UiT The Arctic University of Norway

Department of Arctic and Marine Biology

Phylogeography and gut content of *Priapulus caudatus* in Norway

Maren Christine Hansen

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Abstract

Priapulus caudatus is a marine worm in the phylum Priapulida, commonly known as penis worms. Since it was discovered, *P. caudatus* has had many names that are now considered synonyms. Fossils of Priapulida from the Cambrian period are found all over the world, and extant species show that there have been little changes in their morphology since the Palaeozoic. *P. caudatus* are found in clay/muddy sediment from shallow waters to great depths and records are mostly confined to the northern hemisphere. There is not much knowledge about the role of priapulids in the marine ecosystem, but locally they can be abundant members of benthic communities. Few studies about their diet are available from e.g., the Baltic Sea and Canada, which indicates that extant priapulids feed on a variety of invertebrates like polychaetes, Ophiurida and Amphipoda, but also detritus and meiofauna. Likewise, the priapulid *Ottoia prolifica*, a very abundant species from the middle Cambrian Burgess Shale biota, is found to have a diet consisting of both live prey such as various invertebrates, and detritus present in its habitat. For determining the diet of *P. caudatus* in Norwegian waters, X-ray microtomography was used considering its capability of providing more details than when utilizing traditional microscopy methods. It is a non-destructive way of investigating specimens, with the possibility of also investigating the gut contents of fossil priapulids. Detritus and sediment were found to be the main gut content, with some foraminifers, suggesting it being a detritivorous animal that occasionally feed on other invertebrates. Additionally, DNA was extracted from the gut of specimens from the study site in Tromsdalen to be further analyzed with DNA metabarcoding. Metabarcoding did not work, but COI sequences revealed interesting phylogeographic relationships with potentially cryptic species.

Foreword

Firstly, I would like to express my gratitude to my main supervisor Joel for his encouragement and guidance through this project. He has always been available, assisting me in collecting specimens, guiding me through XMT data and helping with the phylogenetic tree, amongst other things. I am also thankful for my supervisor Andreas, whose expertise has been of great help. His considerations with making sure I have everything I need at the office is highly appreciated. Additionally, I want to thank my supervisor Kim Præbel for his valuable insights and guidance, especially during challenges encountered in the metabarcoding approach.

My main objective in this thesis was to investigate gut contents of *Priapulus caudatus* through XMT and DNA metabarcoding, with the overarching goal of gaining a deeper understanding of what the role of priapulids are in benthic ecosystems along the Norwegian coast. Although metabarcoding failed to provide any data on prey, DNA sequences from the specimens themselves were produced, leading to a shift in focus towards DNA barcoding and phylogeography. Thus, I would like to thank the genetics research group at NFH for their assistance in preparing and processing my data. Special appreciation goes to the bioinformaticians Mads Kristina Reinholdt Jensen and Daniel Kumazawa Morais, for being available to explain my data output. Also, their patience when I didn't understand what I was looking at and asked questions I didn't really know how to formulate is highly appreciated.

Lastly, I extend heartfelt thanks to my friends and family for their emotional support and encouragement. Thanks to my mom, for her love and always supporting my decisions, even if she disagrees with them, and to my dad for his genuine interest and excitement about the project. I am immensely grateful for him introducing me to the ocean and all its wonders at a very young age, igniting my passion for marine biology.

Table of Contents

List of Tables

List of Figures

1 Introduction

1.1 Evolutionary history, current distribution and phylogeography

Priapulida, a group of marine worms belonging to the Ecdysozoans, originated approximately 500 million years ago during the Cambrian period (Huang et al., 2004b; Kroer et al., 2016; Vannier et al., 2010). This era witnessed significant changes in the sediment-water interface was drastically changing going from firm seafloors to softer substrates, allowing for colonization of bioturbators (Bottjer et al., 2000; Kesidis et al., 2019; Vannier et al., 2010). This change made suitable habitat for soft-bodied inhabitants in the sediment, like priapulids, which were notably abundant in the Burgess shale biota (Conway Morris, 1977; Huang et al., 2004a). Fossilized burrow systems show remarkable similarities with those of extant species, underscoring their long evolutionary history as infaunal burrowers (Kesidis et al., 2019; Vannier et al., 2010). Among the vast amount of fossil records, the priapulid *Ottoia prolifica* stands out as a particularly abundant species in the Burgess Shale biota, with studies of its gut contents revealing a varied diet (Vannier, 2012).

