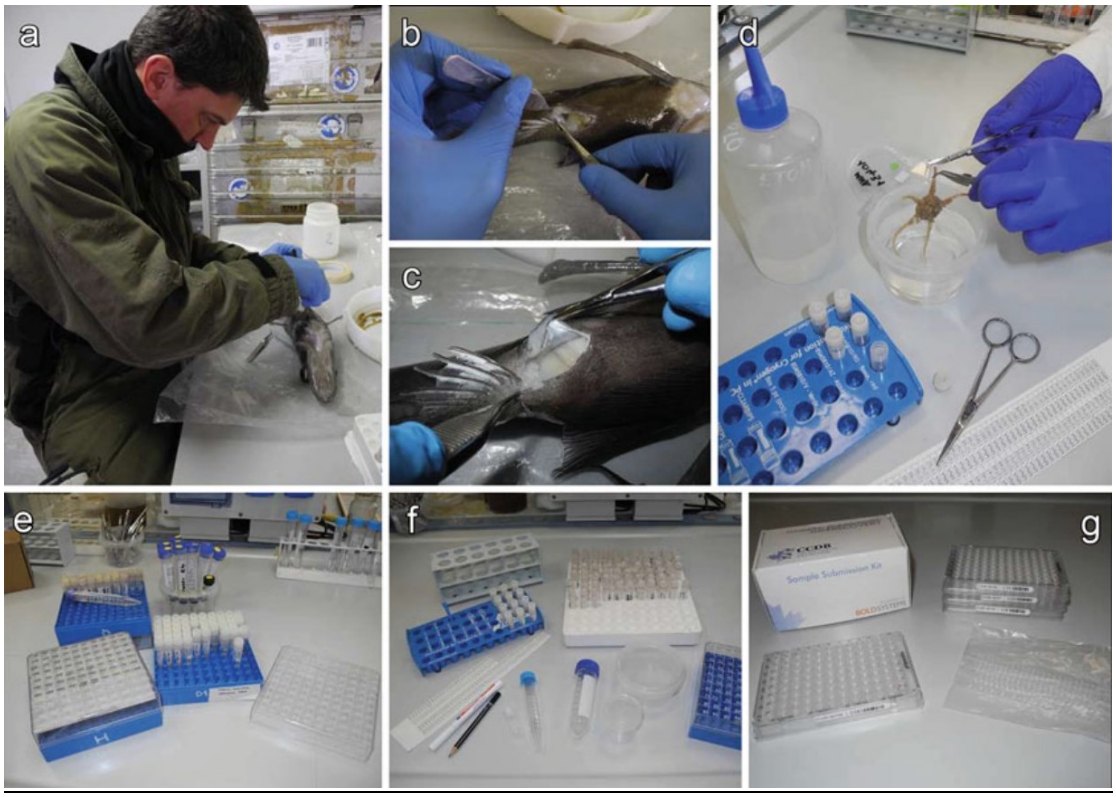


Figure 4 - Tissue clipping and vials to preserve the tissue samples. (a) Tissue clipping from a large fish. In some cases, logistic constraints impose that these operations are done outside the laboratory facilities. In all cases it is mandatory to wear nitrile gloves to avoid contamination. (b

and c) The fish skin is cut to reach muscle tissue. (d) Tissue clipping of the ophiuroid *Ophioplinthus gelida* (Koehler, 1901) with the associated *Iophon* Gray, 1867 sponge covering the disc. In this case, to avoid possible contamination and problems due to the presence of the sponge, a small fragment of an uncovered arm was used. Note the already opened cryovial in a workstation rack and the labels preprinted on archive-quality paper with a size compatible with that of the cryovials. (e) There are several models of racks that enable a compact storage of cryovials and also to trace their position thanks to the numbers reported on the transparent lid. A printed "map" reporting the filed codes can also be added to simplify sample tracking. (f) A set of containers (cryovials, centrifuge tubes, and plastic jars) has to be always available in quantity. Tissue subsamples will be stored in the cryovials and the whole range of sizes will be used to accommodate the specimens. Ethanol-proof markers will be used to indicate field codes on the external side of the containers, while archive paper museum labels will be placed inside the containers. These two ways of marking the samples will ensure that all the needed information is not lost. (g) The standard BOLD submission kit with microplates racks marked with unique barcodes and cap strips (supplied with the sampling kit). These racks will be used in the fully automated sample processing at BOLD.

4 Notes

1. It is rather easy that even the most carefully planned sampling procedure will have to be adapted "on the fly," for example, due to a massive and successful sampling which may run out the available containers for alive specimens storing. In these cases most problems arise from the difficulty to properly mark in a permanent way those spare, free containers that may be available. This can be resolved in having extra labels to be filled in the field (see Note 2). The worst case is the complete absence of a sufficient number of containers that may occur when too many stations are sampled or in the case of particularly successful catches. In these latter cases the pragmatic solution is to do just a very general sorting to pick up most fragile organisms or no sorting at all and bring the bulk sample in the lab for further processing.

2. When jars or barrels have to be labelled in the field and "on the fly" a good way is not to mark their exterior with a marker (which may be a difficult operation due to wet surfaces and, if successful, then requires robust cleaning after sampling), but to put inside the container a piece of waterproof, archive-quality

or museum specification paper, where the needed information can be written with a simple pencil.

3. In polar areas, thanks to the low or below zero temperatures, even if specimens are sorted out of the sediment hours after sampling and not kept in seawater, they still provide good quality materials for DNA barcoding. This situation is generally not met in the tropics, where tissues degenerate quickly after sampling due to the high air temperatures (Schiaparelli, unpublished observation). In such cases also the photography of the selected specimens is much more difficult since, for example, these may be full of sediment (hence have to be cleaned), air bubbles (hence will float if placed again in water) or will be fragmented and dispersed within the sediment (e.g., in the case of polychaetes).

4. In order to quickly move jars and containers these can be stored in boxes with handles. This will help in moving material as needed and store them when moving to the next sampling station. Boxes must be sturdy and with handles large enough to be grabbed even with heavy polar gloves. If possible, dedicate a single box to each station to avoid containers' mixing during sampling.

5. SCUBA diving in polar areas is a dangerous activity restricted to few, well-trained divers. Safety rules and diving procedures may vary for each country and of course will not be treated here. Regardless the rules and restrictions in use, it is necessary to verify well in advance if the choice of sampling materials used to collect the specimens (see Note 6) and if the envisaged sampling schemes are appropriate and feasible. All procedures should be ideally tested in a pilot study, to be performed prior to real sampling, in order to adjust procedures and materials wherever needed.

6. Quite often the choice of containers/jars/drums made by the researchers do not meet the requirements of an underwater use, especially if these have to be handled by divers wearing thick gloves operating at subzero temperatures. Thus,

it is always a good practice to talk in advance with those who will be in charge of the underwater sampling to verify if the choice of materials is correct. It could be possible that changes will have to be done, for example, by bonding together the jars and their lids with small ropes in order to prevent the lids floating, or by arranging a series of centrifuge tubes into a sort of "cartridge belt" in order to keep them together when these are taken out of the sampling bag. Some jars are also hard to be opened underwater where divers operate in cold water and wear very thick gloves, hence middle size (i.e., 100 to 500 cL) jars with large mouths and heavily ribbed lids (to help grabbing while opening and closing the jar) have to be preferred.

7. Usually sampling bags are taken out of the water sometime after divers have ended the dive, hence these will remain on the bottom or attached to a rope for a while. According to the time of the season, diving can take place from a hole in the sea ice or from a rubber boat when there are open water conditions. Either way, bags will have to be pulled quite strongly and may become heavy once outside the water (e.g., with many jars filled with samples and water). In order not to lose them, the sampling bags have to be sturdy and properly attached to ropes with carabiners in a way that will enable to take these out of the water one after the other and process their content accordingly. Again, the rigid environment where these activities take place is of help in maintaining the tissues in a good quality for molecular analyses, even in the case that sample processing will be performed well after sampling.

8. According to the number of people involved in sorting operations it is possible to begin by opposite the corners. Here the goal is to ensure that the entire sample is sorted. By proceeding without an order some areas will inevitably remain unchecked. The unique exception to this procedure is when particularly fragile or brittle organisms are spotted. These have to be isolated immediately to prevent any further damage (Fig. 1d).

9. To accomplish this step, it is necessary to count on the avail ability of

containers of different sizes and volumes. Antarctic benthos is characterized by sponge species of large size, which will have inevitably to be accommodated into large drums (Fig. 1e). Small invertebrates cannot go inside large drums, otherwise will be dispersed or lost. For these tiny specimens a variety of tubes and small jars has to be made available and at hand. Many organisms may produce large quantity of mucus(e.g., the sponge *Mycale (Oxymycale) acerata* Kirkpatrick, 1907 or the fish *Histiodraco velifer* (Regan, 1914)); in such cases single containers have to be dedicated to these taxa.

10. Once closed with their lids, the smaller containers, vials and tubes can also be put inside the drums containing the larger specimens from the same station. In this case it is possible to avoid preparing an extra series of labels for each small container.

11. For example 5 specimens per species; this number has to be decided in advance and may vary according to the study general aims.

12. Many species under stress condition or when placed together with potential predators react by producing copious quantities of mucus or will fragment. Tissues will always be available for analyses, but in some cases body parts could be mixed (e.g., the elytrae of different polynoid polychaetes), hence these mixed fragments will have to be discarded and direct tissue clipping on the specimens has to be done to avoid mistakes.

13. Many Antarctic species are stenothermal and will not survive above zero temperatures for too long. Laboratory conditions are usually designed for researchers' comfort, not to keep alive specimens. It is therefore easy that room temperature will largely exceed species' thermal tolerance if a cool room is not available. Under these unfavorable conditions, for example, nemerteans will suddenly autotomize after some time, while other invertebrates will die. It is therefore a good practice to have access to a fridge at 4 °C in order to avoid any extra stress at this stage.

14. There are several ways to anesthetize invertebrates, but the most common and widespread method is to use of magnesium chloride crystals in the quantity of ~80 g per liter of seawater [9]. The solution has to be made isotonic with the seawater of the sampling area. This step can be checked by using an optical refractometer.

15. Usually it is the same group that has collected the specimens that will process the samples for the molecular study, but it is not always the case. Most problems arise when there is a lack of communication about the meaning and interpretation of the field codes and these are managed after the expedition by a different working group, hence the importance to think of a "Rosetta stone" to enable the understanding of acronyms and field codes in use by other people working on these materials.

16. Preprinted labels (Fig. 2a top) save a lot of time by diminishing handling time, samples contamination and, especially, possible writing errors due to tiredness and lack of concentration. Moreover, problems arising from personal calligraphy or different ways to indicate numbers in different countries will also be eliminated.

17. A simple pattern of field codes could be "number of the expedition-number of the sample" (e.g., XXVIII-00231, Fig. 2a). The first part of the code refers to the number of the expedition and could also include the sampling year (e.g., XXVIII-2021) or the expedition leg (e.g., XXVIII-2021-A). This part will remain stable in all labels. The second part is a consecutive number (i.e., starting with 00001 and going up until the last specimen collected during the expedition). This number has to be linked to station data either in a field book and, later, in an Excel spreadsheet.

18. There are several ways to prepare labels with the field codes to be associated

with the physical samples. In any case these have to be prepared by using archive-quality museum paper. The simplest method, in the field, would be to add the codes manually by using a pencil (Fig. 2b). However, this is a time consuming activity, which does not work when there are hundreds of specimens to be processed. In these cases, preprinted labels represent the ideal solution. They have to be printed on the same archive-quality paper by using a laser printer and can be produced in sizes that fit those of the containers in use.

19. If a subsampling will be necessary (see Subheading 3.6) it is a good practice to print labels in pairs, where one will be placed together with the whole specimen and the other one with the subsample.

20. This operation can be done on the fly, for example, by writing notes in a field book (Fig. 2a in the middle) or, with more calm, by filling an Excel spreadsheet (Fig. 2a, bottom). Usually the first operation precedes the second one. It is mandatory to cross check and revise the matches between labels, vouchers and station data after some entries. It is in fact highly possible that due to the intense activities some mistakes are done in associating labels to specimens or recording the data in the notebook or in the Excel spreadsheets. The photographic documentation (see next section) will also be helpful in resolving possible mismatches in labeling or mistakes in data entry.

21. Petri dishes are the most effective solution to isolate specimens and place all the samples to be photographed in the fridge waiting for their turn.

22. Simple scale bars of a given size (e.g., 1 cm) can be easily made by cutting the archive-quality museum paper of the labels. This paper is strong enough to keep its shape and it is unaffected by immersion. These small-scale bars can be maintained close to the specimen by using inverted forceps (Fig. 3i, k, l) or by using small fishing weights (Figs. 2c and 3m). The latter solution is perfect when very small containers (e.g., petri dishes of 5 cm of diameter) are used.

23. Water in the petri dishes and aquaria has to be changed frequently. It will progressively become dirty and these small particles will affect image quality or will require adjustments in Photoshop to be eliminated from the background. Moreover, some species will release mucus or other secondary metabolites that may trigger alarm responses in the following specimen to be photographed.

24. It is mandatory to decide in advance, taxon by taxon, which parts of the animal may be removed for separate fixation without the risk of selecting parts that may be crucial for future taxonomic identifications based on morphology. It is also important to avoid contamination, for example, by selecting tracts of the digestive system that may be filled with prey tissues. A good practice is to clip tissues (muscles, if present, are the best) not in contact with the external environment (e.g., potentially carrying debris or other organisms) or the digestive tract.

25. This step represents a final quality control where the correspondence with field codes (especially if a new system of codes is introduced just for the subsampling) has to be crosschecked in order to eliminate any ambiguity or mismatch. It is also a good practice to report on the label that will remain with the physical specimen that a subsample has been taken. Samples from which a subsample has been taken have to be clearly indicated in the Excel file.

26. To properly clean a tool it is necessary to carefully remove tissue fragments present on the tool with laboratory paper. Then place the tool in ethanol. When the tool has to be used, take it from the ethanol and pass it on the flame of the Bunsen burner. These passages will eliminate contamination.

27. There are several options for the conservation of tissue sub-samples for molecular analyses. Ethanol is the cheaper and effective solution, while other media are more expensive. Precooled ethanol (e.g., kept at -20 °C before use) is

recommended to make the fixation process more effective.

28. Since the amount of tissue required for analyses can be small, cryovials of 2 mL represent an ideal solution and can be organized in racks for 100 vials. The ratio of fixative vs tissue volume should be around 9:1 (Fig. 4e, f).

CHAPTER 2

Distributional records of sea cucumbers (Echinodermata, Holothuroidea) based on the collection stored at the Italian National Antarctic Museum (MNA, section of Genoa)

1 Introduction

The important diversity of Southern Ocean echinoderms makes them one of the most abundant animal groups in the sea bottoms, where they may represent 95% of the total biomass in deep regions [73]. In the Antarctic region, Holothurians (sea cucumbers) are remarkably diverse with 187 morpho-species reported in O'Loughlin *et al.* 2011 [74]representing ~10% of the total sea cucumbers species listed in the world (1,818 nominal species of holothuroids present in WoRMS 2023 (https://www.marinespecies.org/index.php Accessed 2023-02-02 [75]–[77]). This number is estimated to increase, modern molecular investigations discovered a substantial additional diversity of cryptic species which highlights the need for an integrated identification approach and how our knowledge on this taxon is still severely limited as in numerous species morphological identification alone, based on diagnostic characters such as dermal ossicles, is not always sufficient to distinguish organisms at species level.

Local Southern Ocean holothuroid diversity is known to be high, with 37 species documented in the Weddell Sea, 41 species in Prydz Bay, and 43 in the Ross Sea, from shallow to moderately deep waters (to 1180 m) [74]. High diversity is also reflected in the importance of sea cucumbers in the biota: almost 4% of known Antarctic benthic species are sea cucumbers [78]. The digitization of scattered collections allows to fill in the gaps in the current knowledge, by providing the necessary orientation to taxonomists and other researches regarding the material available for study. The National Antarctic Museum (MNA), founded with the precise aim of preserving, studying and enhancing the findings acquired during the Italian scientific expeditions in Antarctica fulfills its key role of being an indispensable resource for biodiversity studies by providing data over a vast time span [79]. Specifically, it represents a custodian of biodiversity data for the

Ross Sea area with a constant increasing biological collections which conveys material collected during the Italian Antarctic scientific expeditions as well as materials entrusted by foreign research bodies (e.g. the Alfred Wegener Institute - AWI, the British Antarctic Survey - BAS, the National Institute of Water and Atmospheric Research – NIWA and the Muséum national d'Histoire naturelle - MNHN) with which the MNA has constant collaborations and specimens' exchanges for determination and molecular characterization.

Institutional and Museum collection becomes invaluable resources especially for remote and inaccessible areas such as Antarctica where information are still scarce and to date complete faunistic inventory may still be missing. Terra Nova Bay (TNB) may be a case in point for this condition, despite continued research activity has been held in the area by the Italian National Antarctic Program (PRNA) since 1986 a check list of sea cucumbers is still lacking. Given the relevant role of TNB area, this region has been included in the Ross Sea region Marine Protected Area (RSRMPA), established in December 2017 under Conservation Measure 91-05 (2016)[28]. The need to monitor changes in marine communities over time, establishment of conservation plans and monitor the impacts of climate change are urgent actions and cannot be undertaken without the establishment of a reference baseline of the present fauna.

In this work we present an occurrence dataset, with taxonomic and geographic information, of Southern Oceans sea cucumber (Holothuroidea) collected in the framework of ten PNRA expedition and one AWI and stored in the permanent biological collection of the institute. This dataset also represents an Italian contribution to the CCAMLR CONSERVATION MEASURE 91-05 (2016)[28] for the Ross Sea region Marine Protected Area, specifically, addressing Annex 91-05/C ("long-term monitoring of benthic ecosystem functions"). The Italian National Antarctic Museum (MNA) consistently contributes to updating the information in particular relating to the Ross Sea quadrant of the Southern Ocean [e.g. [22], [32], [34], [35], [37], [41], [42], [80]].

2 Project description

Title: Sea cucumbers (Holothuroidea, Echinodermata) from the Italian National Antarctic Museum (MNA) – Genoa section (Italy)

Personnel: Alice Guzzi, Maria Chiara Alvaro, Matteo Cecchetto, Marco Grillo, Valentina Cometti, Nicholas Francesco Noli, Stefano Schiaparelli

Study area description: The distributional information provided in this data paper originates from eleven different PNRA expeditions and one AWI expedition that had a variety of scientific targets and research teams involved. The specimens were collected in the Ross Sea sector of the Southern Ocean in a bathymetric range from 18 to 743 meters of depth and the Antarctic Peninsula with range from 261 to 437 meters of depth (Fig. 1). This dataset contains information on the occurrence of holothuroid specimens collected over a 25-year period starting in 1987. The data was collected through various scientific research projects with different focuses. Most of the events were collected in the Terra Nova Bay area near the "Mario Zucchelli" Italian research station, which stands as a logistic base for the research activities of the Italian scientific program in the area. A smaller number of sampling events come from research projects in the Ross Sea, with most of the sampling stations along the coast of Victoria Land. A few other are located near the Bransfield Strait and South Shetland Islands (Antarctic Peninsula) collected during the AWI PS81 ANT-XXIX/3 Expedition in 2013. The diversity of research objectives is reflected in the different geographical extent of the areas studied.

Design description: Data were assembled by revising all the distributional records of the specimens deposited in the collections of the MNA – Genoa section (Italy). The samples were collected in the framework of 11 PNRA Antarctic expeditions from 1987 to 2017 and one AWI expedition in 2013.

Funding: The specimens were collected during 11 different Antarctic

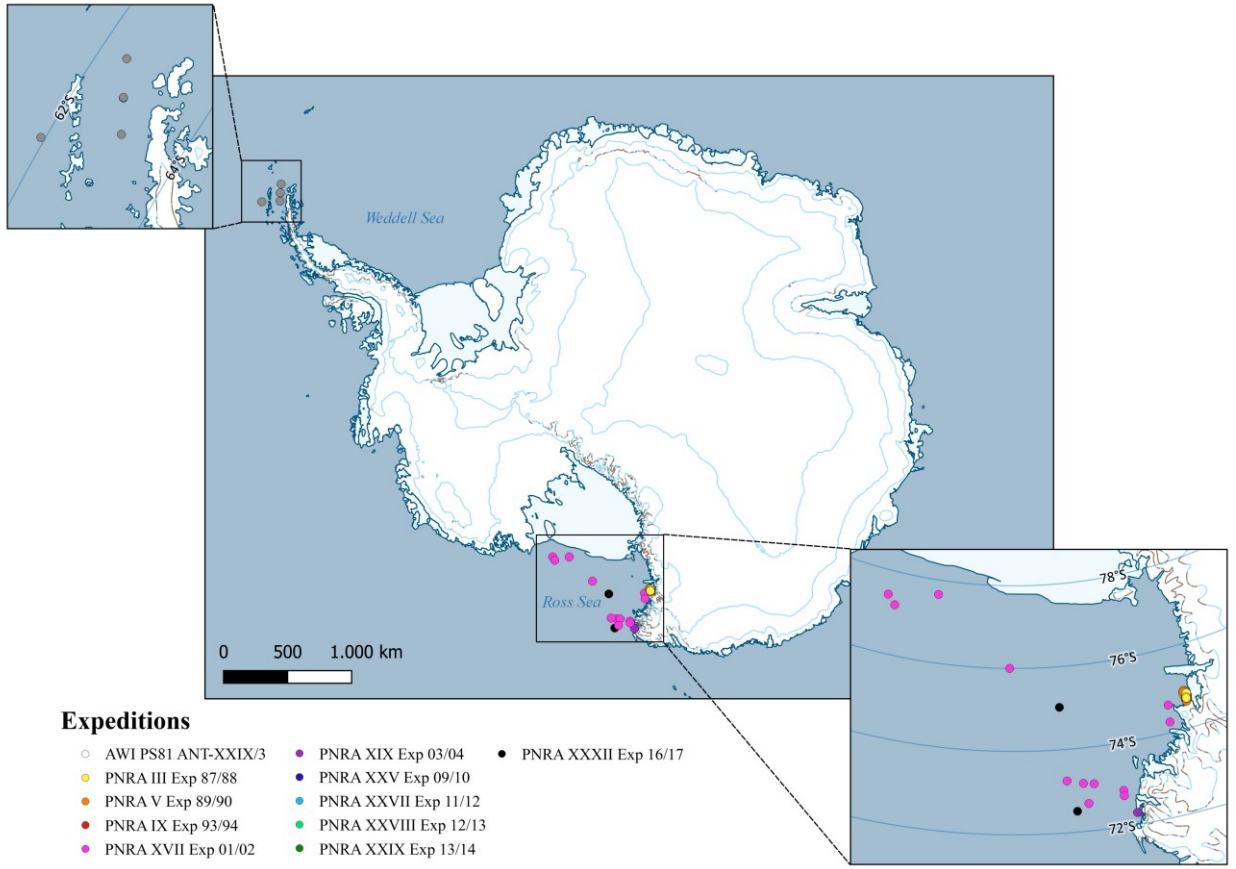


Figure 1 - Sampling stations included in the dataset. The map shows the overview of the two major locations included in the dataset, the Ross Sea and the Antarctic Peninsula. This map was produced using the collection of datasets "Quantarctica" [81] and QGIS [82].

expeditions carried out from 1987 to 2017, one funded by the Alfred Wegener Institute (AWI) and ten by the Italian National Antarctic Research Program (PNRA). The complete list of research projects is reported here (in italic is the project name or category under the AWI/PNRA program, followed by the project code):

AWI "Polarstern" Research Vessel PS81 ANT-XXIX/3 Expedition (2013) [83]

- *Macrobenthic community analysis and biodiversity study* "3.1"

Cruise Report PNRA III Expedition (1987/1988) [84]

- *Necton e risorse da pesca* "2.1.4.6"
- *Oceanografia & Benthos* "2.1.4.3"

Cruise Report PNRA V Expedition (1989/1990) [85]

- *Benthos "*3.2.1.2.5"
- *Oceanografia geologica* "3.2.1.4"

Cruise Report PNRA IX Expedition (1993/1994) [86]

- *Ecologia e biogeochimica dell'Oceano Meridionale* "2d.2"

Cruise Report PNRA XVII Expedition (2001/2002) [87]

- *Processi genetici e significato paleoclimatico e paleoceanografico dei CARBONati marini biogenici in ANTartide – CARBONAN*T "2009/A1.09"
- *L'area marina protetta di Baia Terra Nova: struttura e variazioni a breve e lungo termine* "8.5"

Cruise Report PNRA XIX Expedition (2003/2004) [88]

- *The costal ecosystem of Victoria Land coast: distribution and structure along the latitudinal gradient* "2002/8.6"

Cruise Report PNRA XXV Expedition (2009/2010) [89]

- *L'ecosistema costiero di Baia Terra Nova – Latitudinal Gradient Project* "2006/08.01"
- *Ecologia e ciclo vitale di specie ittiche costiere del Mare di Ross "*2004/08.04"

Cruise Report PNRA XXVII Expedition (2011/2012) [90]

- *Barcoding of Antarctic Marine Biodiversity* – BAMBi "2010/A1.10"

Cruise Report PNRA XXVIII Expedition (2012/2013) [91]

- *Diversità genetica spazio temporale di endoparassiti delle regioni polari: uno studio per la valutazione dell'impatto dei cambiamenti globali sulle reti trofiche marine* "2009/A1.09"
- *Barcoding of Antarctic Marine Biodiversity* – BAMBi "2010/A1.10"

Cruise Report PNRA XXIX Expedition (2013/2014) [92]

- *Barcoding of Antarctic Marine Biodiversity* – BAMBi "2010/A1.10"

Cruise Report PNRA XXXII Expedition (2016/2017) [93]

- *Geochemical signals in Antarctic biogenic carbonates for palaeoceanographic reconstructions*– GRACEFUL "2016/AN2.01"

The publication of this data paper was funded by the Belgian Science Policy Office (BELSPO, contract n°FR/36/AN1/AntaBIS) in the Framework of EU-Lifewatch as a contribution to the SCAR Antarctic biodiversity portal (biodiversity.aq).

3 Sampling methods

Description: This dataset describes the occurrence of Holothuroidea samples stored in the biological collection of the Italian National Antarctic Museum (MNA) – Genoa section, collected in the framework of ten Ross Sea PNRA and one AWI expedition in the Bransfield Strait and the South Shetland Islands (Antarctic

Peninsula) between 1987 and 2017. The data here presented originate from 89 different sampling stations ranging between 18 and 743 meters of depth.

Sampling description: The dataset provides information on sea cucumbers (Holothuroidea) specimens collected from 1987 to 2017 and now part of the permanent biological collection at the Italian National Antarctic Museum (MNA) – Genoa section. The 328 samples composing this Database have been collected in a wide range of scientific projects with variety of scientific objectives employing a variety of sapling gears covering a total time interval of 30 years, for this reason the data presented here does not allow to provide a quantitative dataset but rather an occurrence resource for Antarctic holothuroids. Sampling was performed on a total of 89 different sampling stations Fig. 1 through the deployment of a variety of sampling gears, mainly dredges whose specifications were often not properly described in the expedition's reports (Charcot dredge, Naturalist dredge and Triangular dredge) and Van Veen grabs of different volumes. Other sampling instruments include two towed horizontal nets, one called "Small Hamburg Plankton Net" (Hydrobios, https://www.hydrobios.de/en/), used during the PNRA XXVIII expedition (2012/2013) and composed by a 1 m^2 metallic frame opening and a nylon net of 2 mm of mesh size, and another one called "Multiple Net Mid-Water Tucker Trawl" (Aquatic Research Instruments, http://www.aquaticresearch.com/multi-net_mid_water_trawl.htm) used during the PNRA XXIX expedition (2013/2014) made of three different 900 μm mesh size nylon nets, each one attached to a different metallic frame of 1 m2 opening, which are opened sequentially at different depths. The "Agassiz trawl" employed during the PNRA XIX expedition (2003/2004) was made up of a rigid rectangular steel frame with an opening of about 120 cm in width and 50 cm in height and a bag made of sturdy polyamide yarn net with 8 mm side mesh. A second "Agassiz trawl" with a 3 m^2 opening was used during the AWI ANT-XXIX/3 Expedition (2013). Some samples were also opportunistically collected by long fishing lines, mid water trawls (that touched the bottom due to a failure of the winches), trammel nets, and other fishing nets that provided additional material to standard techniques and a Van Veen grab of 65 l volume. During the PNRA XXV expedition (2009/2010) a few samples were

hand collected by SCUBA diving operations conducted by Stefano Schiaparelli at a maximum depth of 26 meters. A few occurrences listed in the data originate from samples acquired using uncommon methodologies for collection benthic sea cucumbers (e.g., gill nets, trammel nets) and some referred as samples obtained as by-catch from failed deployments. Most of the specimens were collected from the Terra Nova Bay area near the "Mario Zucchelli'' station using small boats and zodiacs, while a smaller number was collected from the Ross Sea using the R/V "Italica". The samples belonging to the AWI ANT-XXIX/3 Expedition (2013) were collected by Alvaro Maria Chiara on board the "Polarstern" Research Vessel from the Bransfield Strait and the South Shetland Islands (Antarctic Peninsula). Once the material has been acquired by the MNA after sorting and shipment activities, all the specimens were classified to the lowest possible taxonomic resolution. The present dataset has been formatted in order to fulfill the Darwin Core standard protocol required by the OBIS scheme (http://www.iobis.org/manual/lifewatchqc/) and according to the SCAR-MarBIN Data Toolkit (available at http://www.scarmarbin.be/ documents/SM-FATv1.zip). Vouchers are now preserved in 90% ethanol (~56% of the entire collection), frozen (~43.5%), or in formalin (~0.3%) Fig. 2.