During the Cambrian period, the emergence of diverse animal phyla, including nematodes and priapulids, reshaped marine ecosystems (Bottjer et al., 2000; Kesidis et al., 2019; Vannier et al., 2010). Kesidis et al. (2019) suggested that priapulid-like scalidophorans were present in both sandy and muddy bottoms, indicated by treptichnids – large, open probing burrows which are trace fossils associated with scalidophoran activity. Most priapulid-like worms from the Cambrian have a worm-like trunk and an introvert with scalid-like structures (Conway Morris, 1977; Schmidt-Rhaesa, 2013), but how closely related extant priapulids are to fossils from Cambrian remain controversial. This is because the typical morphological traits that generally are associated with priapulids are not exclusive to this taxon, as they are also present in other taxa (Budd & Jensen, 2000; Schmidt-Rhaesa, 2013), and most of the old Cambrian fossils may be from the scalidophoran stem group rather than the priapulid crown group. The best characters for investigating a priapulid relationship of the fossils, are the presence of cuspidate teeth and the scalid arrangement which is very particular for priapulids and are clearly present in some species from the Cambrian fauna (Adrianov & Malakhov, 1996; Conway Morris, 1977; Huang et al., 2004a, 2004b), indicating they may be stem lineage representatives of Priapulida (Schmidt-Rhaesa, 2013).

Following the Cambrian period, priapulids experienced a decline in abundance and species richness. The reasons behind this shift remain speculative, but it suggests significant ecological changes occurred post-Cambrian, impacting priapulid populations (Vannier et al., 2010). There are 22 known species of extant Priapulida, where *Priapulus* and *Halicryptus* are the only two macrobenthic genera, the rest are mainly meiobenthic (Schmidt-Rhaesa et al., 2017; Vannier et al., 2010). Populations of extant priapulid species are globally distributed, but most occurrences documented are primarily confined to the northern hemisphere (Figure 1A). The genus *Priapulus* includes the three species *Priapulus abyssorum*, *Priapulus caudatus* (Figure 1D) and *Priapulus tuberculatospinosus*, where *P. caudatus* is the most well-known species of priapulids distributed widely throughout the Arctic, and the Norwegian coast (Figure 1B). However, since *P. caudatus* was discovered, it has had many names that are now considered synonyms (Adrianov & Malakhov, 1996; Paulay, 2024; Schmidt-Rhaesa, 2013; van der Land, 1970). As stated by Kolbasova et al. (2023), all this have raised questions whether *P. caudatus* constitutes a single species or a cryptic species complex, particularly in the light of the findings from the phylogeographic study conducted by Laakkonen et al. (2021), which identified substantial genetic variations among individuals from different regions of the northern hemisphere. The term cryptic species complex is imprecisely used in scientific literature, but is in general used for species that are closely related and often morphologically difficult to identify (Shin & Allmon, 2023).

Kolbasova et al. (2023) investigated *P. caudatus* from throughout the Arctic, where five divergent COI lineages were detected, and sequences from four clades appeared sympatrically. Kimura 2-parameter (K2P) distances were higher than that of similar taxa, ranging from 6.87%-14.35%, indicating a potential cryptic species complex. They also detected genetic diversity among intertidal and subtidal populations, which may be due to fluctuations in intertidal populations during glaciation cycles (Kolbasova et al., 2023). Situated within the Arctic, Northern Norway also has a history of glaciations and other geological events that may have influenced priapulid populations. Parts of this thesis will be devoted to putting priapulid specimens collected around Tromsø within the broader framework established by Kolbasova et al. (2023).

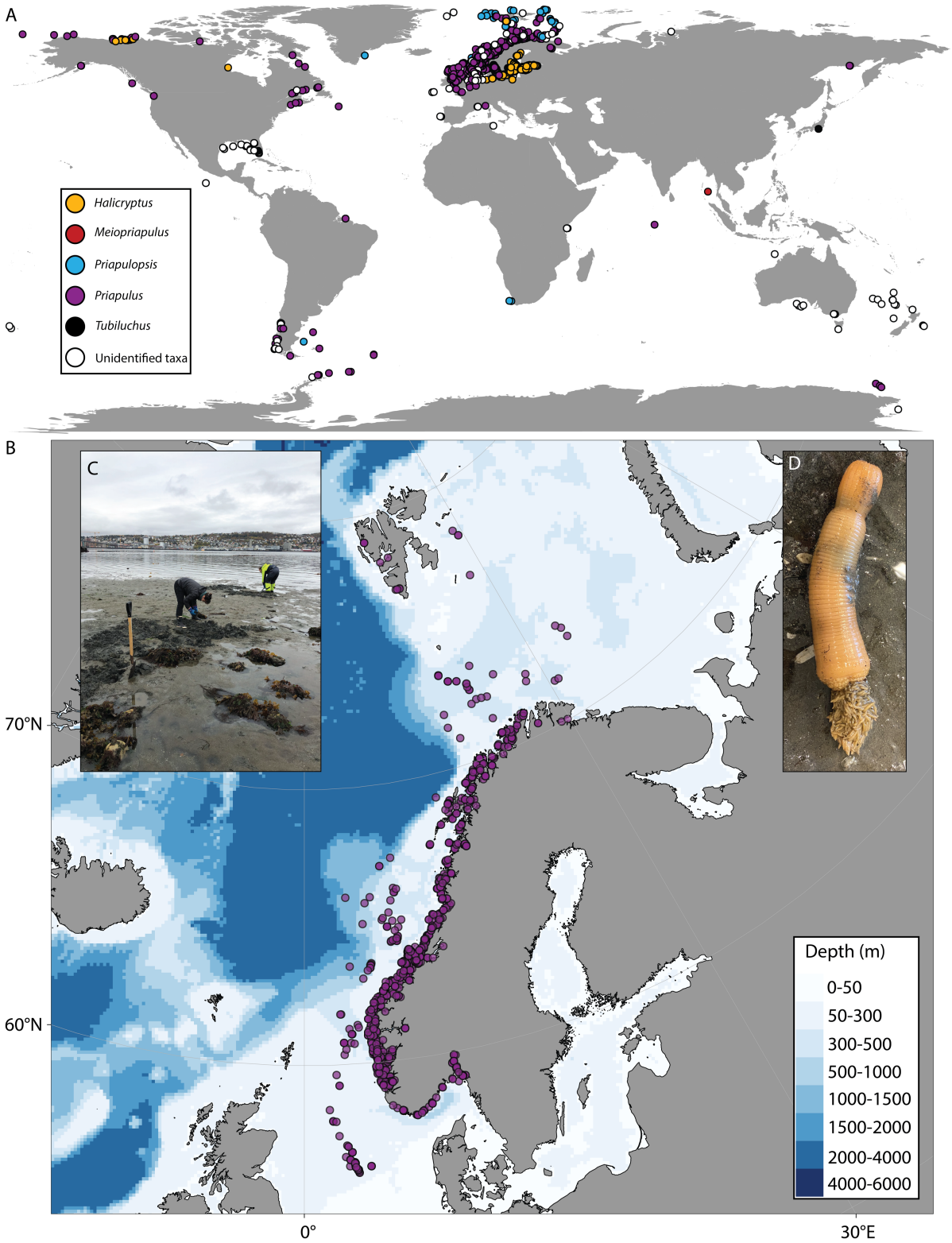

Figure 1: A, Worldwide distribution of the priapulid genera available with coordinates, data set from GBIF.org (14 November 2023) GBIF Occurrence Download https://doi.org/10.15468/dl.tkvh7d; B, distribution of *Priapulus caudatus* in Norway, data set from GBIF.org (21 September 2023) GBIF Occurrence Download https://doi.org/10.15468/dl.3hy529; C, Photo by Joel Wernström showing the muddy habitat where *P. caudatus* specimens were collected; D, A worm collected in Tromsdalen.

1.2 Morphology

Priapulid species exhibit a wide size range from around 1 mm to 400 mm, and undergo growth by a series of molts (Schmidt-Rhaesa, 2013). Their body consists of up to four divisions: an introvert, a neck (mostly restricted to larvae), the trunk (or abdomen), and a caudal appendage (or tail) (van der Land, 1970). The body is flexible with the introvert in the anterior part containing internal short longitudinal muscles, being the most important locomotory organ and can be completely withdrawn into the trunk. The size of the introvert varies amongst the species, and in *P. caudatus* it is very large compared to the trunk length, although in some individuals it is the same length as the trunk (van der Land, 1970). 25 rows of scalids, conical structures organized in series, are found on the introvert. Each series has a species-specific number of scalids, aiding in identification (Adrianov & Malakhov, 1996; Schmidt-Rhaesa, 2013; Schmidt-Rhaesa et al., 2017; van der Land, 1970). The trunk is distinctly annulated with many annuli together with the circular muscles of the body wall, with internal longitudinal long retractor muscles (Schmidt-Rhaesa, 2013). The caudal appendage is a post-anal extension of the trunk with the skin being continuously with the skin of the trunk, and in *P. caudatus* the tail is divided into segments that are barely noticeable. The function of the tail is highly uncertain, but it is thought to be a respiratory organ because of the thin cuticle compared to the cuticle of the trunk and because it has been observed with its tail on the sediment-water interface (Fänge & Mattisson, 1961). However, studies indicate that it is only of important respiratory function in conditions of low oxygen concentration (van der Land, 1970).