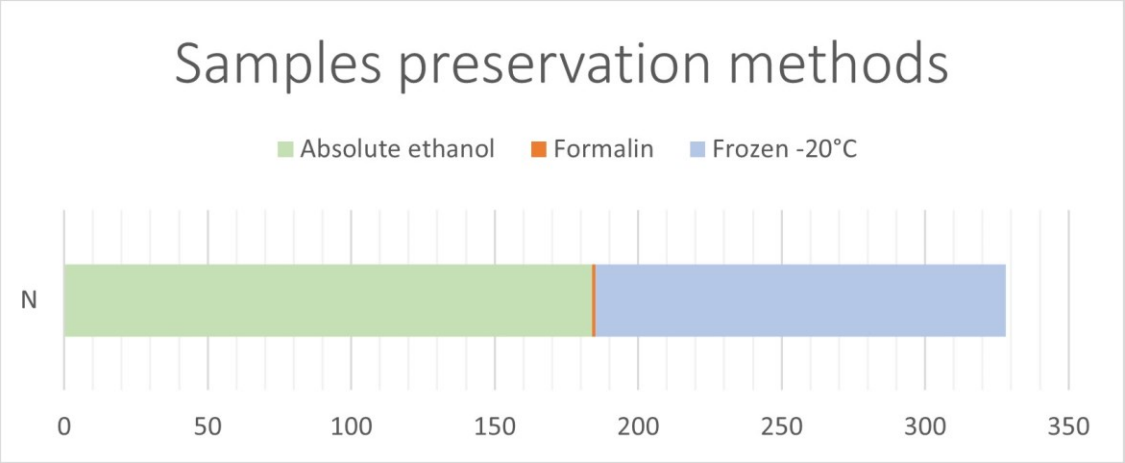


Figure 2 - Number of individuals by preservation method stored at MNA. Specimens preserved in absolute ethanol in green (n=184, ~56%), frozen -20 °C in blue (n=143, ~43.5%) and formalin in orange (n=1, ~0.3%).

Quality control:

- All records were validated.
- Coordinates were converted into decimal latitude and decimal longitude and plotted on map to verify the actual geographical location corresponds to its locality.
- Specimens were identified at the lowest possible taxonomic resolution. The species were classified according to dichotomous keys and the available scientific literature. The classification is based on Miller *et al.* (2017) [94] phylogenetic review that is currently accepted in the World Register of Marine Species database WoRMS 2023 [75].
- All scientific names were checked for typo and matched to the species information backbone of Worlds Register of Marine Species (http://marinespecies.org/) and LSID were assigned to each taxon as scientificNameID.
- Event date and time were converted into ISO 8601 and verified with the field reports.

Step description: The data set provided in this publication is composed of occurrences originating from different expeditions and projects, the treatment and steps adopted to process each sample may differ depending on the expedition, the project and the personnel involved. However, in most cases, the samples were usually sorted in situ on the basis of morphological characteristics and stored directly in formalin or absolute ethanol, to be subsequently identified by researchers at the Italian National Antarctic Museum (MNA) – Genoa section after being acquired in the biological collection. The samples are now stored in absolute ethanol, fixed in formalin or at -20°C.

Most of the records have been identified by Stefano Schiaparelli and Alice Guzzi using original descriptions and taxonomic keys. Identification is made in most cases through scanning electron microscopic (SEM) analysis of the dermal ossicles of sea cucumbers. For ossicles extraction, small portions of tissue were taken from oral tentacles, body wall and tube feet (when available). Each portion was placed on a separate watch glass and covered with several drops of sodium

hypochlorite solution (6-14% active chlorine) (liquid bleach). When the soft tissue was completely dissolved, the ossicles were cleaned with water, air dried, mounted on aluminum stubs, and coated with gold.

The identification of many specimens, corresponding to more than 47% of the entire dataset, was aided by the application of DNA barcoding analyses, including the amplification and sequencing of the cytochrome oxidase I (COI) region, in a collaboration with the Canadian Centre for DNA Barcoding (CCDB) and uploaded to the Barcode Of Life Data System (BOLD System). The results obtained in this context will be the subject of another publication.

4 Taxonomic coverage

General taxonomic coverage description: This dataset focuses on the class Holothuroidea of the phylum Echinodermata (Kingdom Animalia) collected in different research project from the Ross Sea sector of the Southern Ocean and Bransfield Strait (Antarctic Peninsula). Of the seven orders currently sitting within class Holothuroidea, three are represented here, with Elasipodida, Holothuriida, Molpadida and Persiculida absent in this dataset. Determinations here are based on morpho-taxonomic methods and integrated molecular data (COI) if available. Future genetic and morphological work may lead to additions or changes. This dataset looks at over 328 specimens belonging to 7 families, and at least 14 genera and 23 species (Fig. 3). The family represented by the highest number of occurrences was Cucumariidae (220 records), followed by Chiridotidae (34), Psolidae (12), Synallactidae (7). A small proportion of samples, respectively 3 and 1, were also identified as belonging to Ypsilothuriidae and Synaptidae. The database compromise 88 samples only reported as “Holothuroidea”. All the records recorded in this database are based on physical museum vouchers (hereafter “MNA collection records”) curated by the Italian National Antarctic Museum (MNA) – Genoa section. The list presented in this paper represent the first Holothuroidea faunal inventory available for the area of Terra Nova Bay and all the species name marked with asterisk (*) in the table represent new records for the TNB area.

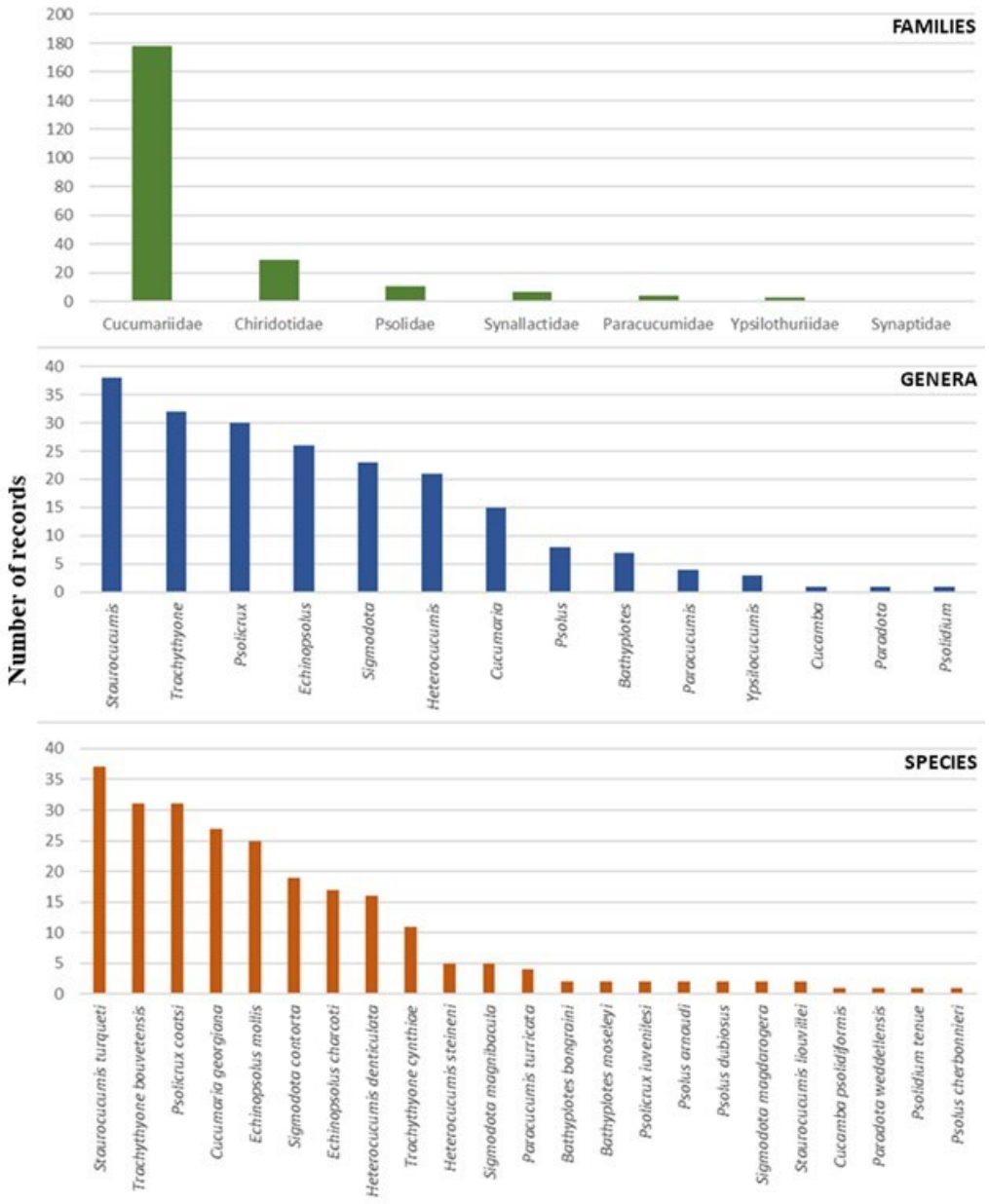


Figure 3 - Number of records identified at the family, genus and species level, for each specific taxon.

Remarks

Miller *et al.* (2017) [94] assessed and revised the phylogeny of extant Holothuroidea. Apodida was highlighted as a sister to the rest of Holothuroidea, the previously paraphyletic Aspidochirotida was split into Molpadida, Dendrochirotida, and Elasipodida (in part) and a new order, Holothuriida, was erected. Here we follow Miller *et al.* (2017) taxonomic groups and currently

accepted in the World Register of Marine Species database WoRMS 2023.

Taxa included (asterisks refer to new record for the area)**:**

Rank	Scientific Name	Common Name
kingdom	Animalia	
phylum	Holothuroidea	
subphylum	Echinozoa	
class	Holothuroidea	Sea cucumber
subclass	Actinopoda	
subclass	Paractinopoda	
order	Apodida	
order	Dendrochirotida	
order	Synallactida	
family	Chiridotidae	
family	Cucumariidae	
family	Paracucumidae	
family	Psolidae	
family	Synallactidae	
family	Synaptidae	
family	Ypsilothuriidae	
species	*Bathyplotes bongraini* *	
species	*Bathyplotes moseleyi*	
species	*Cucamba psolidiformis*	
species	*Cucumaria georgiana**	
species	*Echinopsolus charcoti*	
species	*Echinopsolus mollis**	
species	*Heterocucumis denticulata* *	
species	*Heterocucumis steineni* *	
species	*Paracucumis turricata*	
species	*Paradota weddellensis*	
species	*Psolicrux coatsi* *	
species	*Psolicrux iuvenilesi*	
species	*Psolidium tenue* *	

species	*Psolus arnaudi*	
species	*Psolus cherbonnieri*	
species	*Psolus dubiosus* *	
species	*Sigmodota contorta* *	
species	*Sigmodota magdarogera* *	
species	*Sigmodota magnibacula* *	
species	*Staurocucumis liouvillei* *	
species	*Staurocucumis turqueti* *	
species	*Trachythyone bouvetensis* *	
species	*Trachythyone cynthiae*	

5 Collection data

Collection name: Distributional records of sea cucumbers (Echinodermata, Holothuroidea) based on the collection stored at the Italian National Antarctic Museum (MNA, section of Genoa)

Parent collection identifier: Italian National Antarctic Museum (MNA, section of Genoa)

Curatorial unit: The material presented in this paper are all physical objects belonging to the biological collection of the Italian National Antarctic Museum (MNA) – Genoa section. Vouchers are now permanently preserved in 90% ethanol (~56% of the entire collection), frozen (~43.5%), or in formalin (~0.3%). Samples are stored in ethanol and frozen (-20°C) to maintain condition in order to preserve the DNA quality and integrity. All samples are now stored in the collections of the Italian National Antarctic Museum (MNA) – Genoa section.

Usage licence

Usage licence: Other Data resources

Data package title: Distributional records of sea cucumbers (Echinodermata, Holothuroidea) based on the collection stored at the Italian National Antarctic

Museum (MNA, section of Genoa)

Resource link: https://www.gbif.org/dataset/1dafb239-593d-43b0-a2a0bd3e4bde1b23

Alternative identifiers:

https://ipt.biodiversity.aq/resource?r=mna_echinodermata_holothuroidea

Number of data sets: 2

Data set name: Distributional records of sea cucumbers (Echinodermata, Holothuroidea) based on the collection stored at the Italian National Antarctic Museum (MNA, section of Genoa)

Data format: Darwin Core

Description: The dataset comprises a total of 328 distributional records, each one corresponding to a voucher specimen stored at the Italian National Antarctic Museum (MNA) – Genoa section. These records originate from the sampling activities performed in the context of eleven different research projects that took place during 11 scientific polar expeditions conducted in the Ross Sea and the Antarctic Peninsula. In total, 89 different sampling events were conducted using a variety of sampling instruments and methodologies. The diversity in the scientific goals of each different research project do not allow to provide a quantitative dataset, also considering that many occurrences were gathered as by-catch, still providing basic metadata such as the geographic coordinates, sampling depth and date and a general description of the adopted sampling methodology. For this reason, this resource must be considered exclusively as a contribution to the distributional knowledge of Antarctic Holothuroidea, especially for the Ross Sea, where most of the occurrences are located, and with a special focus on the Terra Nova Bay area. This dataset is published by Italian National Antarctic Museum (MNA) under the license CC-BY 4.0. Please follow the guidelines from the SCAR Data Policy (ISSN 1998-0337) when using the data. If you have any questions regarding this dataset, please contact us via the contact information provided in the metadata or via data-

biodiversityaq@naturalsciences.be. Issues with dataset can be reported at https://github.com/ biodiversity-aq/data-publication/ The occurrences presented in this dataset originated from a multitude of different Antarctic projects funded by the Italian National Antarctic Research Program (PNRA) and one from the Alfred Wegener Institute (AWI). The publication of this data paper was funded by the Belgian Science Policy Office (BELSPO, contract n°FR/36/AN1/ AntaBIS) in the Framework of EU-Lifewatch as a contribution the the SCAR Antarctic biodiversity portal (biodiversity.aq)

Data set name: dnaDerivedData

Description: The dataset comprises a total of 157 DNA derived records, each one corresponding to a voucher specimen stored at the Italian National Antarctic Museum (MNA) – Genoa section and screened for cytochrome c oxidase subunit I (COI) gene. This dataset is published by Italian National Antarctic Museum (MNA) under the license CC-BY 4.0. Please follow the guidelines from the SCAR Data Policy (ISSN 1998-0337) when using the data. If you have any questions regarding this dataset, please contact us via the contact information provided in the metadata or via data-biodiversityaq@naturalsciences.be. Issues with dataset can be reported at https://github.com/ biodiversity-aq/data-publication

The occurrences presented in this dataset originated from a multitude of different Antarctic projects funded by the Italian National Antarctic Research Program (PNRA) and one from the Alfred Wegener Institute (AWI). The publication of this data paper was funded by the Belgian Science Policy Office (BELSPO, contract n°FR/36/AN1/ AntaBIS) in the Framework of EU-Lifewatch as a contribution the the SCAR Antarctic biodiversity portal (biodiversity.aq)

CHAPTER 3

Not all that glitters is gold: barcoding effort reveals taxonomic incongruences in iconic Ross Sea sea stars

1 Introduction

Asteroidea (sea stars) is one of five extant classes belonging to the phylum Echinodermata. The class includes 38 families and approximately 1900 species [95]–[97], making it the second most diverse echinoderm class after the Ophiuroidea [98]–[100]. Sea stars show high ecological diversity and are important components of marine ecosystems where they occur, from the intertidal to hadal depths (9990 m) [45], [101], [102]. In the Southern Ocean (SO), asteroids are well represented, accounting for 15% to 16% of the total number of species reported thereto date [95], [103], [104]. Current diversity estimates for this class south of 45° S count 28 asteroid families, 118 genera, and 299 species [105]. As with other invertebrates thriving in polar environments, Antarctic sea stars have developed specific adaptations (e.g., slow development [106], [107]) and reproductive strategies (brooders vs. broadcasters [105], [108]) that affect distribution patterns and the biogeography of this class in the SO [109].Although many species of sea stars can be identified based on morphological characteristics, their phenotypic diversity at the species level is commonly so high that taxonomic boundaries can be challenging (or even impossible) to morphologically determine [110]–[112].

With the rapid accumulation of samples in museums and the co-occurring decline of taxonomic expertise in recent years [113], cladistics, phylogenetics, and coalescent-based analyses have become key tools for species identification or discrimination. Although some evolutionary relationships between asteroid families and species are still to be conclusively assessed, the implementation of molecular tools and the availability of data during the last 20 years have allowed a great leap in accuracy of knowledge for this taxon (e.g., [114]–[119] Molecular tools differ in effectiveness and interpretation in relation both to the re-search question and the unique evolutionary histories of the taxa [120]. They are proving particularly useful and efficient in the case of Antarctic sea stars. Indeed,

the isolation of the Antarctic continent (which started in the Oligocene) resulted in typically high levels of endemicity in the SO shelf fauna [43], [121], [122]. Use of DNA barcoding has increased since its introduction in 2003 as a routine tool for species identification, to effectively discriminate species and "unmask" those that look similar. In particular, the barcode gap, thanks to interspecific genetic variation being generally higher than intraspecific ones, often allows correct delineation of species [14]. An integrative approach to taxonomy, i.e., by using morphological characteristics as well as one to several genes, is necessary for assessing species richness and species boundaries in many or most situations [123].Few molecular studies have been performed on SO asteroids, and they have focused on the abundant, near-shore genus *Odontaster* (e.g., [21], [124]–[127]), making it one of the most studied echinoderms in Antarctica. This genus occupies a key trophic position in shallow benthic communities of the Southern Ocean [128], [129]. *Odontaster validus* Koehler, 1906, in particular, has been used as a model species in studies in Antarctic water focusing on distribution and abundance(e.g., [130], [131]), metabolism (e.g., [126]), ocean acidification (e.g., [132]), isotopic trophic position (e.g., [133]), and consequences of physical climate change on Antarctic organisms(e.g., [134], [135]).Despite the numerous scientific publications on this model genus, recent updates on *Odontaster* taxonomy [21], [125] highlighted that its diversity might be higher than recorded, even in well-studied areas. Two species within the *Odontaster* genus were fairly recently described from the Antarctic Peninsula region, *O. roseus* Janosik & Halanych, 2010 and *O. pearsei* Janosik & Halanych,2010, and set out the problem of redundant errors due to lack of resources for identification and consistent taxonomic revision. Specifically, these two species should not have to be considered as cryptic species (which display no obvious morphological differences) but are referred to as "unrecognized biodiversity" having clear diagnostic morphological characters (e.g., the number of spines on abactinal plates, spine length, as well as differences in marginal plates and marginal spines) that has escaped previous detection [125]. This pattern of unrecognized species diversity is common in the SO (e.g., [136]–[139]]) and many author have highlighted the efficiency of integrated molecular and morphological techniques

as a fundamental explorative tool to unravel marine biodiversity (e.g., [140], [141]).The Ross Sea area is one of the most productive regions in the Southern Ocean [142];and since December 2017, it has fallen under the protection of the Conservation Measure91-05 (2016), which declared it a Marine Protected Area (RSRMPA). Nevertheless, a specific assessment of the molecular diversity of sea stars has never been performed. Since 1985, the Italian National Antarctic Research Program (PRNA) has coordinated several research activities and gathered extensive biological and oceanographic information, resulting in a rich specimen collection. In this framework, sea stars were targeted by several studies (e.g., [58], [143]), while a first complete faunistic inventory of asteroids from the Terra Nova Bay (hereafter TNB) area (30–500 m depth) was published by Chiantore *et al.* [63]. Chiantore *et al.* [63] identified 15 different sea stars species belonging to seven families, with genus *Odontaster* comprising two species, i.e., *Odontaster validus* Koehler, 1906 and *Odontaster meridionalis* (E. A. Smith, 1876). These two taxa were discriminated by morphological traits, mainly relying on Clark [144]. The Asteroid check list for TNB has not been updated since then. The same two taxa were repeatedly cited in other studies performed in the Ross Sea, especially in the McMurdo area (e.g., [107], [145], [146]). *Odontaster* species are «model species» in a variety of field studies as well as benthic monitoring programs for the Ross Sea region Marine Protected Area by the RSMPA monitoring plan (CCAMLR Conservation Measure 91-05: Ross Sea Region Marine Protected Area. 2016 [28]). The possible presence of unnoticed diversity in the genus *Odontaster* led us to re-evaluate the biodiversity of this genus for the TNB area. Hence, the objective of our study was to perform molecular and morphological analysis on *Odontaster* samples collected by Italian National Antarctic Program (PNRA) and curated by the Italian National Antarctic Museum (MNA, Genoa section).

2 Materials and methods

The study area is Terra Nova Bay, which is commonly ice-free during polar summer months. The region is located on the western margin of the Ross Sea and

stretches from Cape Washington Peninsula (74° 440 S 163° 450 E), in the north, to the floating tongue of the Drygalski glacier (64° 430 S 60° 440 W), arising from David Glacier in the south [30] (Figure 1). The Terra Nova Bay polynya (TNBP), an open water area surrounded by sea ice [147], is a part of both the marine protected area and the Antarctic Special Protected Area (n.161) in the western Ross Sea [148]. The bay comprises a tortuous continental shelf with numerous banks and deep embayments. The mean depth of the shelf is approximately 450 m, with the greatest depths close to the coast and areas up to 1000 m deep in the adjacent basin.

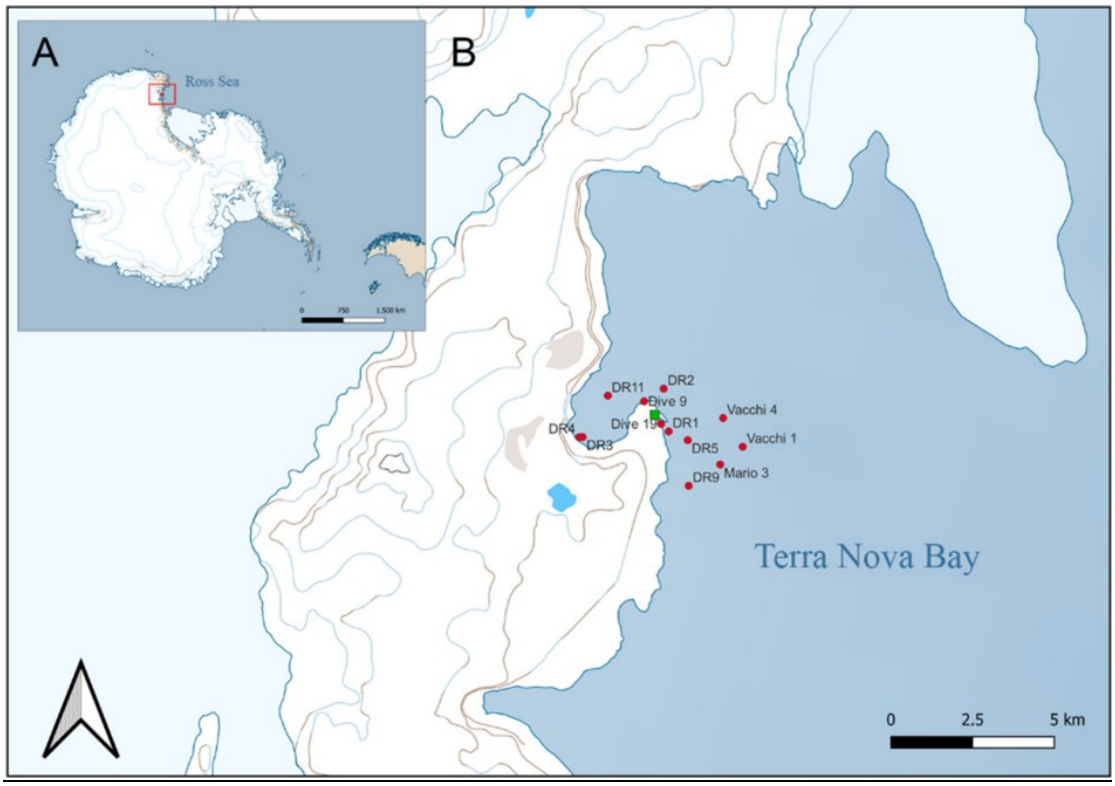


Figure 1 - Antarctica (A) with detail of Terra Nova Bay (Ross Sea) and (B) sampling sites with Mario Zucchelli Station (Italy) highlighted in green

Data presented here were collected in the framework of four different Italian PNRA research projects: - 2006/08.01 ("The coastal ecosystem of Terra Nova Bay" in the Latitudinal Gradient Program—LGP) ("XXV" expedition, 2009/2010). - 2010/A1.10 (BAMBi; Barcoding of Antarctic Marine Biodiversity) ("XXVII" expedition, 2011/2012 and ("XXVIII" expedition, 2012/2013). - 2009/A1.09

(Diversità genetica spazio temporale di endoparassiti delle regioni polari: uno studio per la valutazione dell'impatto dei cambiamenti globali sulle reti trofiche marine) ("XXVIII" expedition, 2012/2013).

2.1 Sampling and DNA extraction

A total of 40 samples belonging to the *Odontaster* genus were analyzed and the distributional data considered here originated from 13 different sampling stations, ranging between 15 and 569 m of depth (Table 1). Sampling was performed through deployments of a variety of sampling gear. Benthic sampling under the Italian PNRA was mainly performed using a rectangular dredge (70 × 30 cm) and an unconventional set of gears for sampling benthic fauna (such as a trammel net and a small Hamburg plankton net) that opportunistically collected benthic specimens due to accidental contact with the bottom during gear deployment "failures". Two samples were photographed and collected by Stefano Schiaparelli during SCUBA diving, performed in the framework of the PNRA "XXV" Expedition (2009/10) along the rocky cliffs of Tethys Bay "Zecca" and Road Bay.

Expedition	Station	Location	Year	Latitude	Longitude	Depth (m)	Sample Vouchers	N
PNRA XXV Exp 09/10	Dive 9	Tethys Bay "zecca"	2009	−74.9027	164.10255	23	MNA-02814	1
	Dive 19	Road Bay	2010	−74.69647	164.12007	15	MNA-02902	1
PNRA XXVII Exp 11/12	DR1	Road Bay	2012	−74.69848	164.12812	100	MNA-03430, 04282, 04283	3
	DR3	Tethys Bay	2012	−74.70005	164.03873	60	MNA-03582	1
	DR4	Tethys Bay	2012	−74.70010	164.03502	198	MNA-04276	1
	DR9	Faraglione	2012	−74.71337	164.14903	150	MNA-03791, 03812, 03825, 03832, 03841, 08034,08035, 08036	8
PNRA XXVIII Exp 12/13	DR5	Road Bay	2013	−74.70087	164.14793	150	MNA-05817, 08037, 08038, 08039	4
	DR9	MZS ('fossa')	2013	−74.68090	164.21433	522	MNA-06116	1
	DR11	Tethys Bay	2013	−74.68872	164.06493	222	MNA-06486, 06489, 06490	3
	Vacchi 1	Tethys Bay	2013	−74.70262	164.20502	569	MNA-05430	1
	Vacchi 4	Tethys Bay	2013	−74.69478	164.18458	454	MNA-06331, 08021, 08022, 08023, 08024, 08025, 08026, 08027, 08028, 08029, 08030, 08031, 08032, 08033	14
PNRA XXIX Exp 13/14	DR2	"Dorsale" MZS	2014	−74.68677	164.12278	94	MNA-08043	1
	Mario 3	Punta Stocchino	2014	−74.70750	164.18167	281	MNA-08042	1

Table 1 - Sampling stations and data. Abbreviations: Mario Zucchelli Station (MZS); number of

specimens (n).

After collection, samples were transferred to the laboratory, and the significant morphological characteristics of the live specimens were photographed to preserve information about the original coloration of the organisms. After that, samples were stored in ethanol (75% Et-OH) or frozen (−20 ◦ C) for subsequent molecular analysis. Thereafter, samples were acquired by the MNA and included in their collections (available online at https://steu.shinyapps.io/MNA-generale/, accessed on 10 January 2022). All specimens were classified to the lowest possible taxonomical resolution [149] on a morphological basis by using the available literature and keys from Fisher (1940) [150] and Clark (1963) [144]. Stefano Schiaparelli, Alice Guzzi, Bruno Danis, and Camille Moreau contributed to morphological identification of specimens. For molecular analyses, a portion of tube feet or arm tip tissue was clipped from each sample for DNA extraction and sequencing of partial cytochrome c oxidase subunit 1 (CO1). The molecular analyses were carried out at the Canadian Centre for DNA Barcoding (University of Guelph, Guelph, ON, Canada). Sequences were uploaded to the BOLD platform (Barcode Of Life Data systems, http://www.boldsystems.org, accessed on 8 February 2022). Primers used for amplification were LCOech1aF1 or LCO1490 and HCO2198 (Table 2).