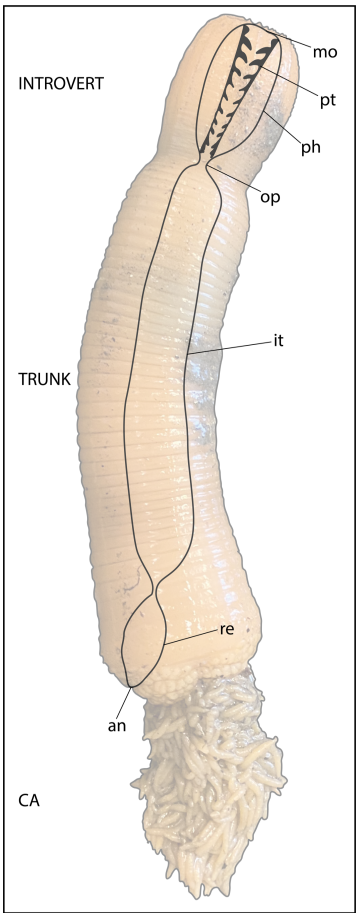

Figure 2: Simple drawing focusing on visualizing the digestive system of *P. caudatus*. Abbreviations: mo: mouth, pt: pharyngal teeth, ph: pharynx, op: oesophagus, it: intestinal tract, re: rectum, an: anus, CA: caudal appedage. Drawing inspired by Fig. 1 from van der Land (1970, p. 11).

The intestinal tract is a straight tube that runs from the terminal mouth opening to an almost terminal anus (Figure 2). In the foregut the pharynx is found, a morphological structure with strong muscles and cuticular teeth. When the introvert is fully everted, the anterior region of the pharynx becomes exposed, potentially facilitating prey capture (Schmidt-Rhaesa, 2013). The shape and size of pharyngal teeth can provide insights into the feeding behavior of priapulid species. Larger priapulids such as *P. caudatus* exhibit

cuspidate teeth, suggesting a predatory diet, while smaller species tend to have pectinate teeth which indicates detritivorous feeding habits (Wernström et al., 2023).

1.3 Ecology

Most priapulid research has been on morphology, taxonomy and systematics, and there is very little knowledge about their ecology and feeding behaviors (Shirley, 1990; Trott, 2017; van der Land, 1970). The studies that have been conducted on the ecology and feeding strategies are old and mostly done in the Baltic Sea and around Canada. Considering that *P. caudatus* is found in extremely high densities in some regions, up to 208 individuals per m^2 (Różycki & Gruszczyński, 1991), it is interesting to learn what their diet looks like along the Norwegian coast as they likely play an important ecological role as predators and possibly prey to commercially important species. *P. caudatus* is found from the intertidal zone down to abyssal depths, mainly in bottoms of sand, mud or silt (Figure 1C) (Schmidt-Rhaesa, 2013; van der Land, 1970).

Ankar and Sigvaldadottir (1981) studied another priapulid, *Halicryptus spinulosus,* in the Baltic Sea where their material was small, but considering the knowledge gap on the feeding behavior of priapulids, they still found it interesting to present the results. They found the most frequent gut content to be detritus, although a significant proportion of specimens had empty guts. They mentioned the carnivore *Glycera alba* as an example of why this might be – this species has a slow digestion time of 24 hours and feeds infrequently (every 5 to 10 days) which is linked to high growth efficiency combined with low energy consumption (Ockeimann & Vahl, 1970 in Ankar and Sigvaldadottir, 1981, p.50). *G. alba* is therefore able to withstand extended periods of starvation, and this energy balance could also be the case for *H. spinulosus*, and other Priapulida (Ankar & Sigvaldadottir, 1981). Because of the little knowledge about the feeding behaviors of Priapulida, Shirley (1990) did a long-term study in Auke Bay, Alaska to collect and present new quantitative data on *P. caudatus*. Auke Bay is around 40-60m deep and mostly consists of muddy substrate with diverse ichthyofauna, macrofauna and meiofauna, and *P. caudatus* were present in all subtidal stations (25-55m) with none present in the intertidal stations. They investigated both larvae and adults, where larvae contained undefined detritus and all adults had only mud in their intestines (Shirley, 1990). It has been suggested in other studies that adults are predators that mostly ingest mud

incidentally along with prey, and larvae are detritus feeders (Shirley, 1990; van der Land, 1970; Wernström et al., 2023), and Ankar and Sigvaldadottir (1981) found significant differences in food choice in relation to body size where bigger specimens chose bigger food items while smaller specimens chose smaller food items.

An observation by Wesenberg-Lund (1929) on *P. caudatus* eating the sylky sea cucumber *Chiridota laevis* indicates that macrobenthic priapulids do have predatorial capabilities. *P. caudatus* is described as an opportunistic predator and scavenger, preying on other invertebrates like polychaetes, ophiurids and amphipods, while Vannier (2012) reported on *Ottoia prolifica* feeding on hyolithids, brachiopods and arthropods as well as scavenging on carcasses and detritus with no evidence of *O. prolifica* favored one feeding strategy over the other. The food selection of some priapulid species is mainly consisting of detritus. Their feeding behaviors are shown to be different dependent on which location they are found. A reason for the variation in gut contents may depend on the food present in the location they are collected as there have been found differences in various habitats (Trott, 1998). As mentioned, learning more about the diet of *P. caudatus* is of importance due to the high local abundances it can have in Norwegian waters. In addition to this, understanding more about how feeding habits have changed in the very morphologically conserved extant priapulids as compared to their Cambrian counterparts, is also interesting from an evolutionary point of view, and was the original idea behind this thesis project.

1.4 This study

Given the knowledge gap in the ecology and feeding behaviors of *P. caudatus*, the main objective of this thesis was to investigate their diet using DNA metabarcoding and X-ray microtomography to gain knowledge about what their role in the marine benthic ecosystem is. Due to the lack of prey sequences from metabarcoding, DNA barcoding also became an area of interest.

Traditional morphological methods used to investigate gut contents such as direct observation of feeding, stomach content analysis (SCA), and stable isotope analysis (SIA), present limitations in studying invertebrate gut contents (Flo et al., 2024; McClenaghan et al., 2015; Rodríguez-Barreras et al., 2020; Urban et al., 2022). Identifying prey items through a

microscope requires expertise in morphology and taxonomy, and the observations will be biased towards big and well-preserved prey. Food items that are too small for microscopic identification or unidentifiable because of degradation and the lack of taxonomic features will be overlooked (Flo et al., 2024; McClenaghan et al., 2015; Pompanon et al., 2012). While SIA offers valuable ecological insights by examining the isotopic composition of tissues (Urban et al., 2022), it is subject to taxonomic limitations (Pompanon 2012).

DNA metabarcoding is an emerging tool for studying DNA in environmental samples like sediment, sea water and gut content (Bush et al., 2019; Miller-Ter Kuile et al., 2021; Watts et al., 2019; Yu et al., 2012), and has the potential of expanding the knowledge of ecological interactions like predation and dynamics (Rodríguez-Barreras et al., 2020). To my knowledge, this method has not previously been employed to investigate the gut contents of priapulids, however, it has been recognized as an effective tool for determining food sources compared to the morphological methods mentioned above (Rodríguez-Barreras et al., 2020; Urban et al., 2022). DNA metabarcoding builds upon the principle of DNA barcoding, a technique used to identify the taxonomic identity of unknown specimens by analyzing genetic markers. DNA barcoding involves extraction of DNA from the specimen of interest, amplifying the target gene (barcode) through PCR, sequencing the PCR amplicons, and comparing the sequences obtained with those in a reference database for taxonomic identification (Berry et al., 2015; Bush et al., 2019; Flo et al., 2024; Urban et al., 2022; Watts et al., 2019). In DNA metabarcoding, DNA is extracted from environmental samples, and primers capable of targeting multiple taxa within the sample are utilized, providing sequence data for all organisms present.

Commonly used DNA metabarcoding markers for diet studies are the mitochondrial cytochrome *c* oxidase subunit 1 (COI) and the nuclear genes 18S and 28S (Berry et al., 2015; Holovachov et al., 2017; Pompanon et al., 2012). In this study, we used the COI gene for assessing gut contents in *P. caudatus*. It is a quite conserved gene that changes at a rate not too fast or too slow, meaning it corresponds well to species and is therefore suitable for species identification in many taxa (Folmer et al., 1994). Andújar et al. (2018) argue in favor of the COI barcode remaining a popular choice for metabarcoding studies in the future.