Region	Direction	Primer	Sequence (5′-3′)	Reference
COI	F	LCOech1aF1	TTTTTTCTACTAAACACAAGGATATTGG	Corstorphine, 2010 [65]
		LCO1490	GGTCAACAAATCATAAAGATATTGG	Folmer et al., 1994 [66]
	R	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al., 1994 [66]

Table 2 - List of used primers for cytochrome oxidase I (COI) amplification in our work. Forward primers (F) and reverse primer (R).

Taxonomic assignation was performed manually in the Barcode of Life database (BOLD) and National Center for Biotechnology Information (NCBI) database BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 8 February 2022) for definitive assignment. A sequence match of >98% to the reference database was considered an “exact” match [151]. Accepted taxonomic names and

classification were obtained from the World Register of Marine Species (WoRMS www.marinespecies.org/, last search 8 February 2022). Chromatograms were edited in CodonCode Aligner v9.0.1 (CodonCode Corporation, Centerville, Massachusetts, USA; http://www.codoncode.com/aligner/, accessed on 8 February 2022), primers were trimmed, and the absence of stop codon in the sequences was checked with the same software. Sequences were aligned using MUSCLE, available within CodonCode Aligner, and checked by eye. Based on current understanding of sea star relationships [152], *Acodontaster conspicuus* (Koehler, 1920) (accession number: DQ380237) was chosen as the outgroup. The model with the lowest BIC scores (Bayesian information criterion) in MEGA X [153] analysis resulted T92 + G (Tamura 3-parameter + Gamma distribution) and is considered to best describe the substitution pattern. The evolutionary history was inferred in MEGA X using the maximum likelihood (ML) method based on the Tamura 3-parameter model [154]. For completeness, a maximum parsimony (MP) tree was also produced in the software. A Bayesian phylogeny was subsequently produced using Mr Bayes [155], [156]. Based on the notion that nonparametric bootstrap frequencies for ML estimates and Bayesian posterior probabilities for clades in phylogenetic trees are not universally equivalent [157] and the possibility of obtaining wrongly supported results with under parametrization in Bayesian inference, the generalized time reversible (GTR) model with gamma(G)-correction was used. Posterior probabilities were calculated by two independent analyses (one cold and three heated chains) using Markov chain Monte Carlo (MCMC) algorithm. Samples of trees and parameters were extracted every 100 steps from a total of 2×10^8 MCMC generations. The first 25% of trees were discarded as the burning and the remaining were used to build a consensus tree. Tracer v.1.6 was used to ensure an appropriate effective sampling size (ESS all > 100). All obtained trees were imported and compared in FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/, accessed on 8 February 2022) for graphic implementation. All sequences were deposited in GenBank (accession numbers: MK811555, MK811610, ON103472-ON103509).

2.2 Species delimitation methods

Throughout our analyses, a phylogenetic species concept, based on the principle that genetic variation between species (interspecific) is greater than the genetic variation within species (intraspecific) [158], was used. Thus, where two or more species are distinct, there should be a lack of overlap between intraspecific and interspecific variation, commonly referred to as the "barcode gap" [159]. To identify the number of molecular operational taxonomic units (MOTUs) within our dataset, we applied four different methods of species delimitation to propose primary species hypotheses. Two were distance-based: (i) Barcode Index Number (BIN) system [67], (ii) Automatic Barcode Gap Discovery (ABGD) [123] (bioinfo.mnhn.fr/abi/public/abgd); and two were tree-based: (iii) Generalized Mixed Yule Coalescent method (GMYC) [160] (species.h-its.org/gmyc), performed using the single threshold method, and (iv) Bayesian Poisson tree process (bPTP) [161] (species.h-its.org/ptp). All sequences were barcode-compliant (n = 40). They received a Barcode Index Number (BIN), which aided species delimitation [67]. The Automatic Barcode Gap Discovery method (ABGD) is an automatic procedure that considers the sequences as hypothetical species based on the barcoding gap. The model employs a two-phase system, which initially divides sequences into operational taxonomic units (OTUs) based on a statistically inferred barcode gap (i.e., initial partitioning), and subsequently conducts a second round of splitting (i.e., recursive partitioning). The default values of 0.001 to 0.1 were explored as intraspecific distances and in ABGD, gap values from 1 to 1.5 were applied. The ABGD analysis (bioinfo.mnhn.fr/abi/public/abgd) was performed with a relative gap width of one and Kimura (K80) as the genetic distance. GMYC requires a fully resolved ultrametric tree as input. The tree-based methods employ a coalescent framework to independently identify evolving lineages without gene flow, each representing a putative species [162]. They can be performed using a single marker and are used to establish a threshold that identifies the separation of intraspecific population substructure from interspecific divergence, and therefore identifies those groups that may be candidate species [163]. The last

species delimitation approach was implemented using a Poisson tree process (PTP), which models the speciation using the number of substitutions to infer putative species boundaries on a given phylogenetic input tree [161]. It assumes that the number of substitutions between species is significantly higher than the number of substitutions within species [161]. Here, we used the Bayesian implementation of the Poisson tree processes model (bPTP) [161], which uses a phylogenetic tree and is based on the phylogenetic species concept. The ML tree was used as input. The bPTP analysis (species.h-its.org/ptp) was applied using 500,000 generations of Markov chain Monte Carlo, a thinning of 100, and a burn-in of 25%. The outgroup (*Acodontaster conspicuus*) was removed in all delimitation analysis.

2.3 Molecular data gathering

To add resolution to our analysis we searched the GenBank and BOLD public sequence database records of *Odontaster* COI sequences from the Ross Sea area to perform a review on all existing classified specimens. The BOLD database regularly synchronizes with GenBank, and there is significant duplication with GenBank records. These duplicated records contain GenBank Accession Numbers, which were checked against the GenBank downloaded entries and removed or added as necessary. GenBank records were given priority over BOLD records because, according to the BOLD handbook (https://v3.boldsystems.org/index.php/resources/handbook, accessed on 8 February 2022), all BOLD records are eventually submitted to GenBank. Any records unique to BOLD should therefore eventually be included in GenBank and would then be removed as duplicates. After downloading respective GenBank and BOLD data, duplicated records from BOLD and GenBank were resolved (keeping the GenBank version in cases of duplication). We also decided to include the COI sequences from Janosik *et al.* [21], [125] (GenBank accession numbers: GQ294339- GQ294396) to ensure we had enough representative sequences from each *Odontaster* species identified. All the data retrieved were combined and we ran the molecular

analyses with the same settings.

2.4 Literature review

We searched the published scientific literature using two techniques: (i) searches in online databases (Wiley Interscience, Sciencedirect and ISI Web of Knowledge, last search 22 February 2022), and (ii) manual searches in specific journals. For the first technique we searched each database using the terms combinations: '*Odontaster*' AND 'Ross Sea' and '*Odontaster*' AND 'Terra Nova Bay'. We searched for these terms under 'full text/abstract' in Wiley Interscience, 'abstract, title, keywords' in ScienceDirect and 'topic' in ISI Web of Knowledge, which includes title, abstract, author keywords, and keywords plus®. In the second technique, we conducted searches using online journal home pages (PlosOne, Antarctic Science, Polar Biology, Marine Ecology Progress Series, Nature, Marine Biology, Deep Sea Research, Frontiers in Marine Science, Hydrobiology and Ross Sea Ecology). The papers we identified through this literature search were included for subsequent analyses, but only if they were peer-reviewed and reported on actual *Odontaster* samples from our study area. Therefore, studies documenting other organisms and comparing them to *Odontaster* from Ross Sea or TNB were not included in our study.

3 Results

A total of 40 specimens were analyzed in the current study and all were correctly sequenced to obtain a final COI sequence length of 628 bp. Of the 40 sequences generated in this study, 17 belonged to *Odontaster* roseus, 16 to *Odontaster* validus, and 7 to *Odontaster pearsei* (Supplementary File S1 and S2). The maximum likelihood and Bayesian analysis results are consistent and reveal three distinct groups corresponding to recognized species of *Odontaster* (Figure 2). Clade I (posterior probability 94.7% ML and value of 1.00 in Bayesian)

comprised individuals of *O. roseus*, Clade II (posterior probability 99% ML and value of 1.00 in Bayesian) comprised *O. pearsei* individuals, whereas *O. validus* individuals were included in Clade III (posterior probability 99.9% ML and value of 1.00 in Bayesian). In our samples, no corresponding sequence matched *O. meridionalis*, a species previously reported from Terra Nova Bay water [63].

3.1 Species delimitation methods

All sequences were barcode-compliant (Table 3) and received a barcode index number (BIN), which aided species delimitation [67]. The other species delimitation methods recovered the same number of secondary species hypotheses, or SSH (Figure 2, Supplementary File S1) three SSH in the total dataset when using ABGD; three SSH using GMYC, and three SSH using bPTP.

BOLD BIN	Species	*n*	Sample Vouchers
AAE2388	*Odontaster roseus* Janosik & Halanych, 2010	17	MNA-02814, 03791, 03812, 03832, 03841, 05817, 06486, 06490, 08024, 08025, 08027, 08028, 08033, 08036, 08037, 08038, 08043
AAK3286	*Odontaster validus* Koehler, 1906	16	MNA-02902, 03430, 03582, 03825, 04276, 04282, 04283, 05430, 06331, 08021, 08022, 08026, 08029, 08030, 08031, 08035
AAO2072	*Odontaster pearsei* Janosik & Halanych, 2010	7	MNA-06116, 06489, 08023, 08032, 08034, 08039, 08042

Table 3 - Samples species partition and associated BOLD BIN. Abbreviations: barcode index number (BIN); number of samples (n).

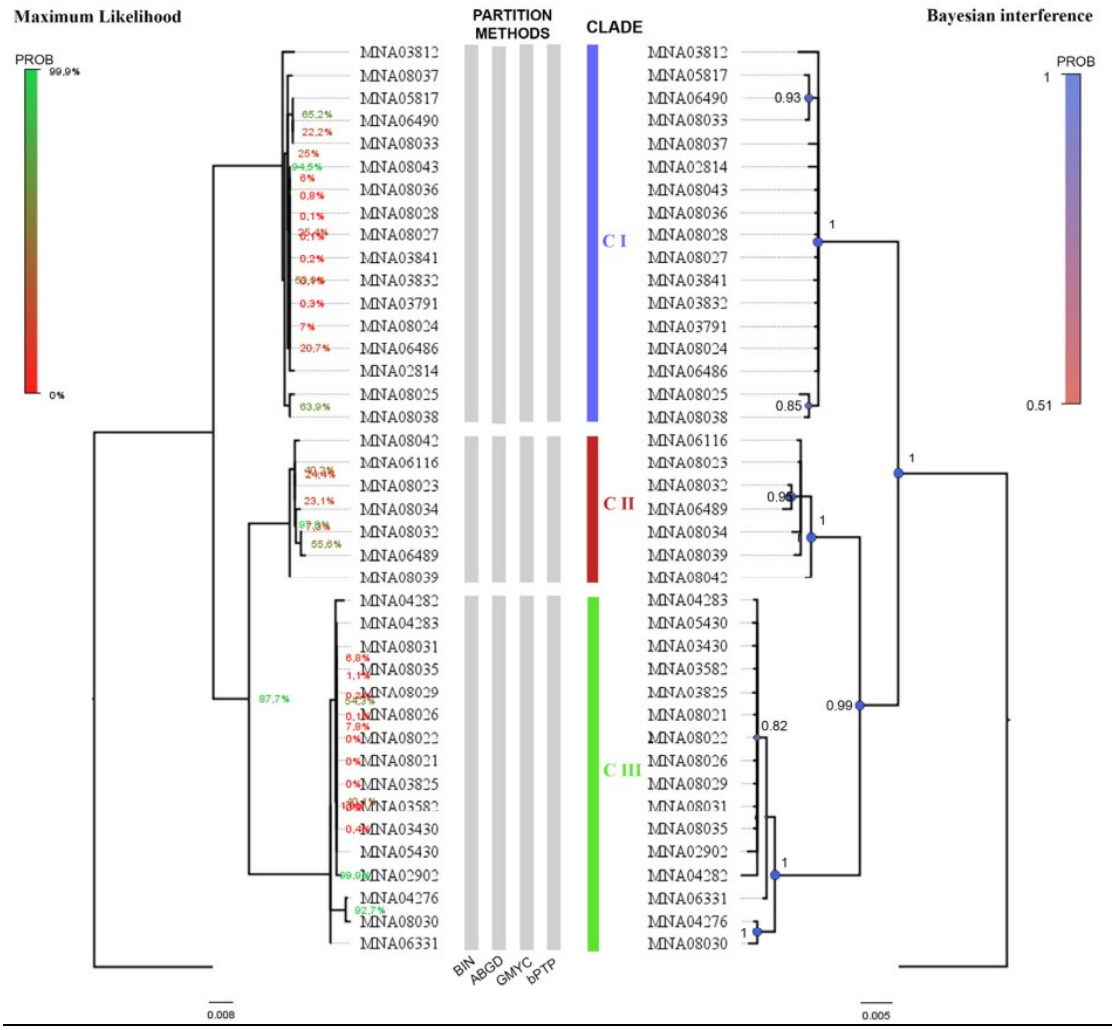


Figure 2 - Tree topology comparison of maximum likelihood (left) and Bayesian interference (right). Posterior probability node values are showed on the tree with corresponding legend for each analysis. BIN: barcode index number; BOLD: automatic species delimitation; ABGD: results from automatic barcode gap discovery method; GMYC: species delimitation from generalized mixed Yule coalescent method; bPTP: species delimitation using Bayesian Poisson tree processes method. Clade I (C I) in the figure corresponds to *O. roseus* Janosik & Halanych, 2010; Clade II (C II) corresponds to *O. pearsei* Janosik & Halanych, 2010; and Clade III (C III) to *O. validus* Koehler, 1906

3.2 Sequences database review

A total of 105 COI sequences (65 obtained from online data repository and 40 obtained by the current work) were combined in a single dataset and analyzed. Tree topology was inferred using maximum likelihood and Bayesian inference

(ML tree available in Figure 3). Species delimitation methods highlighted seven different clades, corresponding to *O. validus*, *O. roseus* (Clade I and II), *O. pearsei*, *O. penicillatus*, and *O. meridionalis* (Clades V and VI, we kept the Janosik *et al.* [35] nomenclature in Supplementary File S2). In Janosik *et al.* [125], Figure 3, GQ294370.1 (Sample ID "As 60") corresponds to *O. penicillatus* (Philippi, 1870) (Clade II) and GQ294363.1 (ID "As37") belongs to *O. meridionalis* (Clade V); in our results, the species identification is inverted (sequence GQ294370.1—Sample ID "As 60" and GQ294363.1—ID "As37") and these are highlighted in red in the tree (Figure 3).

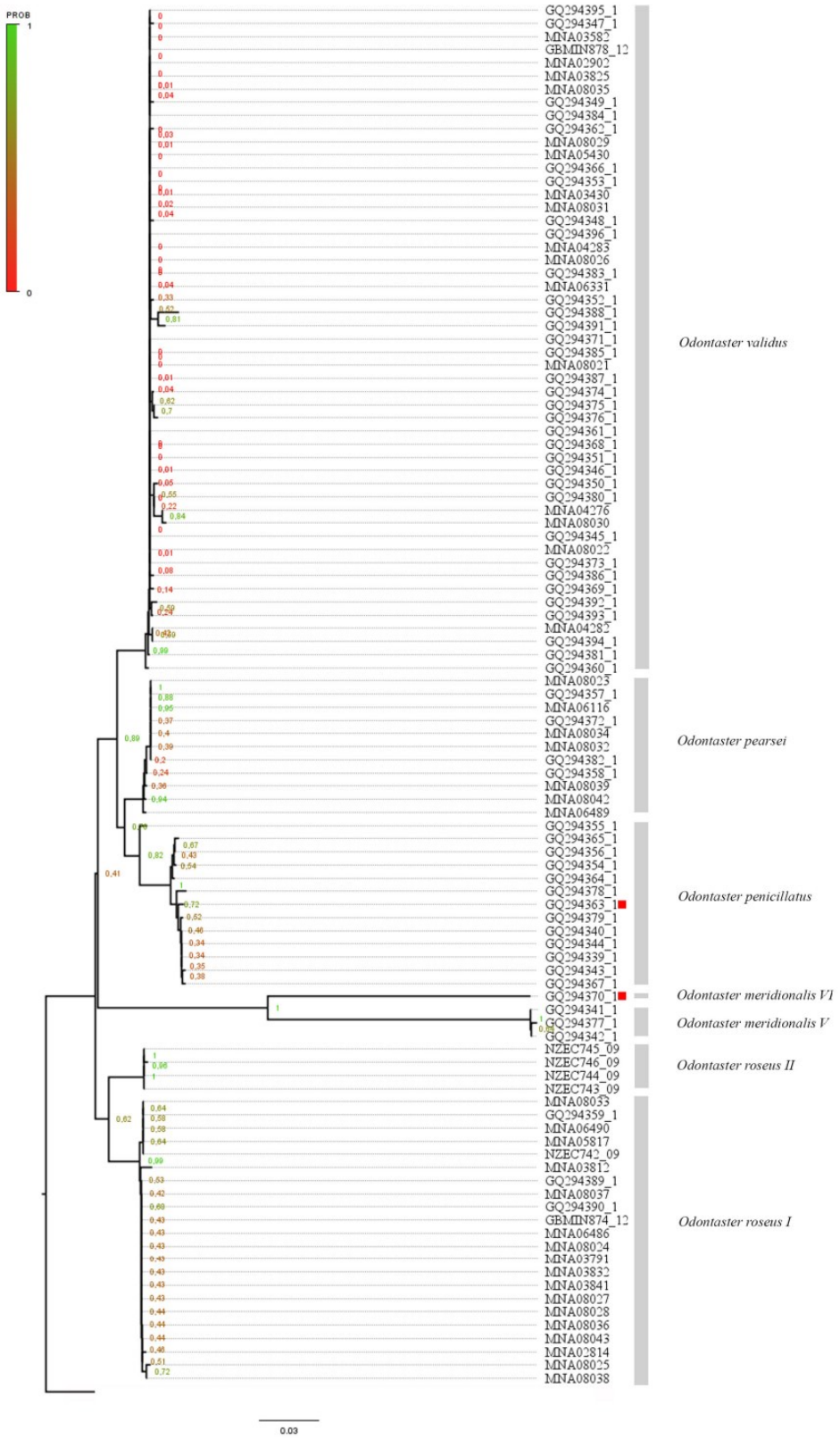


Figure 3 - Maximum likelihood (ML) tree topology of the 105 COI sequences from the *Odontaster* genus. Posterior probability node values is shown on the tree with corresponding legend. Species names are reported near corresponding clades. Sequences GQ294370.1 and

GQ294363.1 (from Janosik *et al.* [125]) are highlighted in red.

From the 65 sequences obtained from online data repository, 13 sequences of *Odontaster* from the Ross Sea area were retrieved (Table 4). Samples "As 33"," As 34", "As 69", "As 70", "As 71", and "As 72" from Janosik *et al.* [125] were not included in the analysis because the sequences were not available in a public database repository. Overall, the analysis (Table 4) highlights many discrepancies between the sequence identification available in the online information systems and the results of our study. From BOLD, four samples from outside the Ross Sea (sample ID: 38186, 38512-1, 38719-1, and 38719-2) reported as *O. meridionalis* define a new clade with affinity for *O. roseus* (here denominated as *O. roseus* II for clarity). One sample from the Ross Sea was identified by us as *O. roseus* but is also incorrectly reported as *O. meridionalis* in BOLD (sample ID: 36438). Of the two *Odontaster* samples reported in Heimeier *et al.* [164] available from BOLD and GenBank, one is correctly identified as *O. validus* (sample ID: A04N.08); the other one is labeled *O. meridionalis*, but we determined that it belongs to *O. roseus* I (sample ID: A02.15T). Our molecular results and current taxonomical identifications are reported in Supplementary File S3. Sequence identification of samples belonging to *O. meridionalis* (Clade V and VI) in Janosik *et al.* [125] remain under investigation. Moreau [101] findings suggested that the sequenced specimens might even belong to another family due to the large (COI) genetic distances involved. Such mismatches between morphological and molecular identifications, however, are a frequent outcome in DNA barcoding.

3.3 Morphological analysis

Following the "*reverse taxonomy*" approach, morphological analyses were conducted for a re-examination of our molecular results on available specimens. The first feature we focused on was life coloration (Figure 4), using pictures of live specimens taken during expeditions. Organisms included in Clades I and II

(corresponding to *O. roseus* and *O. pearsei*) presented a yellow or orange coloration. The yellow coloration of *O. roseus* in our samples differs to the original species description in Janosik and Halanych [21], in which the color, defined as rosy to drab red and tan, of their samples determined the choice of the descriptor "roseus" for the species name. Sample voucher MNA-08042 corresponds to a juvenile organism of *O. pearsei* and presented as pale-yellow coloration, which was slightly different from adults (Figure 4). Clade III, corresponding to *O. validus*, included two different colorations of morphotypes. Some specimens were characterized by the typical dark pink/red color (e.g., MNA-03825, Figure 4) and others had an orange coloration (e.g., MNA-02902, Figures 4 and 5). The co-occurrence in the same areas as species with the same coloration makes rapid identification very difficult, especially during diving or ROV sampling operations (Figure 5). The second step of our morphological analysis focused on skeletal features, such as accessory structures and spines. We based our morphological analysis on the published descriptions and keys from Fisher [150] and Clark [144], with the addition of the unique characters highlighted by Janosik and Halanych [21], who suggest focusing on the number and length of paxillar spines, as well as differences in marginal plates and marginal spines to discriminate *O. roseus* and *O. pearsei* (Figure 6). The main morphological features used to identify species from the original description [21] are as follows: *O. validus* Koehler, 1906: radial paxillae with about a dozen spinelets that are smooth, slender, and tapering; five actinal plate chevrons; actinal plates with up to seven similar, slender spinelets that are even from base to tip; two to three furrow spines. *O. roseus* Janosik & Halanych, 2010: abactinal plates with distinct tabulum crowned with truncate paxillae, comprising 10–12 spinelets per plate; four complete actinal plate chevrons; actinal plates with spines of different lengths (8–10), specifically with one prominent spine in the middle. *O. pearsei* Janosik & Halanych, 2010: abactinal plates with distinct tabulum crowned with truncate paxillae, comprising 16–20 spinelets per plate; three complete actinal plate chevrons; actinal plate with slender tapering (from tip to base) spines of equal length (5 to 8).

Sample ID	Sequence Code BOLD	Sequence Code GenBank	Mined from	Wrong ID	Correct ID	Year	Location	BOLD BIN	Published
36438	NZEC742-09		BOLD	*O. meridionalis*	*O. roseus I*	2008	Ross Sea	BOLD:AAE2388	
38186	NZEC743-09		BOLD	*O. meridionalis*	*O. roseus II*	2008	Out Ross	BOLD:AAE2389	
38512-1	NZEC744-09		BOLD	*O. meridionalis*	*O. roseus II*	2008	Out Ross	BOLD:AAE2389	
38719-1	NZEC745-09		BOLD	*O. meridionalis*	*O. roseus II*	2008	Out Ross	BOLD:AAE2389	
38719-2	NZEC746-09		BOLD	*O. meridionalis*	*O. roseus II*	2008	Out Ross	BOLD:AAE2389	
A02.15T	GBMIN874-12	GU227088.1	GenBank	*O. meridionalis*	*O. roseus I*	2002	McMurdo Sound	BOLD:AAE2388	Heimeier et al., 2010
A04N.08	GBMIN878-12	GU227092.1	GenBank		*O. validus*	2004	Cape Hallett	BOLD:AAK3286	Heimeier et al., 2010
As 68		GQ294374.1	GenBank		*O. validus*	2011	Ross Sea		Janosick et al., 2011
As 86		GQ294384.1	GenBank		*O. validus*	2011	Ross Sea		Janosick et al., 2011
As 87		GQ294385.1	GenBank		*O. validus*	2011	Ross Sea		Janosick et al., 2011
As 88		GQ294386.1	GenBank		*O. validus*	2011	Ross Sea		Janosick et al., 2011
As 33, 34, 69, 70, 71,72					*O. validus*	2011	Ross Sea		Janosick et al., 2011
MNA-3582	TCTNB082-15	MK811555	GenBank		*O. validus*	2019	Terra Nova Bay	BOLD:AAK3286	Rossi et al., 2019
MNA-4276	TCTNB079-15	MK811610	GenBank		*O. validus*	2019	Terra Nova Bay	BOLD:AAK3286	Rossi et al., 2019

Table 4 - A list of all the *Odontaster* sequences from the Ross Sea area available from online databases. Samples ID As 33, 34, 69, 70, 71, and 72 from Janosik *et al.* [35] are listed in the paper but sequences were not available in GenBank.

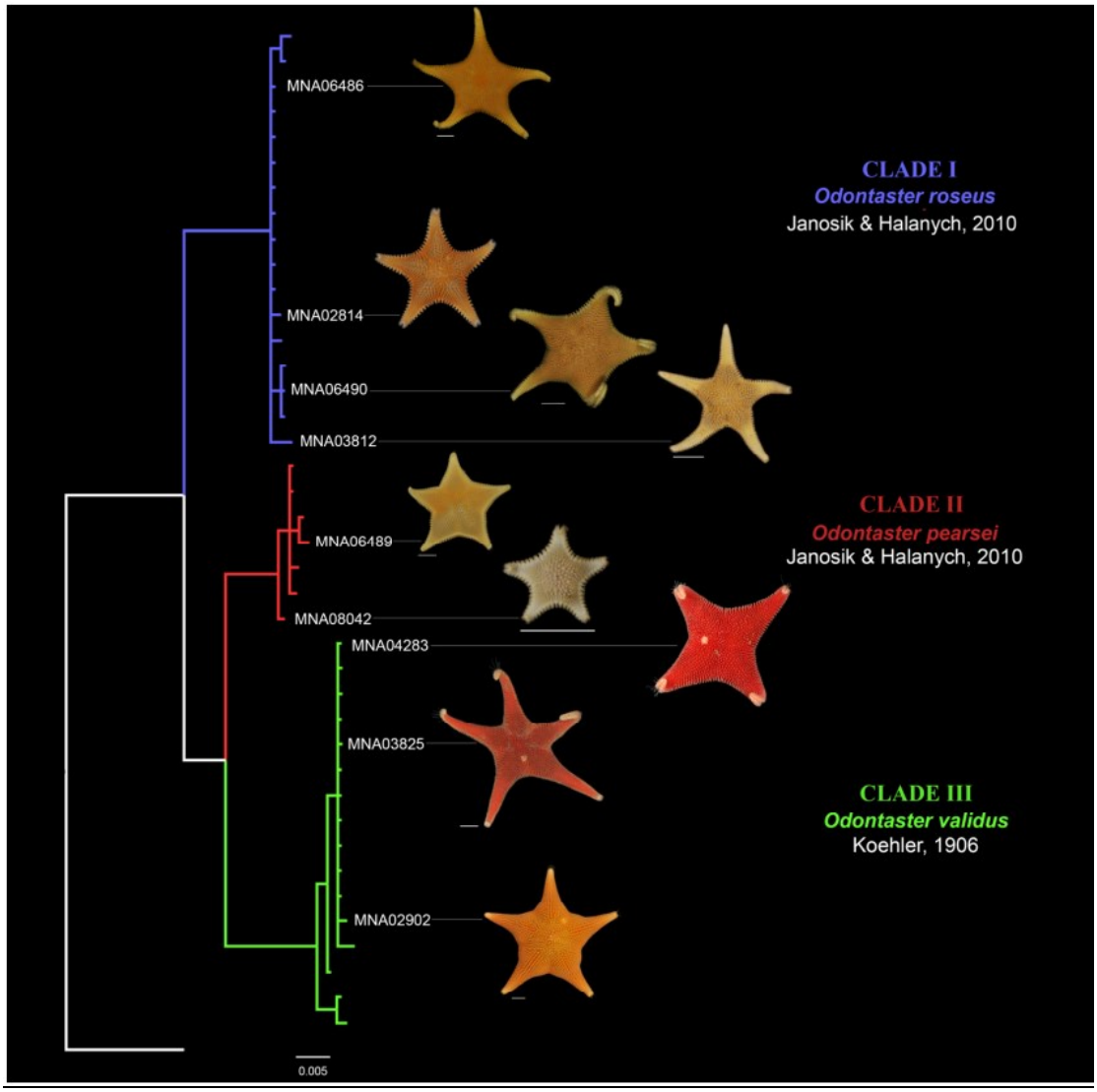


Figure 4 - *Odontaster* morphology variability of selected specimens. In the tree highlighted in blue: *O. roseus* Janosik & Halanych, 2010, characterized by an orange coloration; red: *O. pearsei* Janosik & Halanych, 2010, characterized by an orange coloration; green: *O. validus* Koehler, 1906, with dark pink/red or orange coloration. Scale bar: 1 cm in grey.