Furthermore, a method used in this study for learning about the feeding behavior of *P. caudatus* is X-ray microtomography (XMT), a non-destructive and non-invasive way of investigating gut contents in situ with the capability of giving more details than when utilizing traditional microscopy methods (Olakanmi et al., 2023; Schoeman et al., 2016). By using XMT, specimens can undergo repeated testing while maintaining their integrity, also enabling the investigation of rare and unique specimens without perturbation (Bochaton et al., 2015; Mizutani & Suzuki, 2012). In biology, XMT have been mostly applied on the structural study of bones and teeth, but the development in recent years is also allowing for applications in analyses of soft tissues to provide insights into biological functions of morphological structures, as well as investigating stomach contents (Bochaton et al., 2015; Gutierrez et al., 2018; Mizutani & Suzuki, 2012). Windfelder et al. (2023) utilized micro-computed tomography (micro-CT) to create high-resolution 3D images of the gut compartments of the lepidopteran insect *Manduca sexta*. Their study revealed previously unstudied aspects of gut structure, highlighting the effectiveness of micro-CT for also investigating gut contents. Given the abundance of priapulid fossils available, it is noteworthy that this method has been successfully employed to investigate the gut contents of fossil invertebrates (Kraft et al., 2023). Thus, XMT could be a good candidate for advancing the comparative study of gut contents in fossil and extant priapulids, a topic only marginally explored (Vannier et al., 2010).

Although DNA metabarcoding did not yield COI sequences from prey species, successful sequencing of specimens themselves prompted a shift in focus of this study towards DNA barcoding and phylogeography. Kolbasova et al. (2023) conducted an extensive investigation into the phylogeography and population genetics of *P. caudatus*, identifying five divergent COI lineages and providing a framework for understanding the genetic diversity and historical patterns of *P. caudatus* populations. With my specimens from Tromsdalen and the application of phylogenetics, the inference of trees of relationships, the second objective of this thesis was to explore where these specimens would fall in the clades identified from Kolbasova et al. (2023), enhancing the understanding of phylogeography of *P. caudatus*.

2 Materials and methods

2.1 Collecting samples

2.1.1 Collection site from shore

My primary sampling site for *Priapulus caudatus* was at the shore in Tromsdalen (69°39'15.7"N, 19°00'02.4"E) which is a location known for the worms to be found (Figure 1C). The site consists of a mix of areas with muddy substrate stretching some decimeters deep, and areas with muddy substrate on the top underlain by rocks. All specimens were found in the same area. I sampled some other muddy locations along the shore in Tromsdalen (69°39'10.8"N, 19°00'12.8"E. 69°39'04.3"N, 18°59'14.6"E. 69°38'36.2"N, 18°58'39.2"E and 69°39'55.5"N, 19°01'06.3"E) but there were no signs of priapulids, and in general low diversity of marine macrofauna.

Specimens were collected by wading in the shore at low tide digging in the sediment with shovels (Figure 3A). The first tries were unsuccessful, the tide was too high, meaning I had to dig in the water which made it time consuming and difficult to get the sediment up, see Table 1. The first successful day of finding priapulids the tide was very low, and it was possible to spend much time digging directly in the sand. This made the sand easier to go through, and many times the priapulids were found when I took a block of sand and divided it. The specimens found were stored in a bucket with sea water from the collection site and transported to The Arctic University Museum of Norway.

Figure 3: A, Looking for *P. caudatus* after shoveling in the muddy substrate from collection site in Tromsdalen; B, Preserving samples in 96% molecular-grade ethanol, photo by Joel Wernström; C, Sorting through benthic material after dredging one of the localities explored with RV Helmer Hanssen; D, RV Helmer Hanssen.

Nine specimens were immersed briefly in clean freshwater to remove external contaminants before transferred to Falcon tubes with 96% molecular-grade ethanol (Figure 3B). The tubes were labelled with specimen number, date and sampling location and placed in zip-lock bags which were labelled with current date, sampling location, and my name. The specimens were stored in a freezer at -40 °C. The remaining six specimens were put in a tray with salt water and sediment from the collection site, while waiting for the feeding trials. The tray was put in an incubator at ~8 °C and a light dark cycle of 16/8 hours.

Table 1: Overview of how many worms were found each day of collecting samples and how low the tide was. The column Tide (cm) represents how high the water line is above the zero level for heights in tide tables, i.e. the reference level (https://www.kartverket.no/til-sjos/se-havniva/referanseniva/hva-er-et-referanseniva). The finding of the worm 21/08 and previous findings from this location indicated that it was a good locality to look for more worms.

Date (2023)	Tide (cm)	Number of specimens found	What specimens were used for	Comments
21/08	63	1		Collected for a different project.
23/08	80	0		Investigated different locations with muddy habitat.
19/09	62	0		Investigated two locations but too much wind and waves to be efficient.
21/09	86	0		Also tried another location – in estuary by Tromsdalen harbor.
30/09	15	2	Barcoding, morphology	
2/10	30	9	Culturing	
3/10	50	4	Barcoding, morphology	Big worms compared to earlier findings.
12/10	-	0		Field cruise Helmer Hanssen.
19/10	80	0		
2/11	93	0		
11/12	97	0		The water was higher than expected because of waves, did not manage to dig.