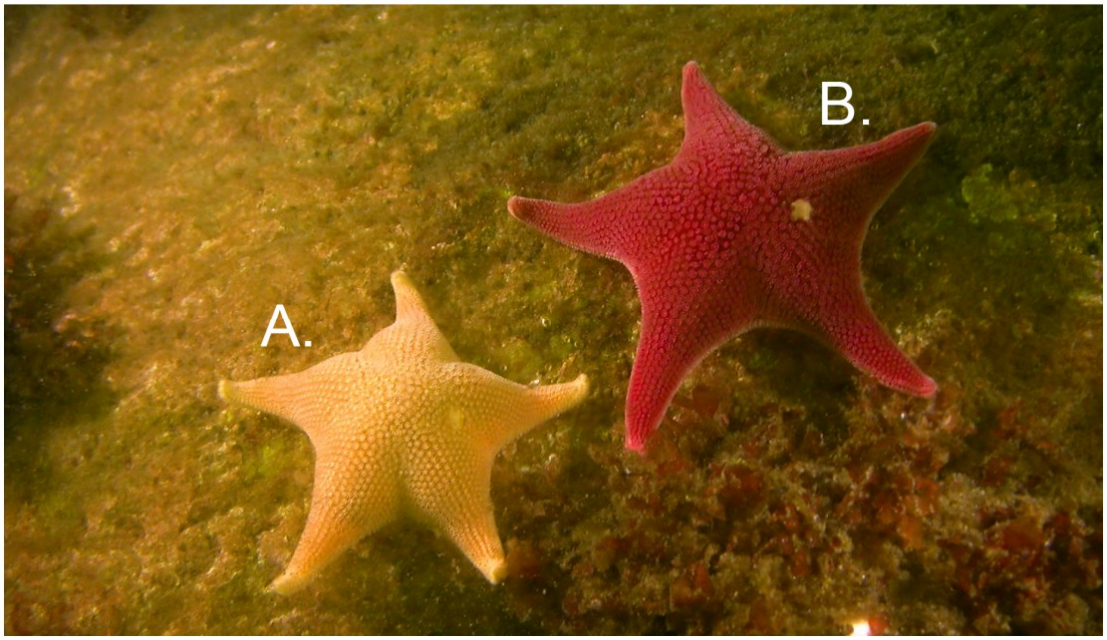


Figure 5 -Two specimens of *Odontaster validus* (A, B) photographed by Stefano Schiaparelli during a dive in Road Bay (Terra Nova Bay area) at ~20 m depth. The orange yellow specimen ((A) in figure) corresponds to the sequenced MNA-02902 (also in Figure 4).

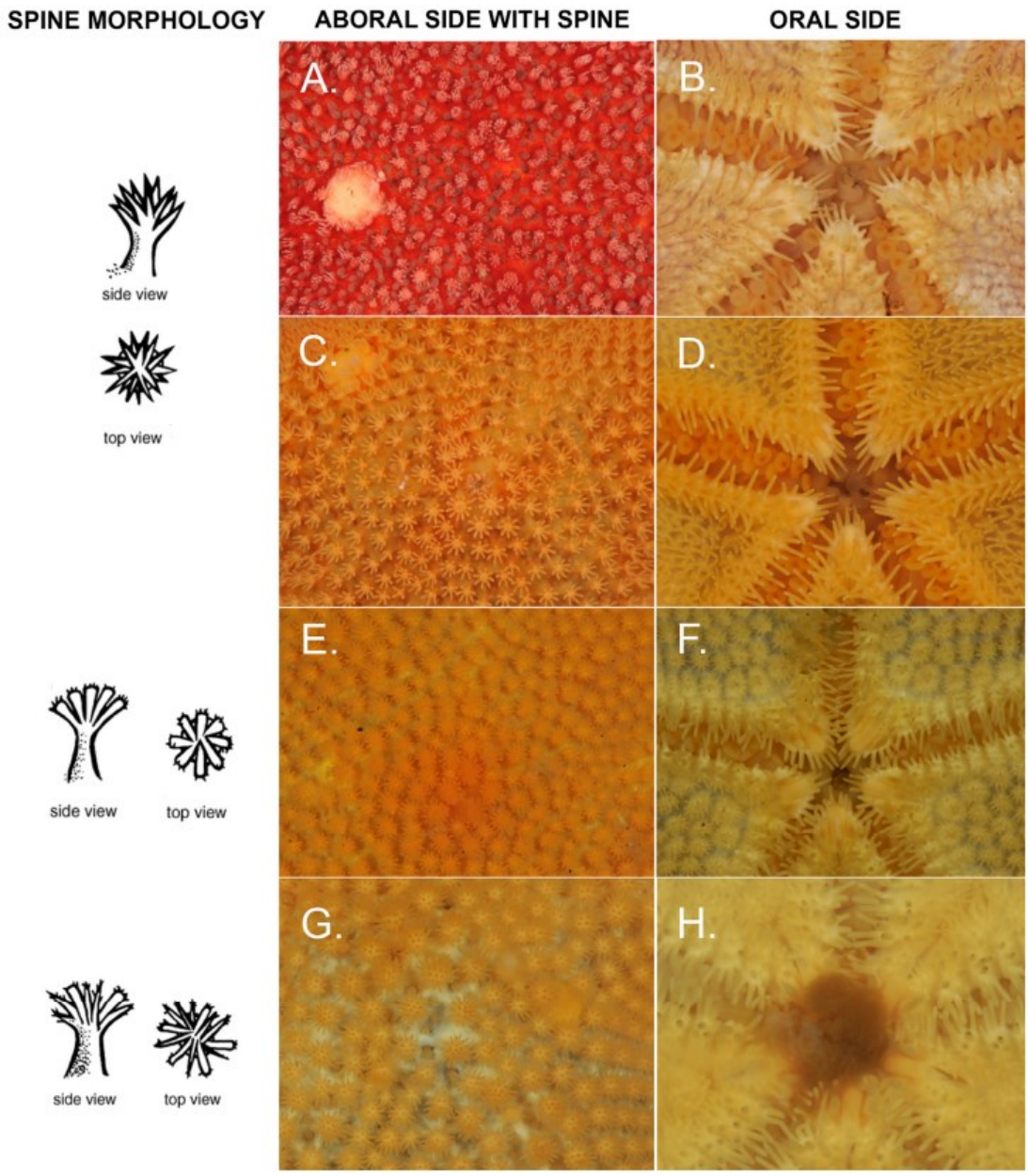


Figure 6 - Photographic details of aboral side with spine on paxillae and oral close up, respectively: (A,B) *O. validus* red morphotype; (C,D) *O. validus* orange morphotype; (E,F) *O. roseus*; (G,H) *O. pearsei*. Drawings of peculiar spine morphology for each species from Janosik & Halanych [21].

The results of the external skeletal structures analysis of our samples were congruent with the species description and in agreement with the species partition resulting from the molecular analyses based on COI (Figure 2). This finding makes the occurrence in the TNB area of the three species robust. As suggested in a previous paper [21], the two species *O. roseus* and *O. pearsei*,

reported for the first time in the Ross Sea with this work, should not be considered cryptic but merely unrecognized biodiversity that escaped identification until now.

3.4 Scientific literature revision of Odontaster in the Ross Sea quadrant

We identified 93 articles that referred to the Ross Sea (Figure 7) that included 43 publications specifically mentioning the Terra Nova Bay area (Figure 8). All these papers were classified according to the main topic treated in the paper. We recognize that there is a possibility that some works, particularly those in the "grey literature", may not have been detected by the research methods we used for this article and, therefore, may not have been included in our review.

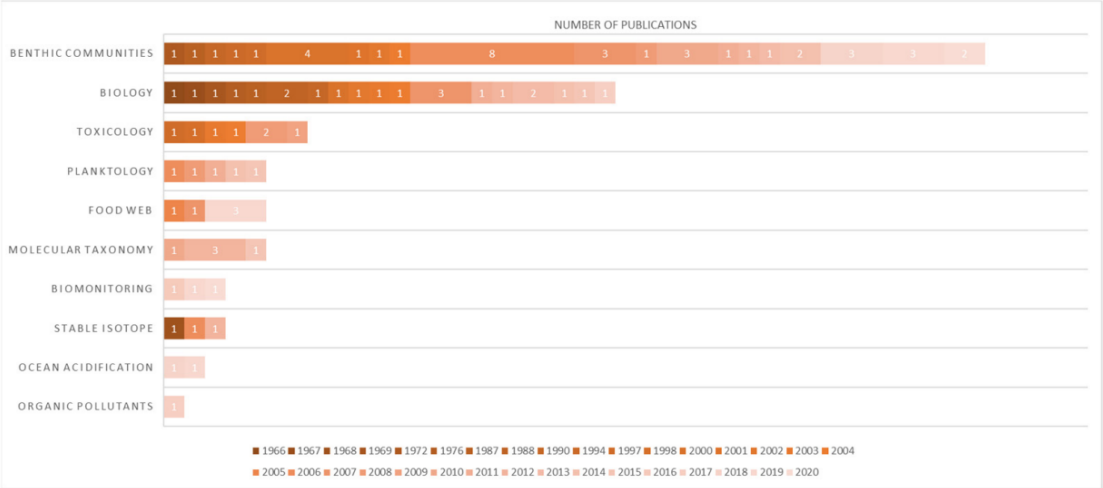


Figure 7 - Graphical ranking representation of the 93 publications analyzed for this work for the Ross Sea. Each paper was classified into a general category. The publications are color-coded based on the year of publication (which runs from 1966 to 2020). The data refer to available literature in February 2022.

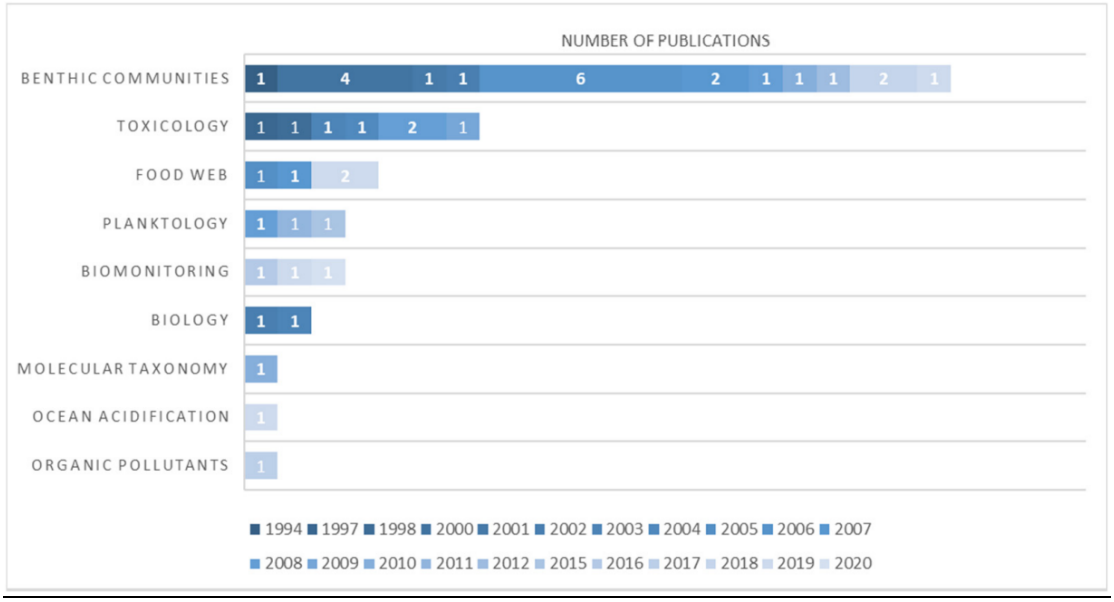


Figure 8 - Graphical ranking representation of the 43 publications analyzed for this work in Terra Nova Bay area. Each paper was classified into a general category. The publications are color-coded based on the year of publication (which runs from 1972 to 2020). The data refer to available literature in February 2022.

Modern scientific investigations of the Ross Sea were initiated during the International Geophysical Year of 1957 and continue today. As a result, the Ross Sea is now one of the most intensively studied regions in the Southern Ocean. Ross Sea studies have greatly benefited from the presence of the McMurdo Station, located adjacent to McMurdo Sound on Ross Island. Research activities are also carried out by the Italian Mario Zucchelli research base, located in Terra Nova Bay 280 km to the north, and at the Scott base, managed by New Zealand [165]. Many publications have followed over the years, and *Odontaster* has certainly represented a frequent subject of study confirmed by the 93 publications we found (Figure 7). In detail: 40 works were identified of benthic communities, 22 of biology, 5, respectively, of planktology, food webs, and molecular taxonomy. Minor contributions were found on biomonitoring and isotopes (3 each), ocean acidification (2), and organic pollutants (1). However, despite these numerous studies and various fields of research, the two lineages of *O. roseus* and *O. pearsei* went unnoticed until now. Most of the works conducted on *Odontaster* in the TNB area were, as expected, focused on the characterization of benthic communities (21), followed by toxicology (7) and

food web (4), with the remaining being planktology and biomonitoring (3), biology (2), molecular taxonomy (1), ocean acidification (1), and organic pollutants (1) (Figure 8). Many scientific contributions highlight the important role which *O. validus* has in the sublittoral ecosystem (e.g., [143]) and the local abundance of this species in the area [132], [166]. Only two papers out of the 43 analyzed applied a molecular approach to determine species identification. Heimeier *et al.* [164] used a combination of different markers (16 s, 18 s, and COI) to identify invertebrate larvae. They reported only the presence of *O. validus* in Cape Hallet and *O. meridionalis* in the Ross Sea. Our analysis of their *O. meridionalis* sequences, however, shows erroneous identification of that specimen (GenBank accession number: GU227088.1), which we found to belong to *O. roseus* (Table 4). The other work was from Rossi *et al.* (2019) [167], which focused on food web structure in TNB ecosystems. In this paper, COI sequences were used to crosscheck morphological determinations. Here, two *O. validus* (GenBank accession numbers: MK811555, MK811610) and one *Odontaster* sp. (MNA-04290, Annex 1) were reported, but the latter was not characterized at the molecular level. In more recent years, two other studies focusing on food web complexity in Terra Nova Bay were published by Signa *et al.* [133] and Caputi *et al.* [168]. In these papers, the species *O. meridionalis* and *O. validus* are considered as key players of benthic food webs, by being apex predators (e.g., Figure 4 in Caputi *et al.* [168]). Here, however, the lack of knowledge of true biodiversity in the area and the absence of molecular identifications led to the incorrect assumption that a "yellow *Odontaster*" is automatically an *O. meridionalis*, perpetuating the misidentifications of this species in the area. The lack of molecular data and/or museum vouchers for these specimens prevents correct determination of which one of the "yellow *Odontaster*" was involved.

4 Discussion

Although many studies have attempted to estimate biodiversity in the Southern Ocean, answering this question is not straightforward. In the present research, the biodiversity of the genus *Odontaster* in the Terra Nova Bay area (Ross Sea)

was investigated in detail. Notably, our work has demonstrated that biodiversity knowledge could be considerably underestimated even in well-studied Antarctic areas and for iconic species. Although sea stars of the genus *Odontaster* are among the most frequently studied organisms in the Antarctic, two previously unrecognized species are reported for the first time from the Terra Nova Bay area (Ross Sea). This study complements the taxonomic and DNA barcoding effort of the Southern Ocean and highlights the necessity of revision even in the case of iconic and common organism. There is considerable scientific literature reporting the presence of *O. validus* and *O. meridionalis* in the Ross Sea. However, the famous proverb "not all that glitters is gold" seems to describe very well the current situation, where "yellow *Odontaster*" were automatically assigned to *O. meridionalis*. The new taxonomic evidence and the revision of public molecular databases showed several incorrect identifications for this genus in the literature. Especially in the shallow waters of Terra Nova Bay and McMurdo, where scientific activity has been intense, the presence of *O. validus* and *O. meridionalis* is widely reported, and they have been the subject of numerous scientific studies and experiments (Figures 7 and 8; Supplementary Files S4 and S5). The identification of these specimens was mostly undertaken using only morphological traits, and the few molecular data show identification errors deriving from morphological recognition. Organism coloration was considered a sufficient trait for species recognition and it is possible that the existence of the well-known "McMurdo identification guide" (http://www.peterbrueggeman.com/nsf/fguide/echinodermata.pdf, accessed on 8 February 2022) [169], widely used in the field especially by research parties working the Ross Sea area, could perhaps represent a common source of these problems. On the other hand, other field guides report only these two species also for the Weddell quadrant of the Southern Ocean (e.g., [170]). Thanks to the scientific contribution of Janosik & Halanych [21] on *Odontaster* from the Antarctic Peninsula, the existence of unrecognized biodiversity even in well-known areas and of iconic widely studied organisms has been brought to light. With our work, based on integrated molecular and morphological data, the presence of *O. validus* has been confirmed in TNB, and we report for the first time

the species *O. roseus* and *O. pearsei*. These species, "as expected", were misidentified until very recently as *O. meridionalis*. In addition, we also report the existence of another "confounding factor", i.e., the presence of orange–yellow morphs of *O. validus*. The data presented here also demonstrate the existence of a yellow morphotype of *O. roseus* that differs from the rosy to drab red and tan coloration in the original species description. These three "yellow" sea star species live sympatrically and thus life coloration is a truly misleading character when "yellow morphs" have to be determined. Correct identifications of *O. roseus* and *O. pearsei* can be easily achieved by using DNA barcoding and skeletal features, especially the number of spines on abactinal plates and spine length, as well as differences in marginal plates and marginal spines. Although in our case the use of morphological traits has made it possible to distinguish the species, particular caution should be employed when the identification of species depends on the morphological characteristics commonly proposed. As reported in the literature [150], [171], different morphological features used to separate species of the genus *Odontaster* in Antarctica are highly variable and sufficiently variable to make them, at best, poor indicators of species-level differences in this genus. Identification is, of course, possible for preserved specimens, whereas the determination of species in ROV images is simply not achievable. This highlights the irreplaceable role and resource of museums as biological specimen repositories and the relevance of their constant effort in curation of preserved specimens. So far, based on our new data and on a thorough check of available COI sequence data available in GenBank, there is no molecular or morphological evidence to sustain the presence of *O. meridionalis* in the Ross Sea. However, the availability of molecular data for the area is still limited and further investigations, especially of offshore "yellow morphs", are necessary. Implementation of analysis of morphological traits and the increasing availability of molecular tools will improve identification of this species to be easier, faster, and more reliable in the future. The revision of the morphological identification is not the only urgent action required to update the scientific information: with the review of the molecular data available online, we observed some incorrect classifications in BOLD and GenBank public databases that will need to be

amended in the future. MOTUs correct taxonomic identification and the use of public sequence databases as exploration tools to evaluate taxonomic identification, the specificity, and robustness of the identification query (to species level or higher taxon) strongly depend on the related reference sequences available. The possibility of misleading identification carried out could have led to erroneous information flow into other science fields with inaccuracies that would persist in the scientific literature. A joint action of revision is fundamental for understanding the current level of diversity, speciation events of the past, and for implementing actions aimed at the conservation of these ecosystems and the species that occupy them. All this information is really important in the study area and in the future monitoring activities that are requested by the conservation measures of Annex 91-05/C [28] of the Ross Sea Marine Protected Area.

The current paper represents a further contribution of the Italian National Antarctic Museum (MNA)—Genoa section, as custodian of biodiversity data for the Ross Sea area. Many contributions to the Antarctic Biodiversity Portal have been published by MNA over the years, with the aim of increasing the knowledge of the area [32], [34]–[37], [41], [42] (http://www.biodiversity.aq, accessed on 8 February 2022). It is desirable that in the next years, all available museum collections will be subject to molecular identifications in order to precisely determine species ranges and occurrences, key data for all monitoring activities.

CHAPTER 4

New Records of echinoids and crinoids from Terra Nova Bay (Ross Sea) based on a reverse taxonomy approach

1 Introduction

The increasing application of integrated taxonomy, coupled with new modeling approaches, requires data to be findable, accessible, interoperable, and reusable in the long term [172]. There is a need to revise the geographic distribution and taxonomic description of many taxa, as it can provide information about changes in the composition of communities in different environments, particularly in sensitive ecosystems such as the polar ones[173].

The most common challenges facing studies or the construction of biodiversity inventories are accurate species identification and the absence of detailed information on the distribution of taxa throughout the different geographical regions of the planet [174]. Morphologic-based identification is the classical approach to taxonomy and is strongly dependent on the level of experience and expertise of the identifier, largely prone to mistakes whenever intraspecific variability has not been previously tested. Furthermore, identifying specimens rapidly in the field is only possible when clear discriminant morphological characters are known. However, the increase in molecular advances has made it evident that this approach comes with some inherent limitations [175]. Taxonomic discrepancies, such as synonymous or cryptic species, are extremely common when a traditional taxonomic approach is used. Neither molecular nor morphological taxonomic methods are sufficient on their own [24] and the number of integrative approach examples to identify species is rapidly increasing (sea stars (e.g. [19], [176]–[181]), brittle stars (e.g. [182]), holothurians (e.g. [20]), fish (e.g. [183])and many more.

With the rapid accumulation of samples in museums and the co-occurring decline of taxonomic expertise in recent years [184], phylogenetics, and coalescent-based analyses have become key tools for species identification or discrimination. In recent years, molecular tools and DNA barcoding in particular

have provided a useful method for fast, efficient, and reliable species identification and discovery [19], [185], [186]. It is based on the concept that intraspecific diversity for the cytochrome c oxidase subunit I (COI) gene is lower than interspecific diversity. The resulting difference is called a "barcode gap" [185]. DNA barcoding not only shortcuts the difficulties of a morphology-based identification, e.g. when diagnostic characters are damaged during collection, but also connects the different stages of animal development [187]. A 658-bp region of COI gene is thus largely used as an effective marker to pinpoint species delimitation boundaries in different groups of marine organisms [15], [18], [19], [182], [188]–[191].

Since the establishment of the Italian research station "Mario Zucchelli", macrobenthic fauna of Terra Nova Bay (TNB) has been widely investigated, with many ecological studies conducted on some of the most conspicuous species of echinoderms, mainly asteroids ([192], [193]). With the introduction in 2016 of the Ross Sea region Marine Protected Area by the RSMPA monitoring plan (CCAMLR Conservation Measure 91-05: Ross Sea Region Marine Protected Area. 2016 [28]) and the inclusion of TNB area in the AMP the accurate description of the benthic communities, already extremely important, has become a priority, with a view to implementing monitoring and conservation plans for species.

However, to date a complete faunistic inventory for echinoderms is still lacking even when continued research has been held in the area. Specifically, the Terra Nova Bay echinoid species inventory was assessed for the first time by Chiantore *et al.*, 2006 [194] with the implementation of morphological taxonomy and report 4 species for the area.

Echinoids are typically large, obvious and fairly easily identifiable animals and are a conspicuous and important element of many marine benthic communities (*e.g*. [195]–[197]) for which a comprehensive database and identification guide to Southern Ocean species has been established [49]. They exploit a wide array of marine habitats, from the poles to the equator and from the intertidal zone to the deep sea, although they achieve greatest levels of diversity and abundance in shallow shelf areas [198], [199]. The Southern Ocean Antarctic Echinoidea

database assembled by David *et al.* 2005, 2005 [49], [200], is an interactive database synthesizing the results of more than 130 years of Antarctic expeditions. It represents one of the most complete collections of information for any Antarctic taxa but still reveals major gaps in the geographic and bathymetric distributions of many species.

Crinoidea is one of the five current classes in the phylum Echinodermata distributed in all oceans. The present-day crinoids consist of two groups, feather stars and sea lilies. Feather stars are ecologically more successfully, with about 570 species occupying diverse habitats from the intertidal to the deep, and from the tropic to the polar sea. On the other hand, sea lilies, with some 80 species, live mostly at depths greater than several hundred meters. This difference is probably due to the stalkless condition in feather stars [201]. Pentacrinoid larvae of feather stars do have stalks, but they abandon the stalks during development. Few species of crinoids are reported in the Ross Sea [48] but a comprehensive list for this taxon is still not available in literature for TNB area.

The objectives of the study are: i) to update the checklist for echinoids of TNB area; ii) to evaluate the first comprehensive inventory for crinoids from the same location. To achieve this goal, we used an integrative approach based on morphological characters and DNA barcoding comparing the obtained sequences with those existing in public databases. In the specific, in the case of echinoderms, object of the present study, sequences are available for 5,147 echinoid and 4,291 crinoid specimens, representing respectively 307 and 203 species respectively on the Barcode of Life Data System, BOLD (Accessed 16 November 2022). The results will serve as a baseline for future works in ecology, monitoring and management of the study area.

2 Materials and methods

The samples available at Italian National Antarctic Museum (MNA) – Genoa section analyzed in this study derives from the Antarctic Peninsula (Figure 1. A) and the Ross Sea sector specifically the Terra Nova Bay area that is part of both

the marine protected area and the Antarctic Special Protected Area (n.161) (CIT 62) (Figure 1. B).

Specimens were collected in the framework of several recent scientific expeditions performed in the Southern Ocean and which are now permanently stored and curated at MNA – Genoa section. The Italian National Antarctic Program (PNRA) expedition "XVII" (2001/2002), "XIX" (2003/2004), "XXV" (2009/2010), "XXVII" (2011/2012), "XXVIII" (2012/2013), "XXIX" (2013/2014), "XXXII" (2016/2017) were all from the Ross Sea and additional samples collected from the Antarctic Peninsula were obtained from the Alfred Wegner Institute (AWI) ANT-XXIX/3, PS81 expedition (2013).

Table 1 - Sampling stations and data. Abbreviations: Mario Zucchelli Station (MZS); number of specimens (N).

Expedition	Station	Location	Year	Latitude	Longitude	Depth (m)	Sample Vouchers	N
AWI PS81 ANT-XXIX/3	163_9	Weddell Sea	2013	-63,79600	-56,31000	550,9	MNA-08430, MNA-08432, MNA-08433	3
	188_4	Weddell Sea	2013	-63,83933	-55,62367	427	MNA-08434	1
	196_8	Bransfield Strait	2013	-62,79667	-57,08917	580	MNA-08435, MNA-08447, MNA-08448	3
	220_2	Bransfield Strait	2013	-62,94533	-58,39383	792	MNA-08431	1
PNRA XVII Exp 01/02	ANT-D9	Tethys Bay	2002	-74,74860	164,12467	113	MNA-08457	1
	Carb 37	Mawson Bank	2002	-73,15133	174,29467	309	MNA-08451	1
PNRA XIX Exp 03/04	H out 3 bis	Cape Hallett	2004	-72,29000	170,44000	258	MNA-00573	1
	R3	Cape Russell	2004	-74,82167	164,19167	330	MNA-00577	1
	R2	Cape Russell	2004	-74,81667	164,30167	364	MNA-07947	1
PNRA XXV Exp 09/10	Dive 19	Road Bay	2010	-74,69647	164,12007	15	MNA-02885, MNA-02886	2
	DR2	Road Bay	2010	-74,70082	164,13762	148	MNA-02896, MNA-02897, MNA-04498, MNA-08490, MNA-08491, MNA-08492, MNA-08493, MNA-08494, MNA-08495, MNA-08496, MNA-08497	11
	DR4	Adelie Cove	2010	-74,76450	164,08202	100	MNA-02937, MNA-08483, MNA-08484, MNA-08485, MNA-08486, MNA-08487, MNA-08488, MNA-08489	8
PNRA XXVII Exp 11/12	DR1	Road Bay	2012	-74,69848	164,12812	100	MNA-03439, MNA-03454, MNA-04497, MNA-04499, MNA-04500, MNA-08446, MNA-08471, MNA-08472, MNA-08473, MNA-08474, MNA-08475, MNA-08476	12
	DR4	Tethys Bay	2012	-74,70010	164,03502	198	MNA-03576, MNA-03577, MNA-08462, MNA-08463, MNA-08464, MNA-08465, MNA-08466, MNA-08468, MNA-08469, MNA-08470	10
	DR9	Faraglione	2012	-74,71337	164,14903	150	MNA-03760, MNA-03766, MNA-03795, MNA-03855	4
	Mooring L	Terra Nova Bay	2012	-74,76130	164,13032	149	MNA-09159	1
PNRA XXVIII Exp 12/13	DR3	Adelie Cove	2013	-74,77468	163,95948	77	MNA-08478, MNA-08479	2
	DR4	Adelie Cove	2013	-74,77430	163,95400	78	MNA-08185	1
	DR5	Road Bay	2013	-74,70087	164,14793	150	MNA-05746, MNA-05747, MNA-05748	3
	DR6	Caletta	2013	-74,76207	164,09623	146	MNA-05905	1
	DR7	Terra Nova Bay	2013	-74,73675	164,17702	240	MNA-08480	1
	DR9	MZS	2013	-74,68090	164,21433	522	MNA-06108, MNA-08074	2
	DR10	Faraglione	2013	-74,71178	164,15802	250	MNA-10419, MNA-10574	2

	DR13	MZS	2013	#########	164,23640	525	MNA-06513, MNA-06514	2
	Mario 1	Terra Nova Bay	2013	-74,70348	164,13550	137	MNA-05676, MNA-05759, MNA-08182	3
	Mario 2	Terra Nova Bay	2013	-74,72597	164,19908	319	MNA-05755	1
	Vacchi 2	Tethys Bay	2013	-74,69677	164,18622	460	MNA-05491	1
PNRA XXIX Exp 13/14	DR3	Tethys Bay	2014	-74,69508	164,08137	60	MNA-08449, MNA-08564, MNA-08565	3
	Mario 1	Terra Nova Bay	2014	-74,70750	164,18167	242	MNA-07963, MNA-07965, MNA-07967	3
	Palamito 2	Terra Nova Bay	2014	-74,70000	164,13333	100	MNA-07964, MNA-08183, MNA-08184	3
PNRA XXXII Exp 16/17	GRC-08	Cape Hallett	2017	-71,98111	172,19383	750	MNA-09365	1

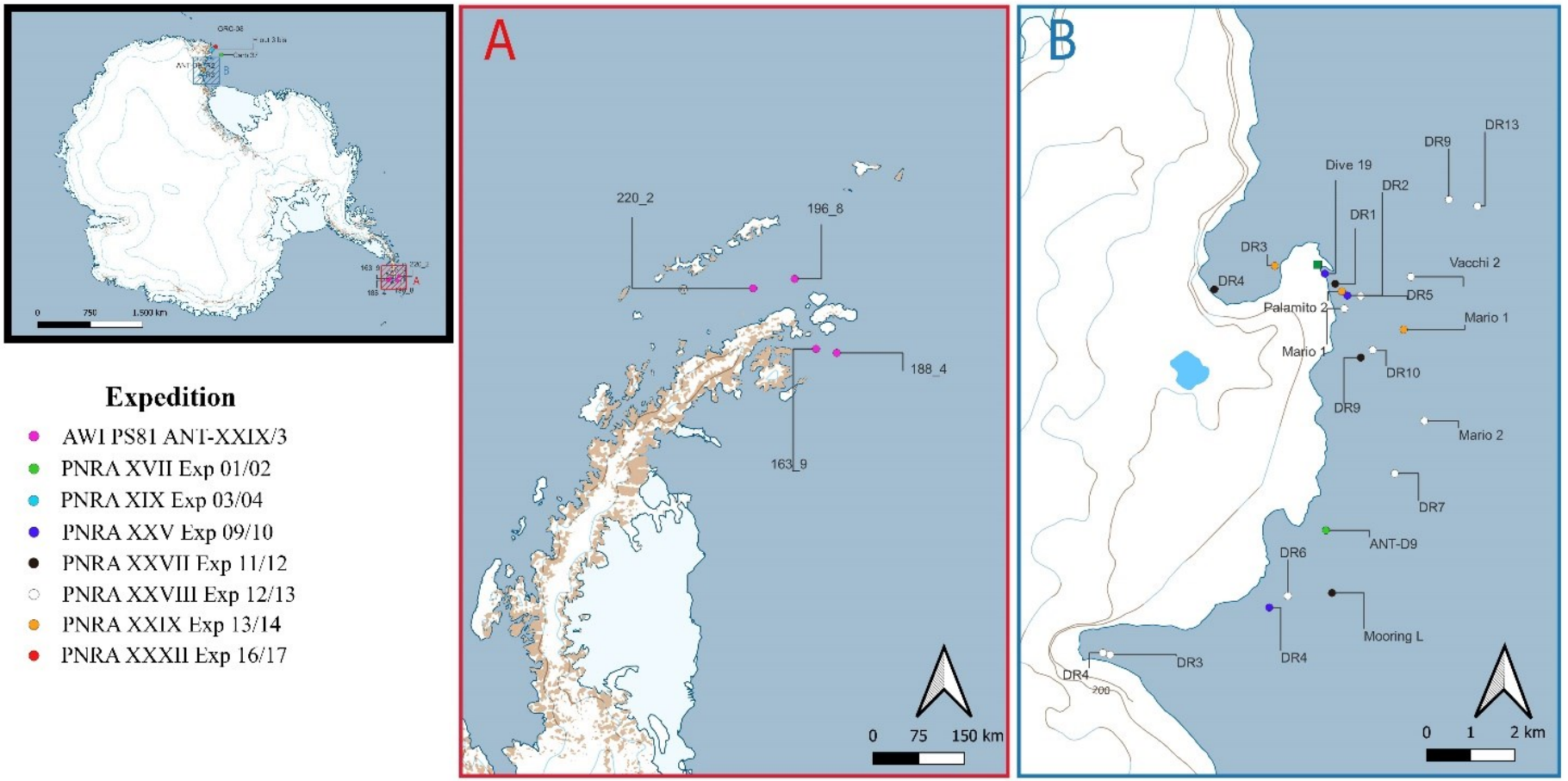


Figure 1 - Antarctica (high left) with highlighted in red the Antarctic Peninsula and in blue the Terra Nova Bay (Ross Sea) sector. (A) sampling station of the Antarctic Peninsula and (B) sampling sites in Terra Nova Bay with Mario Zucchelli Station (Italy) highlighted in green square. Legend is color coded for expedition.

2.1 Sampling and DNA extraction

A total of 92 samples, 70 belonging to echinoids and 22 to crinoids were analyzed and the distributional data considered here originated from 31 different sampling stations, ranging from 15 and 750 m of depth (Table 1). Sampling was performed through deployments of a variety of sampling gear. Benthic sampling under the Italian PNRA was mainly performed using a rectangular dredge (70 × 30 cm), Agassiz Trawl (AGT) and an unconventional set of gears for sampling benthic fauna (such as a Trammel net, Long line and a Gill net) that opportunistically collected benthic specimens due to accidental contact with the bottom during gear deployment "failures". Two samples were collected by S. Schiaparelli during SCUBA diving, performed in the framework of the PNRA "XXV" Expedition (2009/10) along the rocky cliffs Road Bay. Six pentacrinoid larvas were included in the analysis: MNA-03760; 03766; 03795 and MNA-03855 were collected in 2012 with a dredge deployment (remarkably we report that MNA-03766 was associated on a Sabellidae tube), MNA-07967 was collected in 2014 by M. Santoro with a Trammel Net and MNA-09159 in 2012 during the recovery of Mooring L by E. Paschini. All Alfred Wegner Institute (AWI) expedition samples analyzed here were collected by M.C. Alvaro in 2013 with the deployment of AGT.

After collection, samples were transferred to the laboratory and stored in ethanol (75% Et-OH) or frozen (−20 ◦C) for subsequent molecular analysis. Thereafter, samples were acquired by the MNA and included in their collections (available online at https://steu.shinyapps.io/MNA-generale/, accessed on 21 November 2022). All specimens were classified to the lowest possible taxonomical resolution [63] on a morphological basis by using the available literature and keys from Koehler (1926) [202], Clark (1967)[203], Moore (1983)[204]and Speel at al. (1983)[205]. For molecular analyses, a portion of tissue was clipped from each sample for DNA extraction and sequencing of partial cytochrome c oxidase subunit 1 (CO1). The molecular analyses were carried out at the Canadian Centre for DNA Barcoding (University of Guelph,

Guelph, ON, Canada). Sequences were uploaded to the BOLD platform (Barcode Of Life Data systems, http://www.boldsystems.org, accessed on 21 November 2022). Primers used for amplification were LCOech1aF1 or EchinoF1 and HCO2198 (Table 2).

Table 2 - List of used primers for cytochrome oxidase I (COI) amplification in our work. Forward primers (F) and reverse primer (R).

Region	Direction	Primer	Sequence (5'-3')	Reference
COI	F	LCOech1aF1	TTTTTTCTACTAAACACAAGGATATTGG	Corstorphine, 2010 [206]
		EchinoF1	TTTCAACTAATCATAAGGACATTGG	Ward *et al.*, 2008 [18]
	R	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	Folmer *et al.*, 1994 [207]

Taxonomic assignation was performed manually in the Barcode of Life database (BOLD) and National Center for Biotechnology Information (NCBI) database BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 21 November 2022) for definitive assignment. A sequence match of >98% to the reference database was considered an "exact" match [151]. Accepted taxonomic names and classification were obtained from the World Register of Marine Species (WoRMS www.marinespecies.org/, last search 21 November 2022). Chromatograms were edited in CodonCode Aligner v9.0.1 (CodonCode Corporation, Centerville, Massachusetts, USA; http://www.codoncode.com/aligner/, accessed on 21 November 2022), primers were trimmed, and the absence of stop codon in the sequences was checked with the same software. Sequences were aligned using MUSCLE, available within CodonCode Aligner, and checked by eye. *Odontaster validus* Koehler, 1906 (accession number: ON103477) was chosen as the outgroup. The model with the lowest BIC scores (Bayesian information criterion) in MEGA X [153] analysis resulted T92 + G (Tamura 3-parameter + Gamma distribution) and is considered to best describe the substitution pattern. The evolutionary history was inferred in MEGA X using the maximum likelihood (ML) method based on the Tamura 3-parameter model [154]. For completeness, a maximum parsimony (MP) tree was also produced in the software. A Bayesian phylogeny was subsequently produced using Mr Bayes [155], [156]. Based on the notion that nonparametric bootstrap frequencies for ML estimates and Bayesian

posterior probabilities for clades in phylogenetic trees are not universally equivalent [157] and the possibility of obtaining wrongly supported results with under parametrization in Bayesian inference, the generalized time reversible (GTR) model with gamma(G)-correction was used. Posterior probabilities were calculated by two independent analyses (one cold and three heated chains) using Markov chain Monte Carlo (MCMC) algorithm. Samples of trees and parameters were extracted every 100 steps from a total of 2 × 108 MCMC generations. The first 25% of trees were discarded as the burning and the remaining were used to interfere a consensus tree. Tracer v.1.6 was used to ensure an appropriate effective sampling size (ESS all > 100). All obtained trees were imported and compared in FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/, accessed on 21 November 2022) for graphic implementation. All sequences will be deposited in GenBank.

2.2 Species delimitation methods

Throughout our analyses, a phylogenetic species concept, based on the principle that genetic variation between species (interspecific) is greater than the genetic variation within species (intraspecific) [158], was used. Thus, where two or more species are distinct, there should be a lack of overlap between intraspecific and interspecific sequence variation, commonly referred to as the "barcode gap" [159]. To identify the number of molecular operational taxonomic units (MOTUs) within our dataset, we applied four different methods of species delimitation to propose primary species hypotheses. Two were distance based: (i) Barcode Index Number (BIN) system [208], (ii) Automatic Barcode Gap Discovery (ABGD) [185] (bioinfo.mnhn.fr/abi/public/abgd); and two were tree-based: (iii) Generalized Mixed Yule Coalescent method (GMYC) [160] (species.h-its.org/gmyc), performed using the single threshold method, and (iv) Bayesian Poisson tree process (bPTP) [161] (species.h-its.org/ptp). All sequences were barcode-compliant (n = 40). They received a Barcode Index Number (BIN), which aided species delimitation [208]. The Automatic Barcode Gap Discovery

method (ABGD) is an automatic procedure that considers the sequences as hypothetical species based on the barcoding gap. The model employs a two-phase system, which initially divides sequences into operational taxonomic units (OTUs) based on a statistically inferred barcode gap (i.e., initial partitioning), and subsequently conducts a second round of splitting (i.e., recursive partitioning). The default values of 0.001 to 0.1 were explored as intraspecific distances and in ABGD, gap values from 1 to 1.5 were applied. The ABGD analysis (bioinfo.mnhn.fr/abi/public/abgd) was performed with a relative gap width of one and Kimura (K80) as the genetic distance. GMYC requires a fully resolved ultrametric tree as input. The tree-based methods employ a coalescent framework to independently identify evolving lineages without gene flow, each representing a putative species [162]. They can be performed using a single marker and are used to establish a threshold that identifies the separation of intraspecific population substructure from interspecific divergence, and therefore identifies those groups that may be candidate species [163]. The last species delimitation approach was implemented using a Poisson tree process (PTP), which models the speciation using the number of substitutions to infer putative species boundaries on a given phylogenetic input tree [161]. It assumes that the number of substitutions between species is significantly higher than the number of substitutions within species [161]. Here, we used the Bayesian implementation of the Poisson tree processes model (bPTP) [161], which uses a phylogenetic tree and is based on the phylogenetic species concept. The ML tree was used as input. The bPTP analysis (species.h-its.org/ptp) was applied using 500,000 generations of Markov chain Monte Carlo, a thinning of 100, and a burn-in of 25%. The outgroup (*Odontaster validus*) was removed in all delimitation analysis.

3 Results

A total of 96 specimens were analyzed in the current study and all were correctly sequenced to obtain a final COI sequence length of 628 bp. Of the 96 sequences generated in this study, 74 belonging to echinoids and 22 to crinoids. All sequences were barcode-compliant (Table 3) and received a barcode index

number (BIN), which aided species delimitation [208]. The other species delimitation methods recovered different number of secondary species hypotheses, or SSH for sea urchin but are all in agreement regarding the crinoid's investigation (Supplementary File S1). The most problematic method seems to be bPTP, in echinoids SSH investigation it showed overestimation in species partition. The maximum likelihood and Bayesian analysis results are consistent and reveal 13 putative species of echinoids and 4 of crinoids (Supplementary File S2)

3.1 Molecular results

Identification through barcoding requires specimens from the same species to cluster together using the barcode markers. Detailed and high-resolution trees comparison (ML and Bayesian interference) with species partition methods results is available in Figure 2 and Supplementary File S2.

Crinoidea

The 22 crinoids analyzed here were assigned to four morphospecies, all of them corresponding to described species. Our crinoid specimens were grouped into four putative species by the species delimitation methods showing consistency between the analysis. Clade 1 (posterior probability 92% ML and value of 0.97 in Bayesian interference) comprised individuals of *Anthometrina adriani* (Bell, 1908), Clade 2 (posterior probability 97% ML and value of 1.00 in Bayesian) comprised *Florometra mawsoni* AH Clark, 1937 individuals, whereas *Promachocrinus kerguelensis* Carpenter, 1879 individuals were included in Clade 3 (posterior probability 97% ML and value of 1.00 in Bayesian) and *Notocrinus virilis* Mortensen, 1917 in Clade 4 (posterior probability 100% ML and value of 1.00 in Bayesian). Molecular investigation performed on both adult and pentacrinoids stage larva.

Echinoidea

The 70 echinoids studied here represented 13 morphospecies, five of which

were given provisional identifications based on molecular taxonomy: Clades 12, 13 and 16 (*Abatus* sp.), Clade 5 (*Antrechinus* sp.) and Clade 8 (*Ctenocidaris* sp.) due to a lack of matching sequence in the online database (cross check on BOLD and GenBank. Accessed 22 November 2022). COI based species delimitation methods identified: 13 (BIN and GMYC), 12 (ABGD) and 19 putative species (bPTP).

Sequences belonging to Clades 16 and 17 were lumped together by ABGD, in bPTP those sequences are similarly grouped also with Clades 12, 13, 14, and 15. However, bPTP seems to over partition putative species of *Abatus ingens* Koehler, 1926 (Clade 11), *Antrechinus* sp. (Clade 5) and *Abatus (Pseudabatus) nimrodi* (Koehler, 1911) (Clade 10).

Posterior probability node values which are shown on the tree (Supplementary File S2) range from 47% to 100% for ML tree reconstruction and a value included from 0.56 to 1 in Bayesian interference. In our samples, no corresponding sequence matched *Abatus koehleri* (previously *Abatus elongatus)*, a species previously reported from Terra Nova Bay water [55].

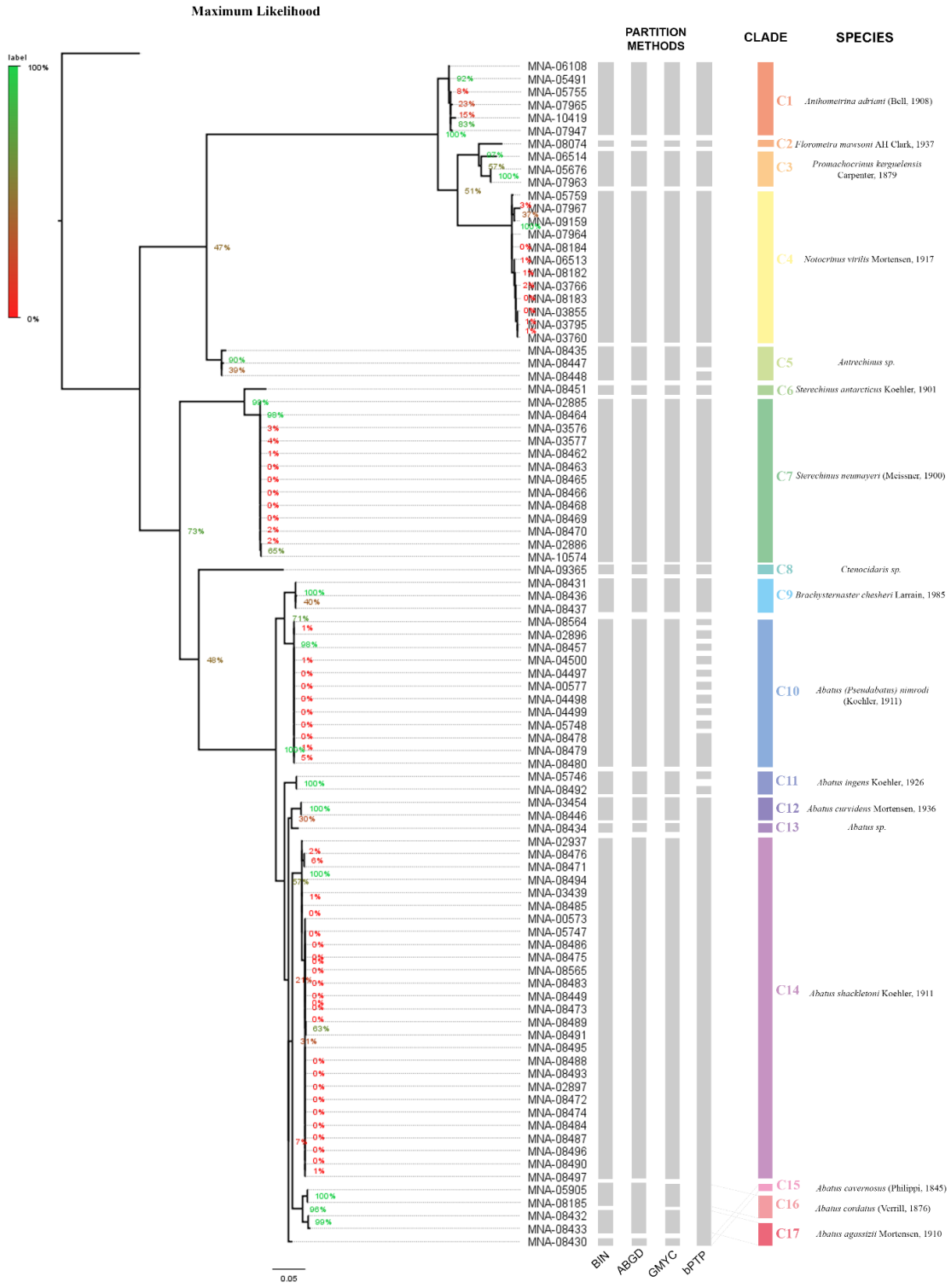


Figure 2 - Tree topology comparison of maximum likelihood. Posterior probability node values are showed on the tree with corresponding legend for each analysis. BIN: barcode index number; BOLD: automatic species delimitation [76]; ABGD: results from automatic barcode gap discovery method [32]; GMYC: species delimitation from generalized mixed Yule coalescent method [77]; bPTP: species delimitation using Bayesian Poisson tree processes method [78].

Table 3 - Samples species partition and associated BOLD BIN. Species occurrence is reported for AP= Antarctic Peninsula, TNB= Terra Nova Bay, CH=Cape Hallett, MB= Mawson Bank, BS= Bransfield Strait, WS= Weddell Sea. Abbreviations: barcode index number (BIN)

Class	BIN	Family	Species	Sample	TNB	BS	WS	CH	MB
Crinoidea	AAA1563	Antedonidae	*Anthometrina adriani* (Bell, 1908)	MNA-05491, MNA-05755, MNA-06108, MNA-07947, MNA-07965, MNA-10419	x				
	AAA1561		*Florometra mawsoni* AH Clark, 1937	MNA-08074	x				
	AAA0604		*Promachocrinus kerguelensis* Carpenter, 1879	MNA-05676, MNA-06514, MNA-07963	x				
	ACE8430	Notocrinidae	*Notocrinus virilis* Mortensen, 1917	MNA-03760, MNA-03766, MNA-03795, MNA-03855, MNA-05759, MNA-06513, MNA-07964, MNA-07967, MNA-08182, MNA-08183, MNA-08184, MNA-09159	x				
Echinoidea	AAC5708	Ctenocidaridae	*Ctenocidaris* sp.	MNA-09365				x	
	AAC4674	Echinidae	*Sterechinus antarcticus* Koehler, 1901	MNA-08451					x
	AAD4804		*Sterechinus neumayeri* (Meissner, 1900)	MNA-02885, MNA-02886, MNA-03576, MNA-03577, MNA-08462, MNA-08463, MNA-084624, MNA-08465, MNA-08466, MNA-08468, MNA-08469, MNA-08470, MNA-10574	x				
	AAL7860	Paleopneustina *incertae sedis* B	*Brachysternaster chesheri* Larrain, 1985	MNA-08431		x			
	AAC7471	Schizasteridae	*Abatus (Pseudabatus) nimrodi* (Koehler, 1911)	MNA-00577, MNA-02896, MNA-04497, MNA-04498, MNA-04499, MNA-04500, MNA-05748, MNA-08457, MNA-08478, MNA-08479, MNA-08480, MNA-08564	x				
	AAH9048		*Abatus agassizii* Mortensen, 1910	MNA-08432, MNA-08433			x		
	ABZ5633		*Abatus cavernosus* (Philippi, 1845)	MNA-08430			x		
	ABY5800		*Abatus cordatus* (Verrill, 1876)	MNA-05905, MNA-08185	x				
	AAH9046		*Abatus curvidens* Mortensen, 1936	MNA-03454, MNA-08446	x				
	AAC8452		*Abatus ingens* Koehler, 1926	MNA-05746, MNA-08492	x				
	AAH9047		*Abatus shackletoni* Koehler, 1911	MNA-00573, MNA-02897, MNA-02937, MNA-03439, MNA-05747, MNA-08449, MNA-08471, MNA-08472, MNA-08473, MNA-08474, MNA-08475, MNA-08476, MNA-08483, MNA-08484, MNA-08485, MNA-08486, MNA-08487, MNA-08488,	x			x	

				MNA-08489, MNA-08490, MNA-08491, MNA-08493, MNA-08494, MNA-08495, MNA-08496, MNA-08497, MNA-08565					
	ADF6219		*Abatus* sp.	MNA-08434			x		
	AAL7859	Urechinidae	*Antrechinus* sp.	MNA-08435, MNA-08447, MNA-08448		x			

3.2 Morphological Analysis

Following the "*reverse taxonomy*" approach, morphological analyses were conducted for a re-examination of our molecular results on available specimens. Observations were carried out under a stereoscopic microscope. For determination to species level, each sea urchin individuals were identified following the Antarctic Echinoidea taxonomic key (http://echinoidea-so.identificationkey.org/mkey.html) by Thomas Saucède and crinoids with available literature from Clark (1967)[203], Moore (1983)[204] and Speel at al. (1983)[205].

For echinoids (Figure 3) our morphological analysis focused on general morphology and skeletal features, such as accessory structures and spines. We particularly focused our attention on pedicellariae morphology, a defensive organ consisting of a head composed of two or more valves hinged to one another, a stem, and sometimes a neck. The four main types of pedicellariae analyzed were: globiferous, dentate, triphyllous, and ophicephalous.

Given the taxonomic relevance of pedicellariae shape morphology for species identification, the small mandibular appendage that articulates on the test was removed from selected samples corresponding to putative species partition highlighted by the molecular analysis. The tissue portion was treated with sodium hypochlorite (NaClO) to remove organic matter. Subsequently, the skeletal elements obtained were washed with deionized water then after with water and ethanol (Et-OH) in increasing proportions until complete washing in 100% Et-OH. This made possible to observe skeletal characteristics in detail under the stereomicroscope in order to obtain the correct identification of the species. With the re-examination of the morphological feature, correct taxonomic assignation could had been accomplished for Clades 12 and 16 (that missed "exact" molecular identification due lack of matching sequence in the online database).

For crinoids (Figure 4) we compared the external morphological features. All diagnostic characters were analyzed in detail, including the cirri, oral pinnules, genital pinnules, arm number, and segments of the cirri and arms under a

stereomicroscope.

Specimens identified in this study showed morphological characteristics corresponding to those described in the literature and molecular species identification was cross-referred with the morphological result.

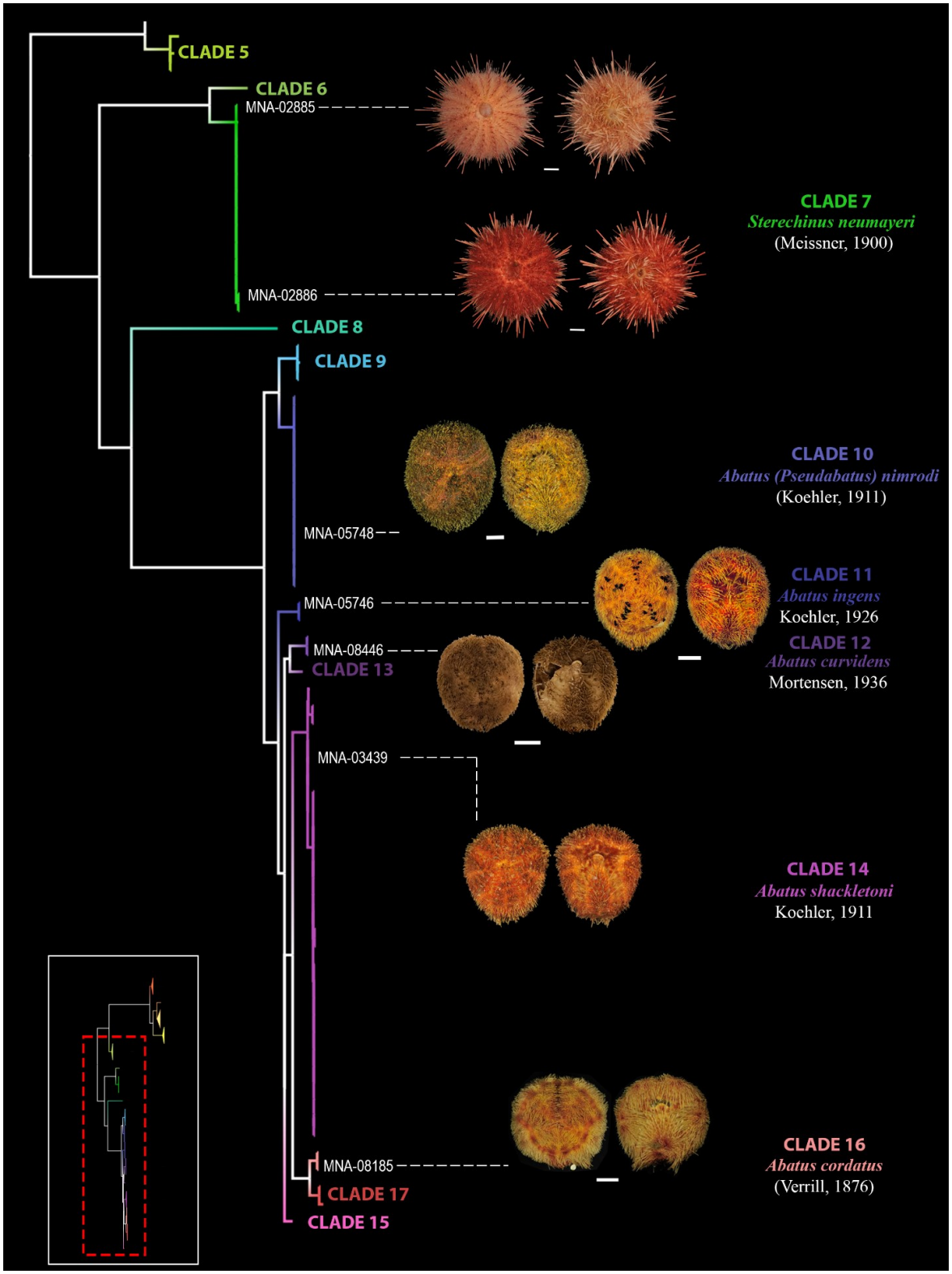


Figure 3 - Morphology variability of selected specimens (left - aboral view, right – oral view). In the tree, the different species identified are highlighted by different colors. The species present in the Terra Nova Bay area are listed on the right. Bottom left is the schematic view of the tree in Figure 2, the portion analyzed in detail in the image is highlighted in red. Clades without the associated species name in the figure are composed of organisms sampled in the Weddell Sea (see Figure 2 and

Supplementary File S2 for full clades name). Scale bar: 1 cm in grey.