2.1.2 Helmer Hanssen

In order to find more specimens, a research cruise was conducted in the vicinity of Tromsø. The research vessel Helmer Hanssen (Figure 3D) sailed around Kvaløya with six planned stops for dredging to find priapulids. The planned stops were based on reports of occurrences of *P. caudatus* from Artsportalen. In the end five stops were made and in three locations the bottom was rocky with shells and kelp (Figure 3C). Two of the locations had muddy sand that were promising to find some priapulids, but it turned out it was too sandy and soft containing only few organisms. Priapulids were found in none of the locations.

2.1.3 Citizen science

A poster about collecting priapulids under their colloquial name "penis worms" (Figure 4) was made and put up at The Arctic University Museum of Norway as well as shared on their Facebook page. This poster was initially made for a citizen science project with the kayak club in Tromsø as they were interested in trying to collect priapulids. The poster was also shared on the Facebook page of the club to engage interest. The secondary school Læring gjennom arbeid (LGA) located near the collection site in Tromsdalen expressed their interest in helping to look for specimens. They saw the project through the social media of the museum but ended up not participating.

Figure 4: Poster calling for help from Tromsø locals in searching for penis worms. The poster starts with a short introduction of the worms, followed by a step-by-step guide on how to collect and where to deliver them. The QR code is for a YouTube video of a live *P. caudatus.*

2.2 Feeding experiments

Of the collected specimens six were kept alive in a tray for feeding experiments. While waiting for the lab, I tried to keep them alive by changing sea water two times per week. Four days after the worms were collected, I changed the sea water, and one worm were observed in the sediment with the caudal appendage on the sediment-water interface while the rest were laying on top. 10 days after the collection of the worms when the water was changed, the tiniest worm was dead as well as one bigger worm, both were removed from the tank. The week after two more worms were found dead when shifting the water. What all dead worms

had in common was that the lower part of the trunk and the caudal appendage turned black. The biggest worm was still alive. The worm that had formerly been in the sediment was now laying on top of it, still alive and looking healthy.

None of the worms survived long enough to be used in the feeding experiments. Since they only stayed on top of the sediment, the light exposure in the incubator might have had a negative effect on their survival. Additionally, it seemed difficult to go through with the experiment because the trays in the lab could not be filled with sediment.

2.3 DNA metabarcoding

The first step of DNA metabarcoding involved extraction of DNA from the gut content of my nine specimens. For this process the DNeasy PowerMax Soil Kit was used following the manufacturer's instructions (see Appendix A2). In addition to my nine specimens, there was a control tube without content. The worms were weighted, and their lengths measured (Table 2), followed by removal of gut content which was added to the PowerBead solutions. The solutions were then vortexed for 1 min. In the extraction protocol, the tubes were supposed to be placed in a vortex adapter and vortexed for 10 min at highest speed, but because the lab didn't have this adapter, I used a sample revolver instead. There was only room for six tubes, so I had them in for 40 min instead of 30, and every 5 min I vortexed the four remaining tubes. After 20 min I switched the tubes, so four was in the sample revolver, and for the remaining 20 min I vortexed the six tubes for 1 min each. They were then centrifuged at 4000 revolutions per minute (rpm) for 3 min at room temperature and transferred to clean collection tubes provided in the soil kit. In the extraction protocol it was stated to centrifuge at 2500 rpm, but it was changed to 4000 rpm. In the protocol, 5 ml of solution C6 were to be added to the center of the spin column membranes, but only 3 ml was added so the solution wouldn't be too diluted. The extracted DNA solutions for each specimen were divided into two tubes of 1.5 ml, one for further processing for DNA metabarcoding and one for backup.

Table 2: DNA concentrations from the DNA extraction including measurements of each specimen, where the caudal appendage is included in length. Sample 10 is a control sample.

Specimen	Length (cm)	Weight (g)	Gut content (g)	DNA concentration (ng/µl)
1	2.8	0.69	0.029	4,1
2	1.7	0.214	0.011	1,5
3	4.5	2.13	0.26	1,1
4	2	0.3	0.025	2,3
5	1.4	0.16	0.037	1
6	5	1.13	0.12	4,1
7	5.2	1.29	0.11	5,4
8	1	0.028	0.7	0,7
9	9	10.4	0.3	0,3
10	0	0	0	0

After the DNA extraction, the samples were delivered to the Genetics research group laboratory in The Norwegian College of Fishery Science at UiT – the Arctic University of Norway, who did the library PCR preparations and sequencing following the protocol found in Appendix A3. The expected amplicon size of COI for my *P. caudatus* specimens was ca. 313 bp. The data was pipelined by the Genetics research group following the protocol in Appendix A3.