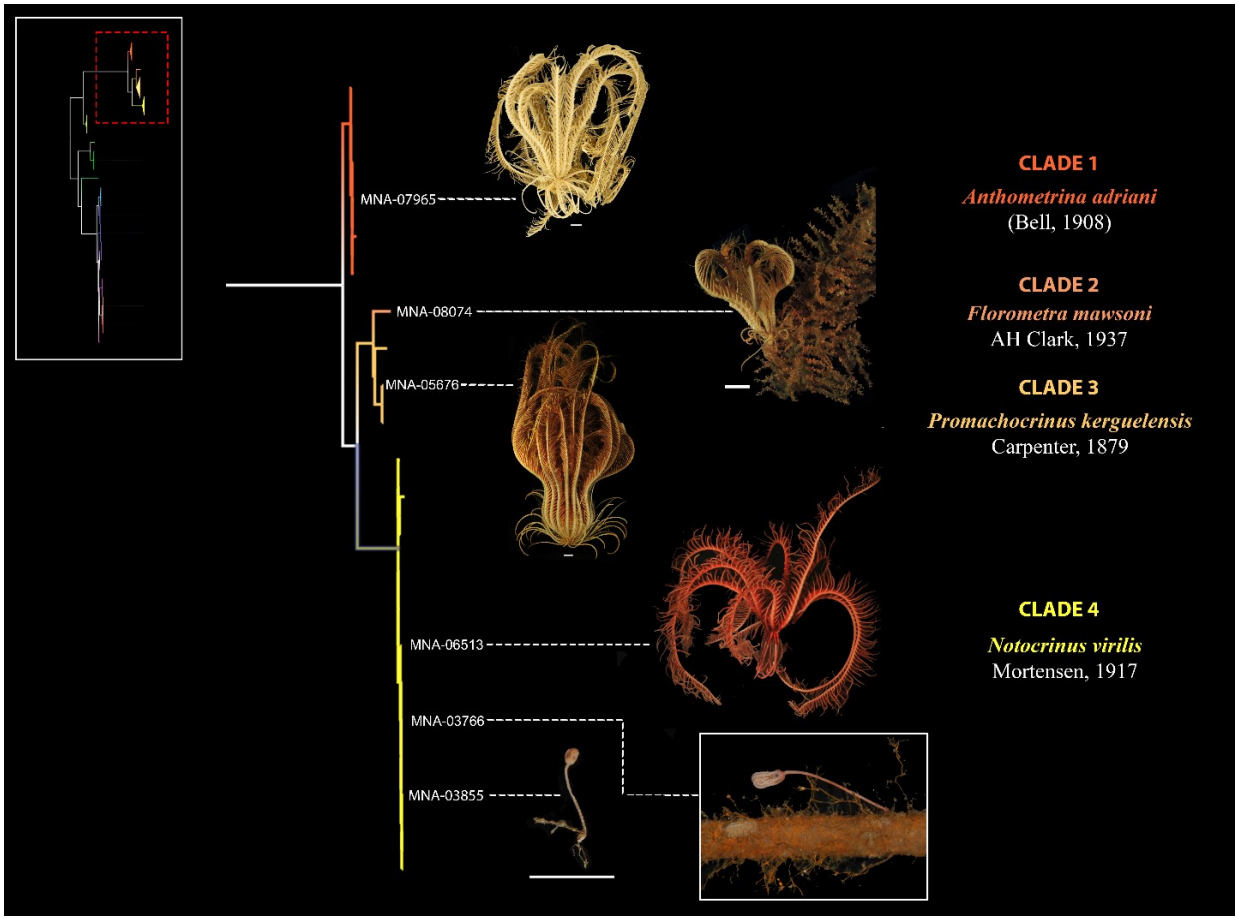


Figure 4 - Morphology variability of selected specimens. In the tree, the different species identified are highlighted by different colors. The species present in the Terra Nova Bay area are listed on the right. Bottom left is the schematic view of the tree in Figure 3, the portion analyzed in detail in the image is highlighted in red. Scale bar: 1 cm in grey.

3.3 Faunistic inventory revision

We integrated our results with the available literature from Chiantore *et al.*, 2006 [194] producing the revised check list (Table 4) and the updated depth range for echinoids species showed in Figure 5. In our analysis, there is no sample that corresponds morphologically or molecularly to the *Abatus koehleri* (previously *A. elongatus*) species identified with classical morphology by Chiantore *et al.*, 2006 [194].

With the aim of double-checking the identification and cross-referring the information in the literature, we searched previously identified samples deposited in the Italian National Antarctic Museum (MNA) - section of the Genoa collection. Unfortunately, a small amount of previously studied and published material has been permanently deposited in the museum collection making the possibility of comparing the results very complex. Sample MNA-00573 is part of the published material from Chiantore *et al.*, 2006 and present original identification label as *A. elongatus*. This sample, in our work, was successfully sequenced, morphologically reviewed and identified as *A. shackletoni* Koehler, 1911. For this reason, we consider the presence of *Abatus koehleri (*previously *A. elongatus*) in the Terra Nova Bay area questionable and the published records in the Southern Ocean Echinoid database as doubtful (e.g. [49], [200]). This modifies the number of previous identified species from TNB area from four to three.

Table 4 - Updated faunistic inventory for echinoids and crinoids of Terra Nova Bay.

Class	**Family**	**Species**	**Depth range (m)**	**Chaintore *et al.*, 2006**	**This work**
Echinoidea	Echinidae	*Sterechinus neumayeri* (Meissner, 1900)	15-380	x	x
	Schizasteridae	*Abatus (Pseudabatus) nimrodi* (Koehler, 1911)	60-150	x	x
		Abatus cordatus (Verrill, 1876)	78-146		x
		Abatus curvidens Mortensen, 1936	100		x
		Abatus ingens Koehler, 1926	148-150		x
		Abatus shackletoni Koehler, 1911	36-380	x	x
Crinoidea	Antedonidae	*Anthometrina adriani* (Bell, 1908)	250-522		x
		Florometra mawsoni AH Clark, 1937	522		x
		Promachocrinus kerguelensis Carpenter, 1879	137-525		x
	Notocrinidae	*Notocrinus virilis* Mortensen, 1917	137-525		x

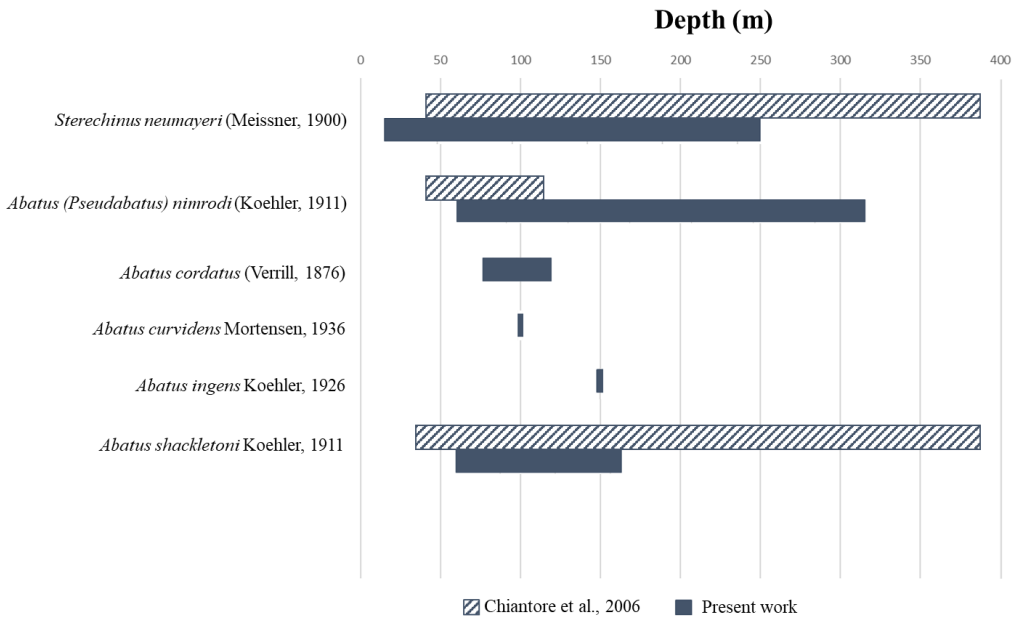


Figure 5 - List of echinoid species found in Terra Nova Bay and updated depth range.

4 Discussion

The identification of species in an ecosystem is the first step in any ecology and conservation measures. It is estimated that only 25% of 0.7 to 1.0 million marine species have been described [209]. In addition to declining taxonomic expertise, only a fraction of the marine biodiversity occurring across the Southern Ocean has been recorded. Prior to documentation, several species were likely to go extinct [210]. The implementation of high-throughput technologies to accelerate the identification of species is therefore important. Our work, together with multiple study results, point out that for many groups, barcoding is a valuable tool for the identification of marine specimens from the Southern Ocean (e.g. [181], [183], [211]–[219]).

Our results strongly stress the importance of DNA barcodes in order to also identify juvenile organisms and larval stage which morphological identification could be challenging. Six pentacrinoid larvae were identified by comparing COI sequences against the reference sequences held in BOLD, confirming that molecular identification can help to assign larva stage to the adults of their species. This work is another demonstration of how important the integration of a classical taxonomic approach in molecular analysis is especially when online

database fail in finding the exact sequence match. The efficiency of identification through barcoding depends on the completeness of the reference database [220], [221], project like “The Barcode of Life” directly [222] and indirectly encourages large-scale molecular studies with a higher focus on quality. The structural need for a voucher specimen provides an opportunity for morphological and molecular studies using the same specimens, and for subsequent controls of the identification. Notably, our work has been shown to be efficient tool also for previously published material and highlight the necessity of a permanent storage of samples for future comparison.

By using a simple DNA barcoding approach, our study contributed to improve our knowledge on the echinoid and crinoid biodiversity found in Terra Nova Bay and will facilitate future taxonomic studies. This standard method supported by the BOLD system allows the analysis of many samples collected by different research groups on large biogeographic scales and allows for a quick and precise investigation.

It confirmed, and sometimes revealed, species that should be investigated further in order to improve their delineation and could describe a diversity that was not noticed so far in an area (*e.g.* [181]). This is of primary importance in our case of study because echinoderms represent a considerable biomass in marine habitats and play a major role in the Antarctic marine ecosystems [53]–[56], [128], [223]–[225]. All this information is vitally important for the study area and in the future monitoring activities that are requested by the conservation measures of Annex 91-05/C [59] of the Ross Sea Marine Protected Area. Two putative species (*Antrechinus* sp. and *Ctenocidaris* sp*.)* defined here based on COI sequences need to be better characterized with integrated taxonomy. Thus, mitochondrial sequencing from type material held at museums would provide valuable information for assigning formal species names to these COI-based putative species. This highlights the key role played by museums, not only as conservation centers for biological collections but also as hubs for sharing information. It is desirable that in the next years, all available museum collections will be subject to molecular identifications in order to precisely determine species ranges and occurrences, key data for all monitoring activities.

Keeping a sound connection between taxonomy and DNA data is of primary importance if future DNA-based investigations are expected to rely on the wide range of observations that are continuously reported for formally described species.

The current paper represents a further contribution of the Italian National Antarctic Museum (MNA)—Genoa section, as custodian of biodiversity data for the Ross Sea area. Many contributions to the Antarctic Biodiversity Portal have been published by MNA over the years, with the aim of increasing the knowledge of the area [32], [34]–[37], [41], [42], [80], [181], [226] (http://www.biodiversity.aq, accessed on 25 November 2022).

CHAPTER 5

Characterization of *Iophon* spp. (Porifera, Demospongiae) in association with the brittle star genus *Ophioplinthus* from Antarctic coasts

1 Introduction

Marine habitats are incredibly diverse and complex ecosystems, with a wide variety of biotic interactions between organisms playing a crucial role in determining their distribution and abundance. In the past, researchers focused on competition, predation, and ecosystem engineering as key factors that influenced the structure of communities [227], [228]. More recently, the importance of symbiosis, mostly the role of parasitism or mutualism, has been identified in altering these interactions (*e.g.* [229], [230]). Symbiotic interactions also represent important ecological and evolutionary drivers, often promoting speciation through host shift (*e.g.* [231]). One area of particular interest for studying marine biodiversity is the Antarctic region. The Southern Ocean, with its relative isolation, high endemism, and vulnerability to disturbances, presents a unique environment for research ([232], [233]) Over the past few decades, there have been extensive investigations into benthic biodiversity in the Southern Ocean [234] and the ecological role of symbiotic interactions has only been recently re-evaluated and found to affect the structure of food webs at a magnitude comparable to that of predation or physical disturbance [235]. In Antarctica there are now several studied cases both for the Weddell Sea (e.g., [236], [237]) and the Ross Sea (e.g., [238]–[241]).

Despite the progress made, our understanding of the forces shaping benthic biodiversity in the Antarctic is still incomplete. It appears that community structure is influenced not only by bathymetry but also by the geographical features of the shelf. Sessile organisms dominate these communities, but there are noticeable variations in biomass and diversity. Certain areas exhibit high abundance and diversity, while surrounding regions resemble arid landscapes with minimal biological activity. One of the main factors controlling distribution

and abundance of sessile organisms in benthic communities is the occurrence of free substrates [242], in the Antarctic continental shelf, space can be considered a limiting resource so that epibiosis represent a spread strategy [243]. In case of scarcity of mineral hard substrates, biotic secondary substrates can provide suitable sites for attachment and therefore new ecological niches for sessile species and this phenomenon is known as ectosymbiosis (i.e., sessile life on biotic substrate, whatever the nature of the relationship: mutualism, commensalism, or parasitism). In the Antarctic, numerous species have been documented as epibionts on substrates [243], [244]; however, these studies were not able to establish the importance of ectosymbiosis in the biodiversity of sessile organisms, mainly because relative proportions of species fixed on living organisms versus those fixed on abiotic substrates were not compared at geographically restricted, homogenous sites. Therefore, it is difficult to determine if ectosymbionts are specific to their living substrate or if they are opportunistic sessile organisms, selecting other organisms as substrate because of the rarity of mineral substrates. Sponges represent one of the most important components of the Antarctic zoobenthos [245], with over 350 known species, both on hard and soft substrata where, owing to their three-dimensional growth, increase habitat heterogeneity [246].

Due to scarcity of hard substrates in the Antarctic sea bottom, sponges adopted peculiar morpho-functional alterations required for soft bottoms colonization, such as the development of stalks as rooting systems or the ability to settle on secondary hard substrata, such as bivalves and hydrozoans [247].

One well-known examples in literature of ectosymbiontic relationships, where two species live together in a close and mutually beneficial association, is between Southern Ocean *Ophioplinthus* Lyman, 1878 (previously including *Ophiolepis* Müller & Troschel, 1840 and *Theodoria* Fell, 1961), which are species rich, widespread and fairly common, and the sponge of the *Iophon* genus. However, even if this interaction is widely reported in the scientific literature, the informations regarding it are still limited. Molecular data analysis show, like many groups, that species diversity for brittle stars belonging to *Ophioplinthus*

genus has been vastly underestimated. Unlike the usual pattern of "cryptic" or unrecognized diversity there are several *Ophioplinthus* "species" there are multiple divergent lineages, most lineages morphologically identical (as far as current studies show), but one of which is morphologically discrete, often formally described as a separate species. When Koehler described the species *Ophioplinthus gelida* in 1900 (as *Ophioglypha gelida*) he did not cite the association with *Iophon* sponge. A few years later, in the 1912 description (as *Ophiurolepis gelida*), he writes about *Iophon flabelo-digitatus* Kirkpatrick, 1907 indicating that it covers the disc and arms in the dorsal area and extends in part through the ventral and suggesting that the presence of the sponge could be a practical identifying character for the species. Mortensen (1936) describes *O. brevirima* (as *Ophiurolepis brevirima*), which also show *Iophon radiatus* Topsent, 1901 in symbiosis. Until then, Mortensen says possibly this species was confused with *O. gelida*. Fell (1961) writes that *I. radiatus*/ *O. gelida* or *O. brevirima* is a parasitic or epizoic relationship. However, this *Ophioplinthus* species have calcareous plates that confers the brittle star a resistant cover to most penetration attempts deterring many possible predators. Fell uses symbiosis as a character to differentiate the species of *O. gelida* from *O. brevirima*, indicating that the infestation is massive in *O. gelida*. Cherbonnier (1962) writes that the morphology of *O. gelida* is in partly due to the presence or absence of *I. radiatus*, highlighting that this relationship can modify the development of *O. gelida*. Other authors who cite the presence of *Iophon* are Madsen (1967) as *Iophon* sp. in both species and Bernasconi & D'Agostino (1974) as *Iophon* sp. in *O. gelida* and *I. spatulatus* (now accepted as *I. unicorne* Topsent, 1907) in *O. brevirima*. For Gutt and Schickan (1998) it's a relationship mandatory for *I. radiatus*, indicating that it would be a proto-cooperation, stage prior to mutualism between *Ophioplinthus* brittle stars and *Iophon* sponges.

The aim of this study is the analysis, identification and comparison of the diversity of *Iophon* species associated with *Ophioplinthus* that utilize the brittle stars body as a substratum. The analysis has been conducted exploiting from the available samples stored in the biological collection at the Italian National Antarctic Museum – Genoa section.

2 Materials and methods

The samples available at Italian National Antarctic Museum (MNA) – Genoa section analyzed in this study derives from different Antarctic areas (Figure 1), in detail: the Antarctic Peninsula (Figure 1.1), Amundsen Sea (Figure 1.2), the Weddell Sea (Figure 1.3) and the Ross Sea sector (Figure 1.4) specifically the Terra Nova Bay area (Figure 1.4.1) that is part of both the marine protected area and the Antarctic Special Protected Area (n.161)[28].

The biological material used in this study is part of the MNA collection (accessible online, https://steu.shinyapps.io/MNA-generale/) collected during the expeditions of the Italian National Antarctic Research Program (PNRA) or materials entrusted to the Museum by foreign institutions such as the Alfred Wegener Institute (AWI, Germany), the British Antarctic Survey (BAS, United Kingdom) and the National Institute of Water and Atmospheric Research (NIWA; New Zealand) with which the MNA has constant collaborations and exchanges.

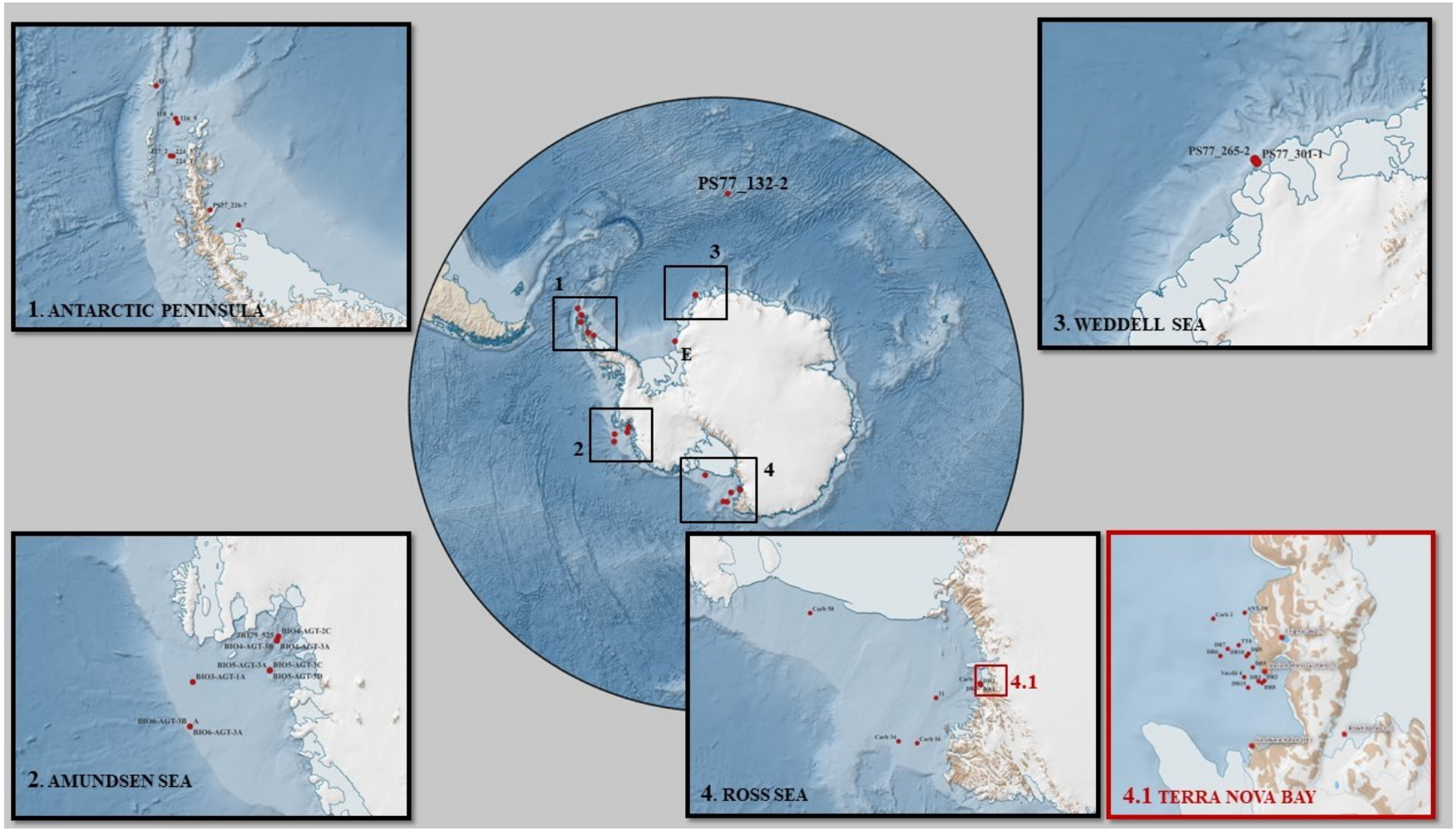
Specimens were collected in the framework of several recent scientific expedition performed in the Southern Ocean and which are now permanently stored and curated at MNA. The Italian National Antarctic Program (PNRA) expedition XVII Exp 01/02, XXI Exp 05/06, XXVII Exp 11/12, XXVIII Exp 12/13 and PNRA XXIX Exp 13/14 were all from the Ross Sea. Other samples were collected from the Alfred Wegner Institute (AWI) ANT-XXIX/3, PS81 expedition (2013), AWI PS 77 (2011) and AWI PS 82 (2013). Additional samples were obtained from expedition of the National Institute of Water and Atmospheric Research (NIWA) NIWA TAN08 (2008) and two British Antarctic Survey (BAS) expeditions JR 144 (2006) and JR 179 (2008).

Table 1 - Sampling stations and data.

Expedition	Station	Location	Year	Latitude	Longitude	Depth (m)	Sponsor
AWI PS 77	PS77_312-2	Bouvet Island	2011	-54.4700	3.1850	296	Alfred-Wegener-Institut (AWI)
	PS77_265-2	East Weddel Sea	2011	-70.7940	-10.6700	633.5	Alfred-Wegener-Institut (AWI)
	PS77_301-1	East Weddel Sea	2011	-70.8510	-10.5880	225.7	Alfred-Wegener-Institut (AWI)
	PS77_226-7	Larsen Ice Shelf	2011	-64.9140	-60.6210	226.2	Alfred-Wegener-Institut (AWI)
	PS 77_F	Larsen B	2011	-65.9586	-60.5688	530	Alfred-Wegener-Institut (AWI)
AWI PS 82	PS 82_E	Filschner Trough	2013	-76.9695	-32.9429	210	Alfred-Wegener-Institut (AWI)
AWI PS81	116_9	Bransfield strait	2013	-62.5632	-56.4635	248.4	Alfred-Wegener-Institut (AWI)
	224_3	Bransfield strait	2013	-63.0088	-58.5945	261	Alfred-Wegener-Institut (AWI)
	118_4	Bransfield strait	2013	-62.4325	-56.2877	437	Alfred-Wegener-Institut (AWI)
	227_2	Bransfield strait	2013	-62.9305	-58.6848	564	Alfred-Wegener-Institut (AWI)
JR 144	D	Elephant Island	2006	-61.3339	-55.1947	199.54	British Antarctic Survey (BAS)
JR 179	A	Amundsen Sea	2008	-71.3486	-110.0060	523	British Antarctic Survey (BAS)
	B	Amundsen Sea	2008	-71.3482	-109.9984	496	British Antarctic Survey (BAS)
	BIO4-AGT-3A	Amundsen Sea	2008	-74.4100	-104.6550	510.84	British Antarctic Survey (BAS)
	BIO3-AGT-1A	Amundsen Sea	2008	-71.8100	-106.3300	590.35	British Antarctic Survey (BAS)
	BIO4-AGT-2C	Amundsen Sea	2008	-74.4770	-104.2570	1150.58	British Antarctic Survey (BAS)
	BIO4-AGT-3C	Amundsen Sea	2008	-74.4050	-104.6110	505.71	British Antarctic Survey (BAS)
	BIO4-AGT-3B	Amundsen Sea	2008	-74.3990	-104.6300	496.63	British Antarctic Survey (BAS)
	BIO5-AGT-3C	Amundsen Sea	2008	-73.9860	-107.3900	541.75	British Antarctic Survey (BAS)
	BIO5-AGT-3A	Amundsen Sea	2008	-73.9750	-107.4220	557.8	British Antarctic Survey (BAS)
	BIO6-AGT-3A	Amundsen Sea	2008	-71.3480	-109.9980	485.72	British Antarctic Survey (BAS)
	BIO5-AGT-3D	Amundsen Sea	2008	-73.9800	-107.4080	544.05	British Antarctic Survey (BAS)
	JR179_525	Amundsen Sea	2008	-74.3989	-104.6295	527	British Antarctic Survey (BAS)
	BIO6-AGT-3B	Amundsen Sea	2008	-71.3420	-109.9980	480.95	British Antarctic Survey (BAS)

NIWA TAN08	31	Ross Sea	2008	-74.5905	170.2757	283	National Institute of Water and Atmospheric Research (NIWA)
PNRA XVII Exp 01/02	ANT-D9	Ross Sea	2002	-74.7486	164.1247	113	Italian National Antarctic Research Program (PNRA)
	Carb 16	Ross Sea	2002	-73.1160	173.4272	436	Italian National Antarctic Research Program (PNRA)
	Carb 2	Ross Sea	2002	-74.7512	164.2315	218	Italian National Antarctic Research Program (PNRA)
	Carb 34	Ross Sea	2002	-73.2427	175.6392	389	Italian National Antarctic Research Program (PNRA)
	Carb 58	Ross Sea	2002	-77.6513	-171.3608	461	Italian National Antarctic Research Program (PNRA)
PNRA XXI Exp 05/06	DR1	Ross Sea	2006	-74.6879	164.1425	145	Italian National Antarctic Research Program (PNRA)
PNRA XXIX Exp 13/14	DR2	Ross Sea	2014	-74.6868	164.1228	94	Italian National Antarctic Research Program (PNRA)
	DR7	Ross Sea	2014	-74.7224	164.2122	260	Italian National Antarctic Research Program (PNRA)
	DR6	Ross Sea	2014	-74.7183	164.2421	271	Italian National Antarctic Research Program (PNRA)
	TT6	Ross Sea	2014	-74.7228	164.1732	205	Italian National Antarctic Research Program (PNRA)
PNRA XXVII Exp 11/12	DR9	Ross Sea	2012	-74.7134	164.1490	150	Italian National Antarctic Research Program (PNRA)
PNRA XXVIII Exp 12/13	DR10	Ross Sea	2013	-74.7118	164.1580	250	Italian National Antarctic Research Program (PNRA)
	DR15	Ross Sea	2013	-74.6850	164.1814	302	Italian National Antarctic Research Program (PNRA)
	DR5	Ross Sea	2013	-74.7009	164.1479	150	Italian National Antarctic Research Program (PNRA)
	DR8	Ross Sea	2013	-74.6856	164.1321	105	Italian National Antarctic Research Program (PNRA)
	Vacchi 4	Ross Sea	2013	-74.6948	164.1846	454	Italian National Antarctic Research Program (PNRA)

Figure 1 - Antarctica (central) with highlighted the main sampling locations: 1) Antarctic Peninsula, 2) Amundsen Sea, 3) Weddell Sea, 4) Ross Sea area and in red the Terra Nova Bay (Ross Sea) sector (4.1).



The 166 specimens of the brittle stars belonging to *Ophioplinthus* genus were analyzed and the distributional data considered here originated from 41 different sampling stations, ranging from 94 and 1150.58 m of depth (Table 1). Sampling was performed through deployments of a variety of sampling gear. Benthic sampling under the Italian PNRA was mainly performed using a rectangular dredge (70 × 30 cm), Agassiz Trawl (AGT) and an unconventional set of gears for sampling benthic fauna (such as a Trammel net, Long line and a Gill net) that opportunistically collected benthic specimens due to accidental contact with the bottom during gear deployment "failures". Samples from AWI, BAS and NIWA were all collected using Agassiz Trawl.

After collection, samples were transferred to the laboratory and stored in ethanol (75% Et-OH) or frozen (−20 ◦C) for subsequent molecular analysis. Thereafter, samples were acquired by the MNA and included in their collections (available online at https://steu.shinyapps.io/MNA-generale/, accessed on 10 February 2023). All specimens were classified to the lowest possible taxonomical resolution on a morphological basis by using the available literature and keys from Topsent (1901)[248], [249], Goodwin *et al.*, (2012) [250], Rios *et al.*, (2004) [251].

For molecular analyses, a portion of tissue was clipped from each brittle star sample for DNA extraction and sequencing of partial cytochrome c oxidase subunit 1 (CO1). The molecular analyses were carried out at the Canadian Centre for DNA Barcoding (University of Guelph, Guelph, ON, Canada) (unpublished results).

Subsequently, the available *Ophioplinthus* samples were subdivided on the basis of the presence or absence of associations with *Iophon* sponge. Of the 166 samples analyzed, 95 present a sponge cover (from partial to massive coverage). To carry out the morphological analysis of the taxonomical elements of the porifera (spicules), a portion of the sponge needed to be removed. Given the destructive sampling of the material, before carrying out the removal each brittle

star was photographed from the oral and aboral side to maintain information on the amount of sponge covering on the organism.

The spicule complement and skeletal architecture were examined under light microscope; spicule preparations and hand-cut sections of sponge portions were performed following the methodologies in Núñez Pons *et al.*, (2022)[252] . Taxonomic decisions were made according to the revision of Demospongiae of Morrow and Cárdenas (2015)[253] and the classification present in the World Porifera Database (WPD)[254]

For each spicule type, measurements were obtained from 30 spicules, and were reported as the range of the smallest length - (mean ± standard deviation)- largest length x smallest width-(mean ± standard deviation)- largest width. The slides for microscope analysis are permanently stored in the biological collection at Italian National Antarctic Museum – Genoa section.

3 Results

A total of 96 *Iophon* specimens were analyzed in the current study, morphological analyses were conducted with the examination of different elements: general shape, color, surface structure, skeleton elements. In the specimens studied, the presence of *Iophon* has different degrees of development, indistinctly in the two species, but numerous specimens of *O. gelida* collected did not exhibit the association with a *Iophon* sponge, while all the *O. brevirima* specimens are in symbiosis with *Iophon* sponge. We were able to identify two species, *I. unicorne* Topsent, 1907 and *I. flabellodigitatum* Kirkpatrick, 1907.

For each one of the 96 samples analyzed, the morphologies of the various spicules were observed and size measurements were made for each element. Summary of mean measurements (and standard deviation) for identified species is given in Table 2. We particularly focused our attention on the macrosclera styles and tylotes, and the microsclera, anisochelae and bipocillae (Figure 2).

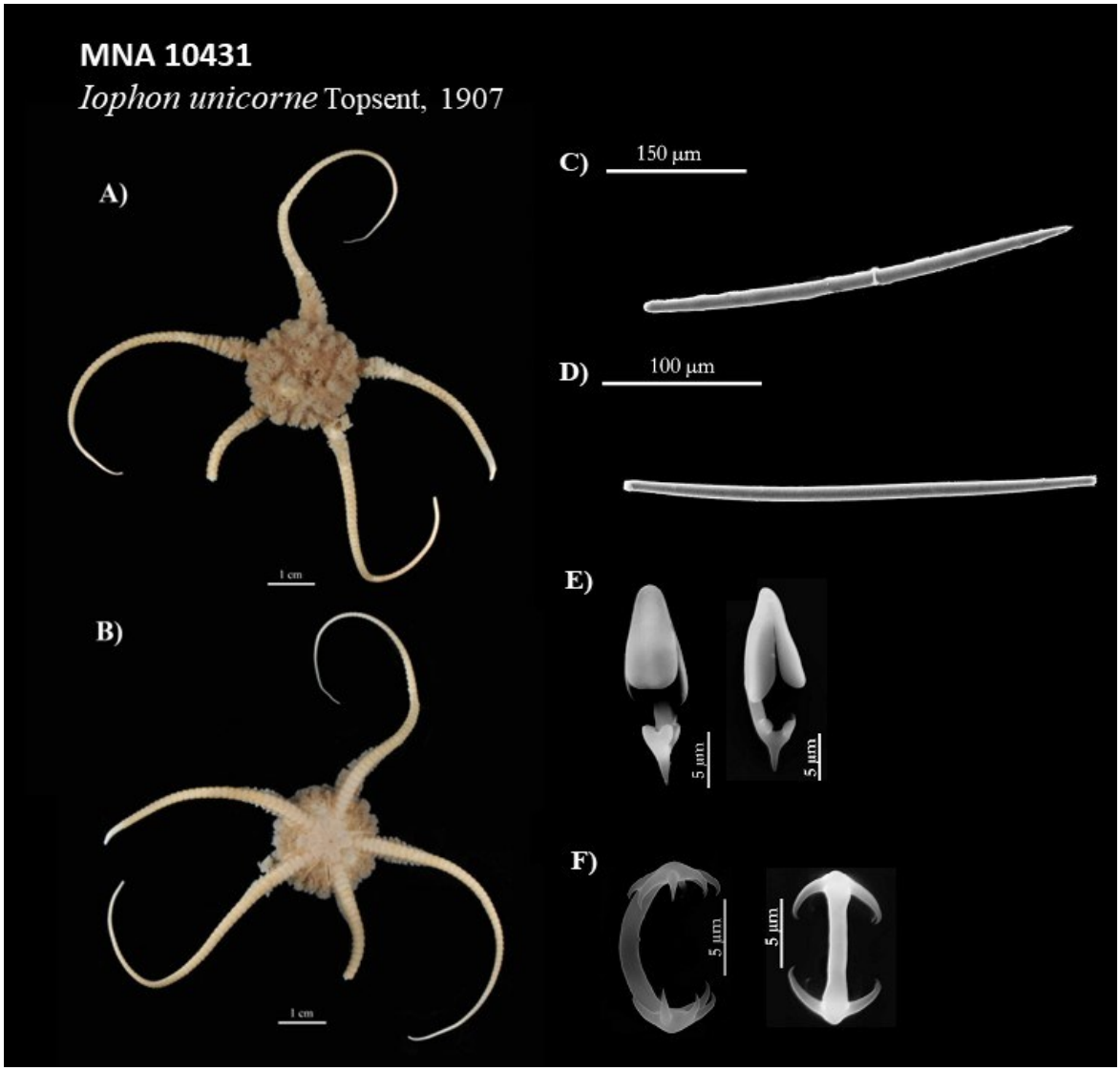


Figure 2 - Selected specimen MNA 10431 corresponding to *I. unicorne* associated to *Ophioplinthus* brittle star, aboral (A) and (B) oral view. Scale bar: 1 cm in white. General morphology of spicules of *Iophon* genus sponges. Megascleres: styles (C) and tylote (D). Microscleres: anisochele (E) and bipocilli (F). Scale bar different of each skeletal element.

In addition to the morphological observation of the skeletal elements, we used graded orbital that allowed us to measure the spicules and compare the measurements with the original species descriptions reported in the scientific literature, in order to identify the sponge species present in our samples.

	MEGASCLERES				MICROSCLERES	
	STYLE		TYLOTE		ANISOLCHELAE	BIPOCILLAE
species	Average length (± st. dev.)	Average width (± st.dev.)	Average length (± st. dev.)	Average width (± st.dev.)	Average (± st. dev.)	Average (± st. dev.)
I. unicorne	393.62 (± 33.30)	15.10 (± 2.52)	250.12 (± 24.93)	10.23 (± 2.01)	18.63 (± 1.42)	13.05 (± 1.04)
I. flabellodigitatum	446.11 (± 22.63)	19.31 (± 2.16)	312.06 (± 24.96)	10.68 (± 1.08)	18.04 (± 0.92)	12.06 (± 1.00)

Table 2 - Average measurements (with associated standard deviation) of the skeletal elements analyzed for the two species of *Iophon* identified.

I. unicorne and *I. flabellodigitatum* differ mainly on the morphology of the tylotes and mesures of spicoles in general (Figure 3). Specifically, *I. flabellodigitatum*, presents a tylotes with a pointed end, whereas *I. unicorne* has a tylotes with both rounded and indented ends.

The ectosomal tylotes (achantotylotes) of *I. flabellodigitatum* differ from those of *I. unicorne* in having one end with a very large spine, but otherwise have similarly formed and sized spicules. Topsent (1907) recognized that the oxea-like choanosomal spicules in this species were superficially similar to oxea but considered the micron to be ornamentation, similar to basal spination of styles found in other *Iophon* species, and consequently regarded them as modified acanthostyles. This has been followed by Rios (2006)[251] who terms the spicules styles with mucronate ends. *I. unicorne* is widely distributed in the Antarctic with records from Antarctic Peninsula, Bransfield Strait, Bellinghausen Sea, Ross Sea, Kerguelen Island, South Orkney Islands, South Shetland Islands, and Weddell Sea ([248], [249], [251], [255]–[258]). Burton (1929) [259] considered the majority of Antarctic species of *Iophon* to be synonyms, so it is not clear if his specimens include this species.

Our analysis also reported a difference between the external morphology of the two *Iophon* sponge, in the original description by Kirkpatrick of 1907 (as *I. flabello-digitatus* and *I. spatulatus*), in free form the sponge species present arborescent growth, in our samples the sponges associated with *Ophioplinthus* brittle stars assumes encrusting or massive growth form.

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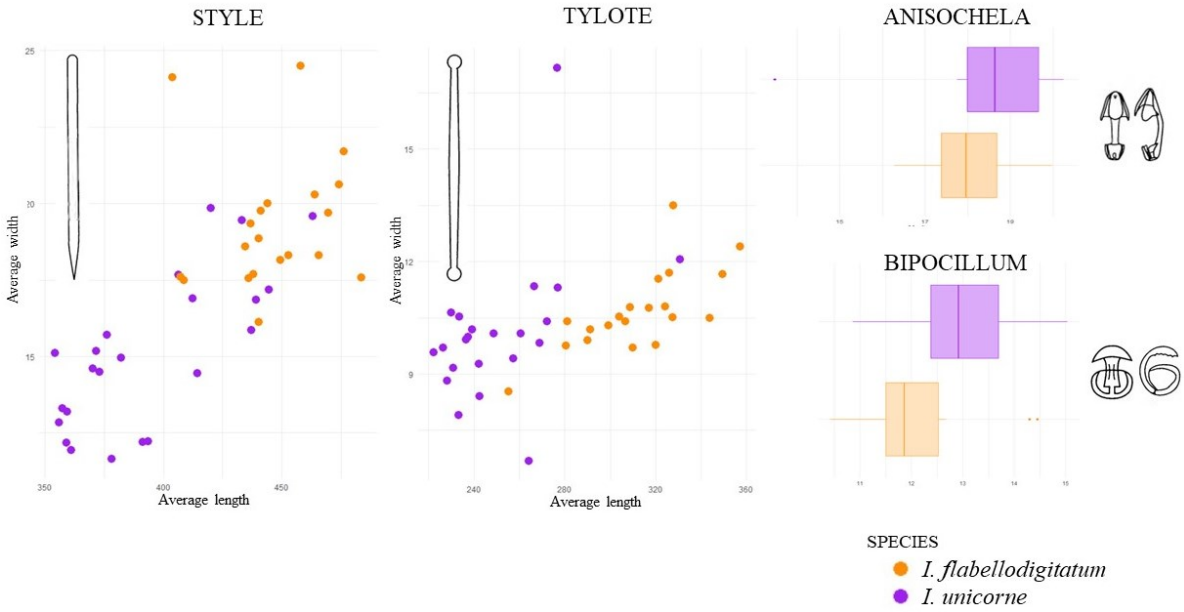

Figure 3 - Scatterplots of the relationship between length and width of styli and tylote. Box pot of anisochele and bipocilli measurements (all measurements expressed in µm). *Iophon unicorne* (in purple) and *Iophon flabellodigitatum* (in orange). General morphologies of spicules adapted from "Thesaurus of sponge morphology"[260].

We compared the measurement of the spicules obtained in our analysis, and we observed a variation dimension related to a longitudinal gradient on the styles width with the maximum dimension reached in the Terra Nova Bay area (Ross Sea) and the minimum at the East Weddell Sea for *I. unicorne*. This pattern seems to replicate in *I. flabellodigitatum* samples however we only have observations from two location (Figure 4). This characteristic could be dependent on the quantity of silica dissolved in water, as a greater quantity of silica allows the formation of spicules with larger dimensions.

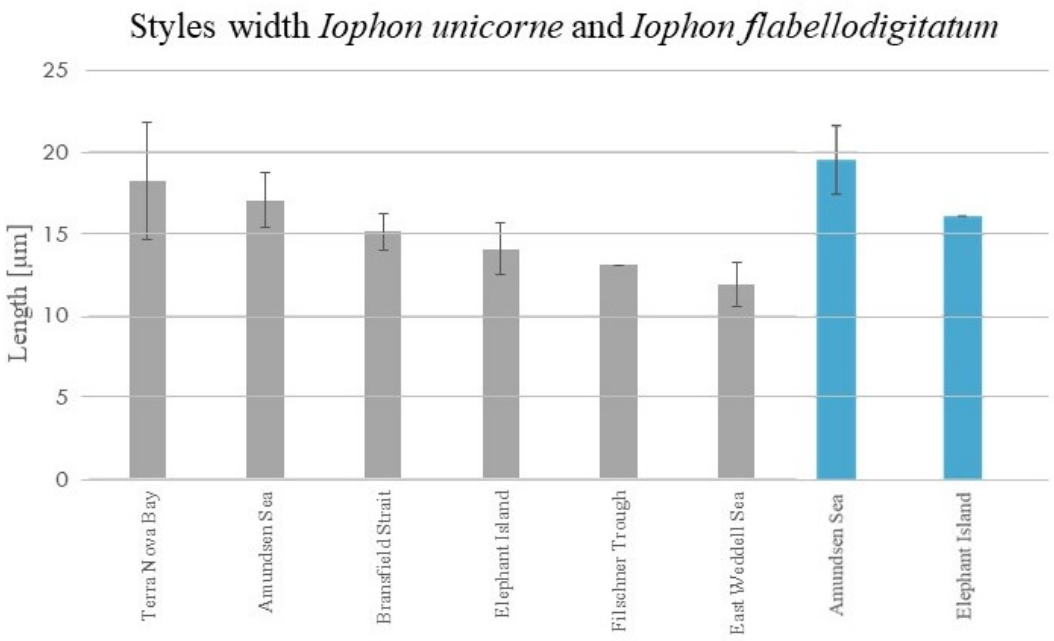

Figure 4 - Dimensional variation of the styles in relation to the sampling location

4 Discussion

In order to comprehend the intricate mechanisms involved in symbiotic processes, which encompass specialized physiological adaptations, it is imperative to delve into experimentation. Although it may lie beyond the scope of this work, understanding the life cycles and evolutionary paths taken by hosts and symbionts necessitates an exploration of the diverse array of hosts and their respective symbiotic relationships [261]. Even though it is acknowledged that the symbiotic interactions mentioned in this context still lack definitive explanations, we shall strive to provide an approximation that interprets the cited observations.

Out of the *Ophioplinthus* brittle stars examined in this work, the significant proportion of 57.8% were found to be engaged in symbiotic associations highlighting the importance of symbiotic interactions within the Antarctic brittle star population. These interactions occur with sponges of *Iophon* genus, accounting for 53.5% of the observed interactions with *I. unicorne* and 46.5% with *I. flabellodigitatum*. In the specimens studied, the presence of *Iophon* has

different degrees of development, indistinctly in the two species, but numerous specimens of *O. gelida* collected did not exhibit the association with a *Iophon* sponge, while all the *O. brevirima* specimens are in symbiosis with *Iophon.* Previous research saw that the *Iophon* presence on *Ophioplinthus* brittle stars could led to modifications of the plaques growth [262] that affect the appearance of the disc and could let lead to host identification errors. Smirnov (1984) observed that *Iophon* can modify the ambulacral pores of *O. brevirima*, although it is not always present. However, in our work we didn't observe this anomaly growth. The material analyzed in this work comes from four main sampling areas of the Southern Ocean: Ross Sea, Weddell Sea, Amundsen Sea and Antarctic Peninsula. Of the two symbiotic sponge species identified, *I. flabellodigitatum*, was found on samples almost exclusively from the Amundsen Sea, while *I. unicorne* appears to have a more ubiquitous distribution.

The morphological analysis allowed us to highlight the presence of the two sponges, which present a peculiar growth pattern when associated with the brittle star that differ from the original description in the scientific literature. We were able to identify what seems to be a correlation between the locality and the dimension of the styles in both *Iophon* species identified. However, the data are still limited and extensive specific work is needed on this subject, which will better highlight this correlation (if present). For what concern our result we can only speculate that the reason is due to the presence of silicate in the water but there could be many other factors that influence these observations.

Ophiuroids are among the most important Antarctic benthic groups, in terms of abundance and ecological roles, from shallow sublittoral habitats to continental shelves and the deep sea [263]–[267]. Brittle stars are not new to study of symbiotic association; in general the most commonly observed hosts of brittle stars include sponges, cnidarians, bryozoans, sea urchins and algae [268]–[272] which may provide refugia from predation, habitat and feeding space.

Specifically, Southern Ocean *Ophioplinthus* Lyman, 1878 (previously including *Ophiolepis* Müller & Troschel, 1840 and *Theodoria* Fell, 1961), which is species rich, widespread and fairly common, represents optimal candidate to study this

organism's interaction. *Ophioplinthus gelida* (Koehler, 1901) has a circumpolar distribution Fell (1961)[263], Madsen (1967)[273], Bernasconi & D'Agostino (1974)[274] and has been found from 36 to 2844 m deep and is one of the dominant brittle stars of the Antarctic benthos.

Previous works reports the association of *Iophon radiatum* with *Ophioplinthus* sp. as one of the more common, however none of our samples show the presence of *I. radiatum*. Therefore, we register the first record of *I. flabellodigitatum* on *O. brevirima* and the first record of *I. unicorne* on *O. gelida*. In Table 3 we reported the updated list of sponge-brittle star associations listed in the scientific literature for the Southern Ocean with updated information produced in this work.

There are no specific studies where the degree of symbiosis that these species present is established. For Gutt and Schickan (1998)[244] it is a mandatory relationship for *Iophon radiatus*, indicating that it would be a proto cooperation, a stage prior to mutualism. If we consider that they are species with ecological success due to their wide distribution and abundance, since both species are Antarctic and Magellanic circumpolar and can be found massively, in some cases as dominant in Ross and Weddell Sea. Relationships between epibiotic sponges and their hosts are generally suggested to increase the fitness of these latter protecting them from predation. Anyway, Antarctic sponges are usually exploited as a trophic source [245], [247] playing a negative effect on their hosts.

The possible advantages that the association would provide for *Ophioplinthus* (understood as protection against predators) could perhaps come from the secondary metabolites that porifera habitually use to, together with their spicule structure, discourage predation by some predators. Thus, and taking into account the terminology used, although some authors have cited this relationship as parasitism, it cannot be understood, since it would occur due to the trophic dependence of the symbiont from the living tissues or internal fluids of the host (the brittle star), and there is no evidence to support this relationship between the two organisms). There seems to be no common opinion as to which, if only one, species of *Iophon* enters the symbiosis.

Scientific research mentions the presence of *Iophon radiatum* symbiosis to be considered as parasitic on spines of the pencil sea-urchins, by increasing their porosity and leading to their detachment. This aspect should be better explored in future research considering that *I. radiatum* is not known as a boring species [275].

Porifera	**Ophiuroidea**	**Relation**	**Reference**
Iophon sp.	*Ophioplinthus brevirima*	Epibiosis	Mortensen 1936, Fell 1961, Madsen 1967, Bernasconi 1974, Hunter 2008, Martín Ledo 2010
Iophon sp.	*Ophioplinthus gelida*	Epibiosis	Koehler 1912, Koehler 1922, Mortensen 1936, Fell 1961, Cherbonnier 1962, Madsen 1967, Bernasconi 1974, Jangoux 1987b, Gutt 1992, 1995, 1998, Hunter 2008, Martín Ledo 2010
Iophon radiatum	*Ophioplinthus* sp.	Epibiosis	Barthel & Gutt 1992, Gutt & Schickan 1998
Rosella sp., *Pseudosuberites* sp.	*Ophioplinthus gelida, Ophioplinthus brucei, Ophiomastus conveniens, Ophiocten megaloplax, Amphiura angularis protecta, Amphiura deficiens*	Epibiosis	Kunzmann 1996
Rosella sp., *Scalymastra* sp.	*Astrotoma agassizii*	Epibiosis	Gutt 2000
Porifera spp.	*Astroclamys sol*	Epibiosis	Smirnov 1984
Iophon unicorne	*Ophioplinthus gelida, Ophioplinthus brevirima*	Epibiosis	**This work**
Iophon flabellodigitatum	*Ophioplinthus gelida, Ophioplinthus brevirima*	Epibiosis	**This work**

Table 3 – Table of reported porifera associated with brittle stars listed in scientific literature with updated information produced in this work.

With due approximation, two factors can be considered that could influence this high number of symbionts: a morphological one (an appropriate disc for fixation), and a numerical one, of host availability in the environment (facilitate the probability of encounter). One reason highlighted by Martin Ledo

(2010)[262] could be due to the morphology of *O. gelida* itself, since it has a disc formed by thick plates, making it almost rigid and, therefore, it assumes a stable surface, a condition that does not occur in other groups, such as the species of the families Amphiuridae and Ophiacanthidae, which have unstable flexible discs. If we add this to the fact that the plates have a rough surface with concentric lines, ideal for facilitating the anchoring of larvae, *O. gelida* becomes a good candidate for epibiosis. However, it must be taken into account that ophiuroids are echinoderms, and their plates form an endoskeleton that present an epithelium above it, therefore, to access the plates, a series of immunological barriers would have to be passed or settle directly on the organic part.

THESIS CONCLUSIONS

Given the peculiar conditions of the Antarctic environment and the need to define a standard work protocol that allows the comparison of the collected data, as well as the acquisition of all the relevant information, the first chapter presents guidelines for DNA barcoding approach from sampling to laboratory procedures in remote areas and hostile environment. In the guidelines we pay a specific attention to the treatment of the sample, which would require specific steps that are often skipped of overlooked. It is necessary that the procedures are carried out to avoid possible contamination of the material and in the shortest possible time to avoid possible degradation of the organisms. Particular emphasis is given to the photographic documentation of the fresh samples, a phase which is often omitted to speed up the lab processing phases but which allows, at a later stage, to obtain important information related to the appearance of the organism (e.g. its colour), which can be totally lost once the specimens are stored in museum collections, that are instead of great relevance in species definition and that can be used for scientific publications.

My research delved into the diversity of echinoderm species in the Terra Nova Bay area, utilizing a method known as "*Reverse Taxonomy*." This approach involved integrating molecular DNA barcodes (COI) with morphological analysis of preserved samples from the Italian National Antarctic Museum (MNA) – Genoa section.

This work specifically targeted a core of 360 echinoderm samples representative of the five currently existing classes of echinoderms (specifically 40 Asteroidea, 22 Crinoidea, 74 Echinoidea, 166 Ophiuroidea and 328 Holothuroidea), selected from a much larger set of samples stored at the MNA. Molecular screening (COI barcoding) was performed on 136 specimens, representing 19 different species, with a successful extraction, amplification and sequencing for all selected specimens.

This collection marks the initiation of a comprehensive revision of the Terra Nova Bay area fauna, based on sequences published in public repository to serve

as reference DNA barcodes for further studies. In fact, the substantial molecular data produced through DNA barcoding opens up avenues for comparative morphological studies and meta-analysis, offering valuable insights for ecological and molecular-based research. The use of a DNA barcode library is only efficient if it contains reference sequences with correct associated species identifications. Additionally, under-representation of geographical coverage within a species, may lead to an incomplete picture of its intraspecific genetic diversity [276]. Due to recent advances in sequencing technology and computational software, the implementation of DNA barcoding and barcode-based studies such as eDNA and metabarcoding are increasing [277]. DNA barcoding and DNA metabarcoding are two distinct molecular biology techniques used for species identification, each with its specific applications and methodologies. DNA barcoding is primarily employed for the identification of individual species and involves sequencing a short, standardized DNA region from a specific gene, often using Sanger sequencing. It is suitable for samples with known or low diversity and is commonly used in taxonomic and systematic studies, as well as biodiversity assessments. In contrast, DNA metabarcoding is geared towards the simultaneous identification of multiple species within complex samples, such as environmental DNA. This technique targets multiple DNA regions using high-throughput sequencing platforms like Illumina or Ion Torrent, making it well-suited for studying diverse communities in ecological and environmental contexts. While DNA barcoding is more focused on individual species, DNA metabarcoding provides a broader perspective on community composition and dynamics, contributing to our understanding of biodiversity in various research contexts. The application of this molecular techniques for species identification facilitated the development of comprehensive reference libraries that store the molecular sequences of specific genes, such as the widely used cytochrome c oxidase subunit I (COI) gene. Specifically, DNA barcode libraries do not only aid in species identification but are also implemented in other domains such as population genetics, phylogenetics, and community-based studies [278].

This work goes one step further by applying the "*reverse taxonomy*" approach, hence gaining the best from a detailed molecular analysis, which is then

combined with morphological data to obtain correct and stable species determinations and highlighting possible mismatches in sequence libraries. Furthermore, it reconfirms the validity of DNA barcoding for the identification of both adult organisms and juvenile forms (particularly larval stages) whose morphological identification is complex. With this work we want to put particular emphasis on the relevance of the biological collections and the great resource of information kept within the natural history museums (or research institutes), given the considerable relevance, simplicity and ease of comparison of the results obtained from the application of investigations based on molecular techniques (specifically DNA barcoding). A systematic screening program of biological samples preserved in collections is desirable for the future in order to fill important missing data in molecular libraries and make the use of information and the reliability of identifications even more effective [279].

The results strongly suggest that a constant effort is needed to increase our knowledge and must go in parallel with a review of the previous information deposited in the scientific literature with the application of the most modern investigative technologies to specimens present in museum collections. The results show how an erroneous identification can lead to an overestimation or underestimation of biodiversity in a given area, with obvious impact also of numerous scientific fields and research effort where a correct taxonomy is the key (e.g. *Abatus koehleri*, previously *A. elongatus*, in Chapter 4).

This work provides an update of knowledge and species lists regarding the five classes belonging to the phylum Echinoderms (Asteroidea, Holothuroidea, Ophiuroidea, Crinoidea and Echinoidea). This is only the beginning of unravelling the echinoderms biodiversity in the Terra Nova region as more locations (e.g. those more far from the research station) and less accessible areas (e.g. deeper communities) could still host an undiscovered diversity. With this study we again emphasized the need to combine morphology and genetics in species identification to gain the most accurate estimate of biodiversity.

Previous studies assessed Echinoderms diversity for the study area focused on sea stars, sea urchin and brittle stars [34], [63]. Specifically in this work, we

addressed the absence, till now, of a checklist for Holothuroidea (sea cucumber) and Crinoidea (feather stars) belonging in the area and the revision of the present information's in literature. Consequently, we report 15 new species records of sea cucumbers (Chapter 2) and 4 of crinoids for the area (Chapter 4).

With the aim of cross-referring the information obtained with this work with the one previously archived in the literature, I searched previously identified samples deposited in the MNA collection. Unfortunately, only a small amount of previously studied and published material has been permanently deposited in the museum collection making the possibility of comparing the results quite complex and very limited. Sample MNA-00573 is part of the published material from Chiantore *et al.*, 2006 [63] and present original identification label as *A. elongatus*. This sample, in our work, was successfully sequenced, morphologically reviewed and identified as *A. shackletoni*. For this reason, we consider the presence of *Abatus koehleri* (previously *A. elongatus)* in the Terra Nova Bay area questionable and the published records in the Southern Ocean Echinoid database as doubtful and could fall within the case of overestimation of biodiversity due to the limitations of morphological identification.

A review of the state of knowledge was necessary due to potential error in the classification in the *Odontaster* genus after the results presented by Janosik & Halanych, (2010) [21] due to the limitation of morphological approach previously employed. The two species, *Odontaster roseus* and *Odontaster pearsei*, have been repeatedly misidentified as *O. meridionalis* in the Ross Sea sector which leads us to believe that, due to the increase in the number of reference sequences during the last decade, the existence of a genetic entity called *O. meridionalis* needs to be further investigated (Chapter 3). Several publications highlight the occurrence of *O. meridionalis* in the Ross Sea quadrant. For instance, Caputi *et al.* (2020) [168] attribute to this species the role of omnivorous predators alongside *O. validus*, but their determination is wrong and the apex predator role remain unassigned. Consequently, it is mandatory to base any ecological paper on a good taxonomy, prioritizing accurate species identification.

In this work, attention has also been focused on the characterization of the

association between *Ophioplinthus* genus brittle stars and sponges of the *Iophon* genus. Therefore, we register the first record of *I. flabellodigitatum* on *O. brevirima* and the first record of *I. unicorne* on *O. gelida* (Chapter 5). The molecular characterization of sponge organisms is currently underway at the laboratories of the Anton Dohrn Zoological Station (Naples, Italy) and this information could shed further light on the association between these two organisms. Furthermore, thanks to the "LinnéSys: Systematics Research Fund" grant awarded by The Linnean Society of London (London, UK), 3D Microtomographys will be undertaken using a Skyscan 1172 micro-CT system (Bruker®), Hamamatsu 100/250 X-ray source and Hamamatsu C9300 11 Mpx camera at the Department of Geosciences – University of Padua (Italy). To unravel the morphological, ecological and evolutionary implications of the relationship between brittle star and sponge we will use this innovative technique to understand i) how the sponge adheres to the brittle star; ii) if the presence of the sponge determines a change in the morphology of the brittle star; iii) if the presence of the *Iophon* sponge causes a change in the porosity of the plates in the brittle star.

Overall, DNA barcoding stands as a foundational technique and a valuable tool to address the limitations of morphological taxonomy (arising from both cryptic and unidentified diversity). Nevertheless, only the synergistic use of molecular and morphological techniques, exemplified by the "*reverse taxonomy*" approach, emerges as the ideal procedure to provide the foundations of any ecological and conservation effort.

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Supplementary Materials

Chapter 3

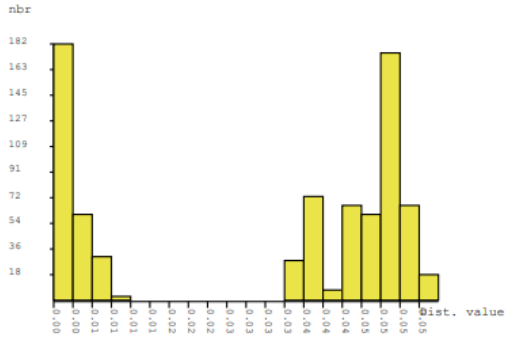
Supplementary Materials S1

PARTITION SPECIES ANALYSIS

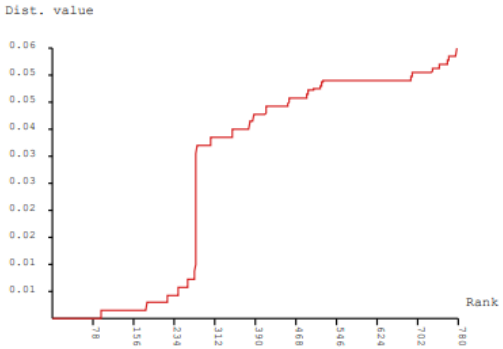
B1) ABGD

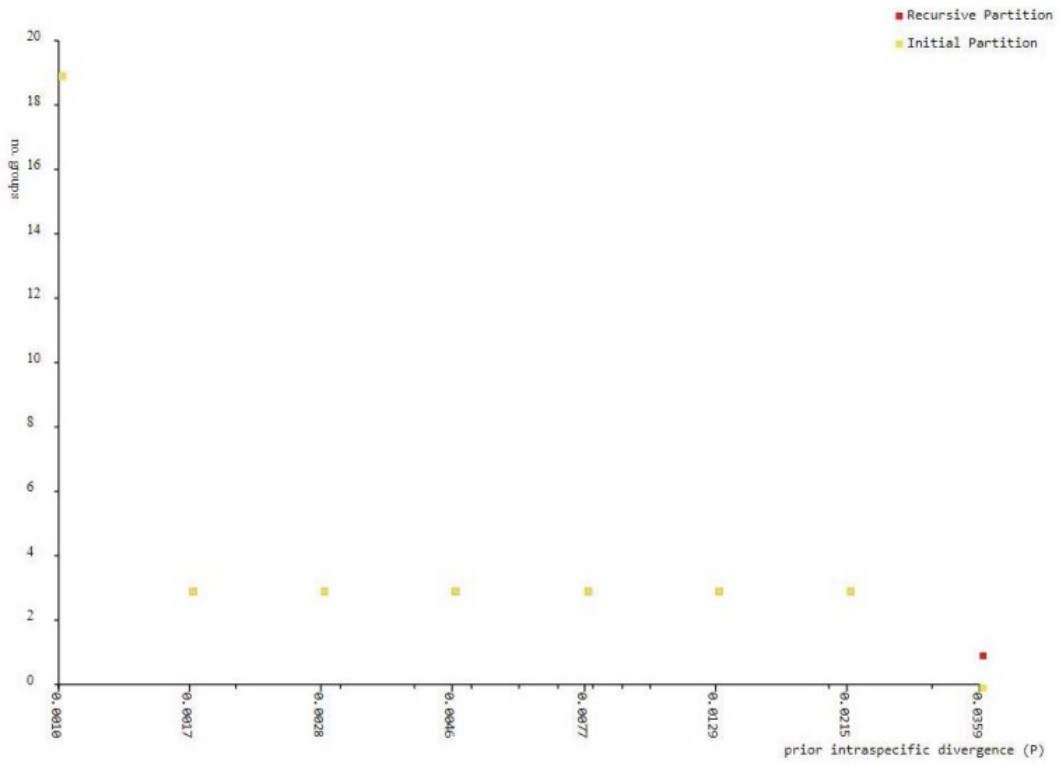
Group	N	Sample ID	Species
Group[1]	17	MNA02814 MNA03791 MNA03812 MNA03832 MNA03841 MNA05817 MNA06486 MNA06490 MNA08024 MNA08025 MNA08027 MNA08028 MNA08033 MNA08036 MNA08037 MNA08038 MNA08043	*O. roseus*
Group[2]	16	MNA02902 MNA03430 MNA03582 MNA03825 MNA04276 MNA04282 MNA04283 MNA05430 MNA06331 MNA08021 MNA08022 MNA08026 MNA08029 MNA08030 MNA08031 MNA08035	*O. validus*
Group[3]	7	MNA06116 MNA06489 MNA08023 MNA08032 MNA08034 MNA08039 MNA08042	*O. pearsei*

Histogram of distances



Ranked distances





B2) bPTP

Species 1 (support = 0.653)
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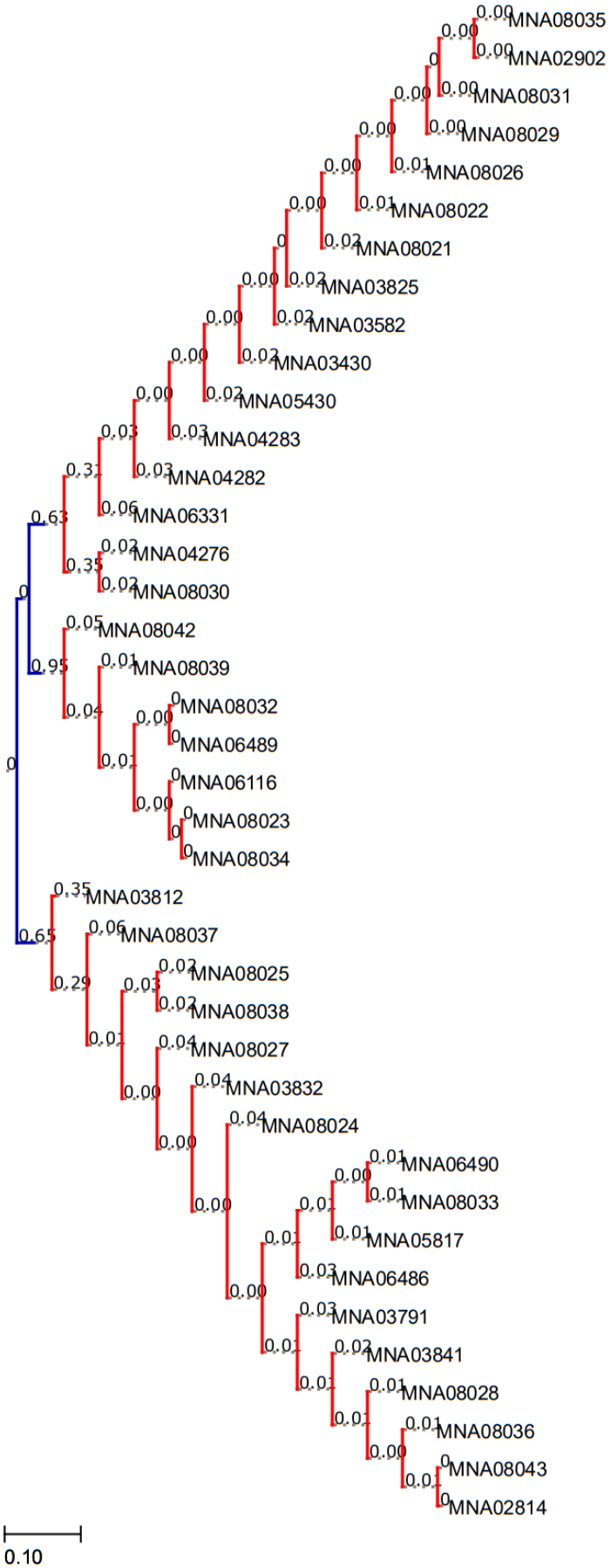
91,MNA03841,MNA08028,MNA08036,MNA08043,MNA02814

Species 2 (support = 0.633)
MNA08035,MNA02902,MNA08031,MNA08029,MNA08026,MNA08022,MNA08021,MNA03825,MNA03582,MNA03430,MNA05430,MNA042
83,MNA04282,MNA06331,MNA04276,MNA08030

Species 3 (support = 0.947)
MNA08042,MNA08039,MNA08032,MNA06489,MNA06116,MNA08023,MNA08034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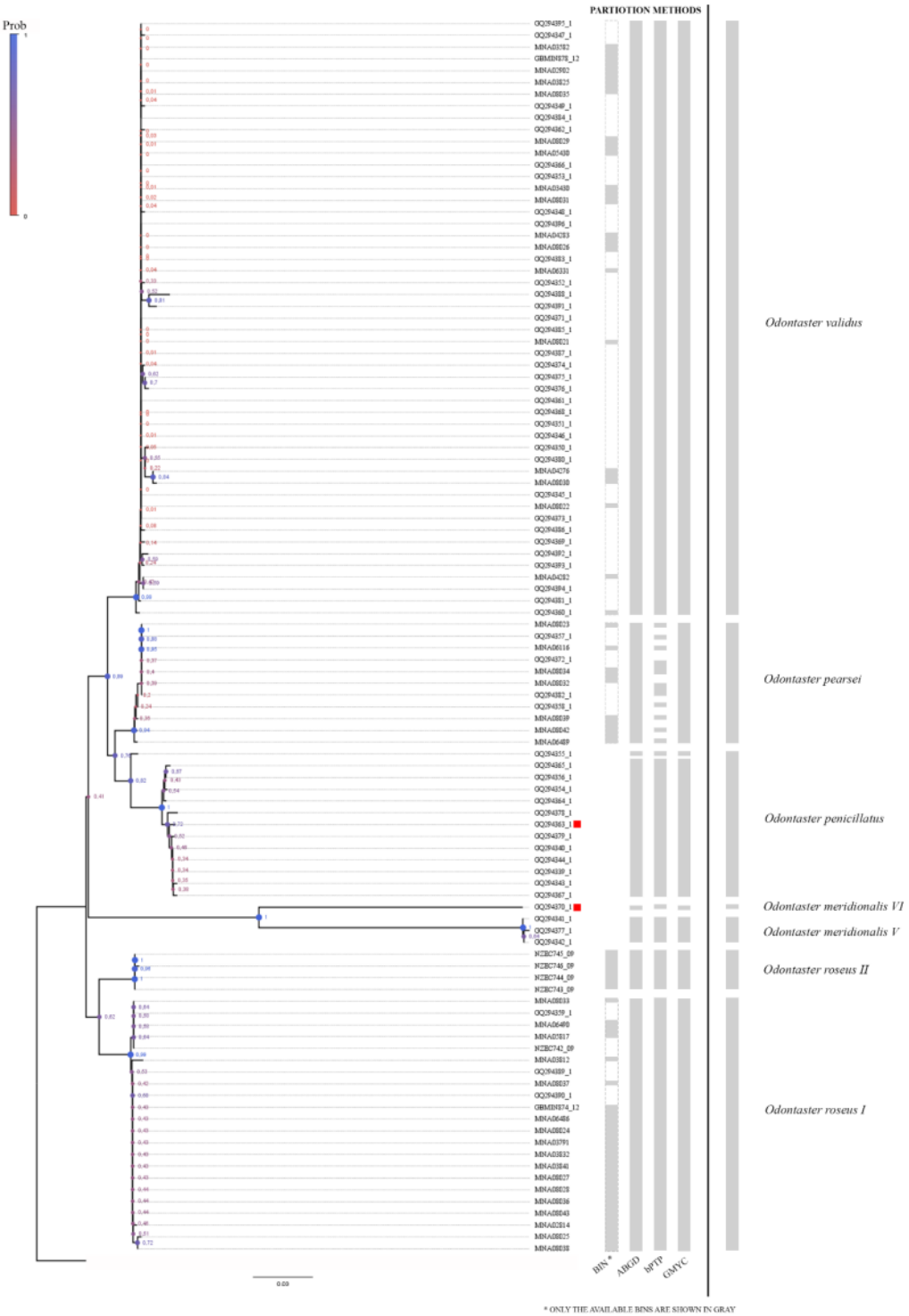
Supplementary Materials S2

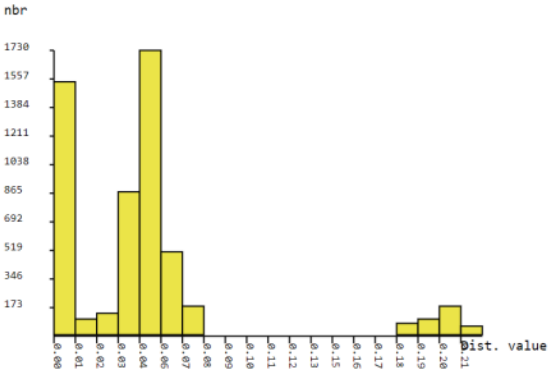
C1) Tree topology with species partition results

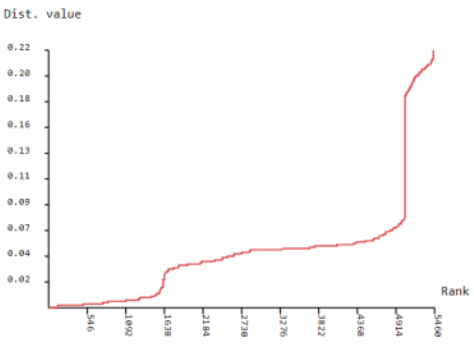


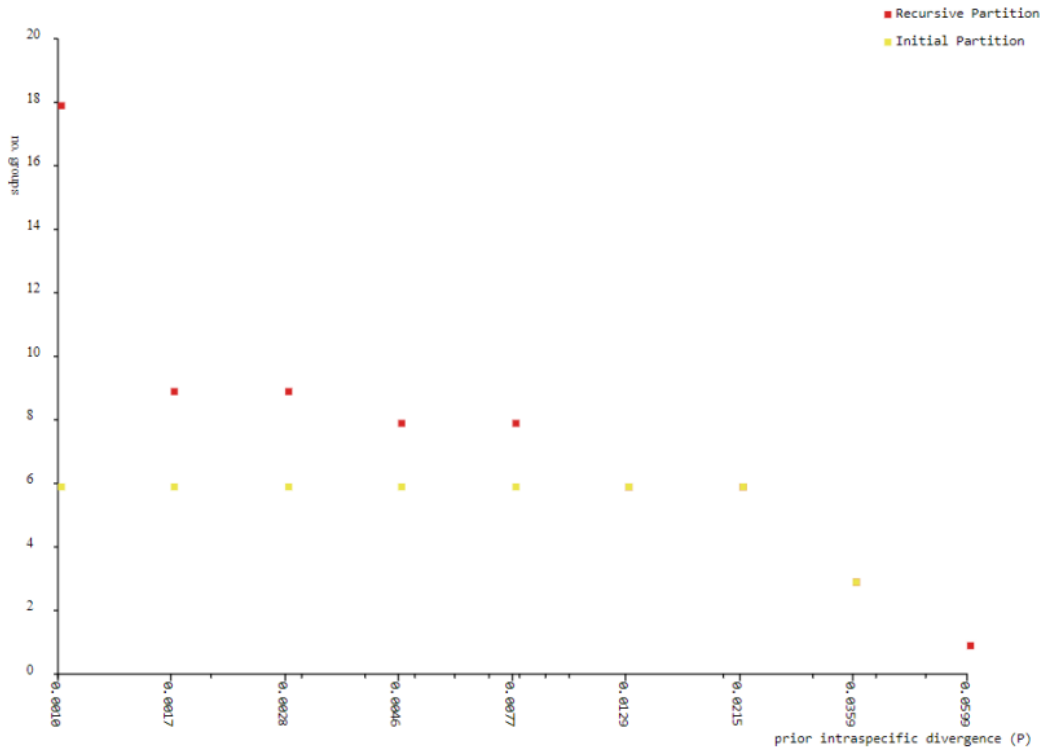
Supplementary Materials S3

C2) ABGD results

GROUP	N	SAMPLE ID	SPECIES
Group[1]	3	GQ294377_1 GQ294341_1 GQ294342_1	*O. meridionalis V*
Group[2]	1	GQ294370_1	*O. meridionalis VI*
Group[3]	4	NZEC743_09 NZEC744_09 NZEC745_09 NZEC746_09	*O. roseus II*
Group[4]	22	GQ294389_1 MNA03812 NZEC742_09 MNA05817 MNA06490 MNA08033 GQ294359_1 MNA02814 MNA08037 GQ294390_1 GBMIN874_12 MNA06486 MNA08024 MNA03791 MNA03832 MNA03841 MNA08027 MNA08028 MNA08036 MNA08043 MNA08025 MNA08038	*O. roseus I*
Group[5]	51	GQ294388_1 GQ294381_1 GQ294360_1 GQ294391_1 GQ294361_1 GQ294375_1 GQ294376_1 GQ294368_1 MNA04276 MNA08030 GQ294392_1 GQ294371_1 MNA04282 GQ294350_1 GQ294386_1 GQ294384_1 GQ294396_1 GQ294362_1 GQ294366_1 GQ294369_1 GQ294395_1 GQ294353_1 GQ294393_1 GBMIN878_12 MNA02902 GQ294385_1 GQ294345_1 MNA04283 MNA05430 MNA03430 MNA03582 MNA03825 MNA08021 MNA08022 MNA08026 MNA08029 MNA08031 MNA08035 GQ294351_1 GQ294349_1 GQ294387_1 GQ294352_1 GQ294373_1 GQ294374_1 GQ294383_1 GQ294347_1 GQ294348_1 GQ294380_1 GQ294394_1 GQ294346_1 MNA06331	*O. validus*
Group[6]	11	GQ294358_1 MNA06489 GQ294382_1 MNA08032 MNA08034 MNA08039 MNA08042 GQ294372_1 MNA06116 MNA08023 GQ294357_1	*O. pearsei*
Group[7]	1	GQ294355_1	*O. penicillatus*
Group[8]	12	GQ294378_1 GQ294364_1 GQ294365_1 GQ294354_1 GQ294356_1 GQ294363_1 GQ294343_1 GQ294340_1 GQ294379_1 GQ294344_1 GQ294339_1 GQ294367_1	*O. penicillatus*







C3) bPTP

GROUP	SUPPORT	SAMPLE ID	SPECIES
Species 1	support = 1.000	GQ294370_1	*O. meridionalis VI*
Species 2	support = 0.806	GQ294342_1,GQ294377_1,GQ294341_1	*O. meridionalis V*
Species 3	support = 1.000	MNA06489	*O. pearsei*
Species 4	support = 0.504	MNA08032,GQ294382_1	*O. pearsei*
Species 5	support = 1.000	MNA08042	*O. pearsei*
Species 6	support = 1.000	MNA08023	*O. pearsei*
Species 7	support = 1.000	GQ294357_1	*O. pearsei*
Species 8	support = 1.000	MNA06116	*O. pearsei*
Species 9	support = 0.970	MNA08034,GQ294372_1	*O. pearsei*
Species 10	support = 1.000	GQ294358_1	*O. pearsei*
Species 11	support = 1.000	MNA08039	*O. pearsei*
Species 12	support = 0.084	MNA03430,MNA04283,MNA02902,GBMIN878_12,GQ294385_1,MNA08026,MNA08031,GQ294384_1,GQ294345_1,GQ294362_1,GQ294352_1,GQ294387_1,GQ294391_1,GQ294388_1,MNA06331,GQ294346_1,GQ294383_1,GQ294373_1,MNA08030,MNA04276,GQ294350_1,GQ294380_1,GQ294394_1,MNA04282,GQ294347_1,GQ294374_1,GQ294381_1,GQ294360_1,GQ294348_1,GQ294386_1,GQ294393_1,GQ294392_1,GQ294371_1,GQ294353_1,MNA08029,MNA08021,MNA08035,GQ294351_1,GQ294368_1,GQ294395_1,GQ294366_1,GQ294375_1,GQ294376_1,MNA08022,MNA05430,GQ294369_1,MNA03825,MNA03582,GQ294349_1,GQ294396_1,GQ294361_1	*O. validus*

Species 13	support = 0.467	MNA03812,MNA08038,MNA08025,MNA06490,GQ294359_1,MNA05817,MNA08033,NZEC742_09,MNA08036,MNA03832,GQ294390_1,MNA06486,MNA08028,MNA03841,MNA08027,MNA08037,GBMIN874_12,MNA03791,MNA08024,MNA08043,MNA02814,GQ294389_1	*O. roseus I*
Species 14	support = 0.687	NZEC746_09,NZEC745_09,NZEC743_09,NZEC744_09	*O. roseus II*
Species 15	support = 0.953	GQ294355_1	*O. penicillatus*
Species 16	support = 0.895	GQ294365_1,GQ294354_1,GQ294364_1,GQ294356_1,GQ294378_1,GQ294344_1,GQ294340_1,GQ294367_1,GQ294339_1,GQ294343_1,GQ294379_1,GQ294363_1	*O. penicillatus*

Supplementary Materials S1

PARTITION SPECIES ANALYSIS

1) ABGD

Group	N	Sample ID	Species
Group[1]	12	MNA-07967 MNA-07964 MNA-05759 MNA-08184 MNA-09159 MNA-06513 MNA-08182 MNA-08183 MNA-03766 MNA-03855 MNA-03795 MNA-03760	*Notocrinus virilis* Mortensen, 1917
Group[2]	6	MNA-05491 MNA-06108 MNA-05755 MNA-07965 MNA-10419 MNA-07947	*Anthometrina adriani* (Bell, 1908)
Group[3]	1	MNA-08074	*Florometra mawsoni* AH Clark, 1937
Group[4]	1	MNA-06514	*Promachocrinus kerguelensis* Carpenter, 1879
Group[5]	2	MNA-05676 MNA-07963	*Promachocrinus kerguelensis* Carpenter, 1879
Group[6]	3	MNA-08435 MNA-08447 MNA-08448	*Antrechinus* sp.
Group[7]	1	MNA-08451	*Sterechinus antarcticus* Koehler, 1901
Group[8]	13	MNA-08464 MNA-02886 MNA-10574 MNA-02885 MNA-03576 MNA-03577 MNA-08462 MNA-08463 MNA-08465 MNA-08466 MNA-08468 MNA-08469 MNA-08470	*Sterechinus neumayeri* (Meissner, 1900)
Group[9]	1	MNA-09365	*Ctenocidaris* sp.
Group[10]	12	MNA-08564 MNA-08457 MNA-04500 MNA-04497 MNA-00577 MNA-02896 MNA-04498 MNA-04499 MNA-05748 MNA-08478 MNA-08479 MNA-08480	*Abatus (Pseudabatus) nimrodi* (Koehler, 1911)
Group[11]	3	MNA-08436 MNA-084311 MNA-08437	*Brachysternaster chesheri* Larrain, 1985
Group[12]	4	MNA-05905 MNA-08185 MNA-08432 MNA-08433	*Abatus cordatus* (Verrill, 1876) + *Anthometrina adriani* (Bell, 1908)
Group[13]	2	MNA-05746 MNA-08492	*Abatus ingens* Koehler, 1926
Group[14]	2	MNA-03454 MNA-08446	*Abatus curvidens* Mortensen, 1936
Group[15]	27	MNA-02937 MNA-08489 MNA-08491 MNA-05747 MNA-00573 MNA-08486 MNA-08495 MNA-08476 MNA-08471 MNA-08475 MNA-08488 MNA-08494 MNA-08565 MNA-08490 MNA-08493 MNA-08485 MNA-08483 MNA-02897 MNA-03439 MNA-08449 MNA-08472 MNA-08473 MNA-08474 MNA-08484 MNA-08487 MNA-08496 MNA-08497	*Abatus shackletoni* Koehler, 1911
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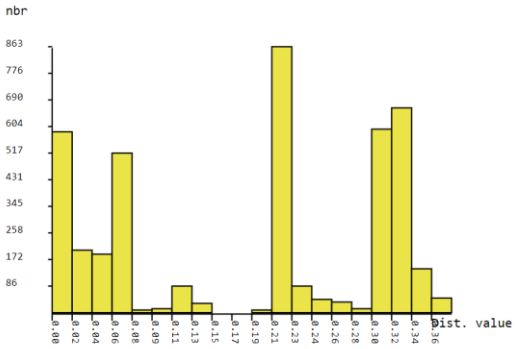
Group[17]	1	MNA-08434	*Abatus* sp.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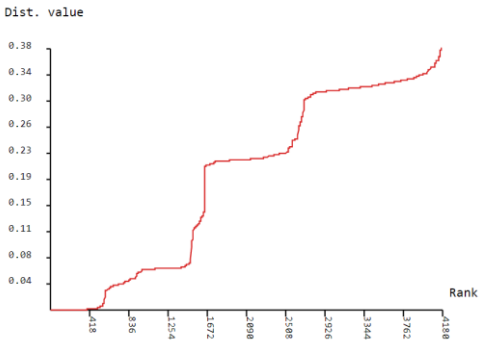
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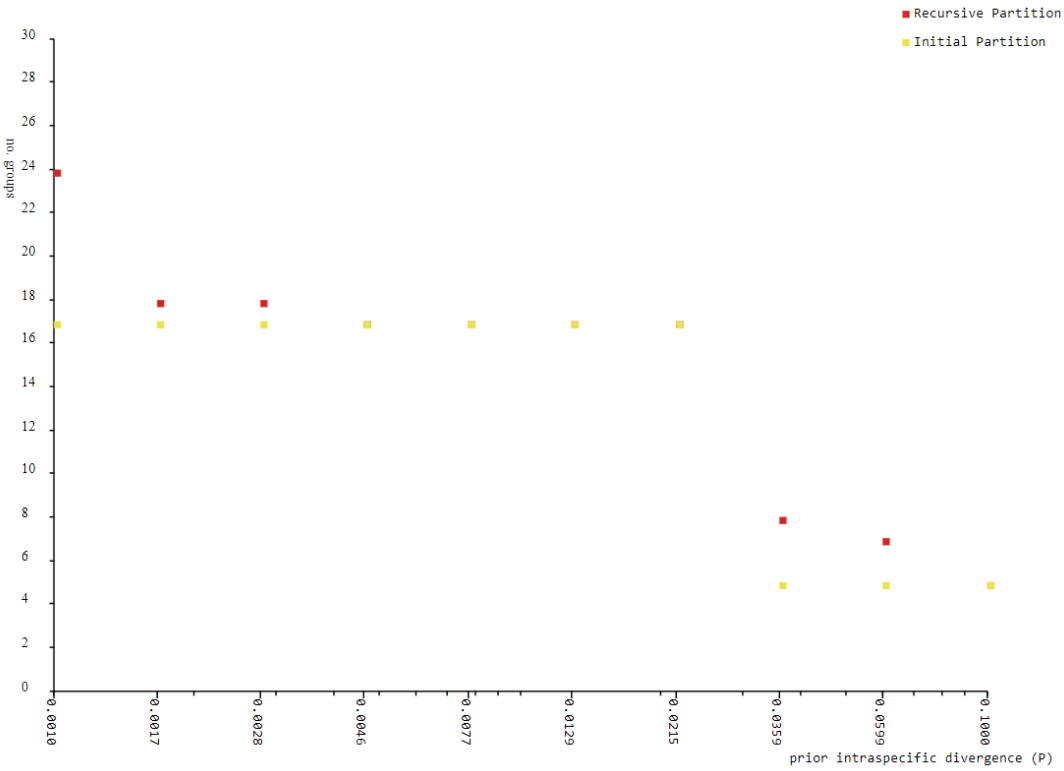
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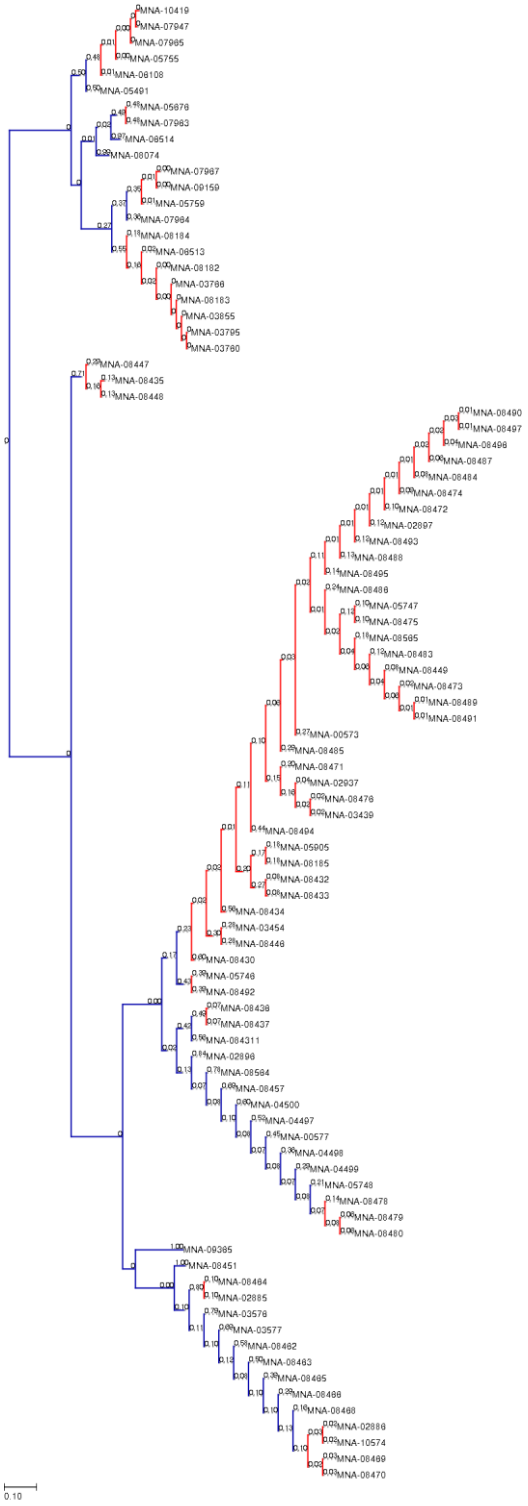
1) bPTP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Supplementary Materials S2