2.4 X-ray microtomography

X-ray microtomography data of *Priapulus caudatus* gut contents was acquired prior to the start of the research project by Joel Wernström, using a Zeiss Xradia 620 Versa X-ray microscope at the Luleå University of Technology, Sweden. Prior to scanning, priapulid specimens were either stained with Lugol's solution for 24 hours and embedded in candle

wax or left unstained and stored in 70% ethanol. X-ray scans were individually adjusted to compensate for differences in sample preparation protocol, specimen sizes and tissue contrast, and were run under either 50 kV/5W/5s exposure time or 40 kV/3W/10s exposure time using a 4x objective. All scans were run with binning 2 applied. 3D renderings of *P. caudatus* gut content were reconstructed in the software VGStudio MAX 2022.3.

To begin with the tolerance on the tool were changed to get the best contrasts so necessary features would be possible to extract. Because of poor contrasts it was important to extract small sections at the time for getting the details. The first 3D rendering was of the cuticle of a *P. caudatus*, and to add voxels the tool region growing was used, varying the tolerance to get most the structure needed without extracting too much. When all voxels for the cuticle was extracted, the tools opening/closing, erode/dilate and smoothing were used to make the visually best 3D rendering. Erode/dilate takes granular structure and puts the voxels together based on how close they are to get a finer structure. The smoothing tool was used to get a better visual, and different strengths of the tool were tested. For internal organs such as gonads and intestine, including gut contents, the tool region growing was used followed by coloring of the different components to give a better visual.

2.5 DNA barcoding

2.5.1 Phylogeography

With DNA barcoding I wanted to place the specimens collected in Tromsdalen in the phylogeographic framework presented by Kolbasova et al. (2023). In addition to the COI sequences from my specimens, I obtained *P. caudatus* COI sequences downloaded from the BOLD database (https://boldsystems.org/index.php).

The sequence analyses were done in Geneious Prime version 2023.2.1 (Biomatters Ltd, New Zealand). The sequences were aligned using the MUSCLE algorithm, and the alignment was then manually trimmed. From the alignment a tree was built using the Geneious Prime MrBayes plugin (Huelsenbeck & Ronquist, 2001), and run with a GTR+G+I model, four MCMC chains runs for 1.100.000 generations and sampled every 1000 generations. The first 250 000 trees were discarded as burn-in.

The sequences on the resulting Bayesian tree were colored based on their locations found in BOLD. The specimens for this project were from the northern Norwegian Sea, but they were colored as the Barents Sea according to the map of Arctic Large Marine Ecosystems (LME) from Protection of the Arctic Marine Environment (PAME) (PAME, 2016). Additionally, I colored the main clades in the tree with the same color codes as in Kolbasova et al. (2023).

2.5.2 Morphological investigations

Each specimen was brought under the microscope Zeiss Discovery V12 stereo microscope to count the number of scalids in each series. The microscope was used to take photographs that could be investigated further. Most of the specimens could not be photographed as the introvert was too damaged after dissections. This investigation was done in addition to making the phylogenetic tree for looking after any similarities/differences between individuals both on a morphological and genetical level.

3 Results

3.1 Citizen science

The kayak club delivered two specimens which turned out to be annelids (the blow lugworm *Arenicola marina*). No additional specimens were obtained through citizen science.

3.2 DNA metabarcoding

The run of my data failed to provide any COI sequences of prey species, making it impossible to analyze the sequencing dataset. Too much inorganic material in relation to organic matter made the samples too diluted for processing the small amount of DNA extracted (Table 2). However, DNA from the *P. caudatus* specimens themselves was possible to obtain, making these data possible to process further in a phylogenetic study.

3.3 X-ray microtomography

X-ray microtomography was used to make 3D renderings of the cuticle from the specimen being investigated, as well as internal organs (Figure 5A and B). The gut content of two specimens were investigated where one had some gut contents (Figure 5C) and the other had a very filled gut (Figure 5D). It was not possible to extract some specific food items in the gut

for 3D renderings since the particulate matter was too small to be resolved well with the scanning parameters that had been used. When scrolling through the X-rays it was possible to see what looked like Foraminifera, as seen in Figure 6A, with a 3D rendering of a Foraminifera (Figure 6B) sourced from Figure 7B in Heřmanová et al. (2020, p. 11) for comparison, in addition to structures looking like detritus and inorganic material.

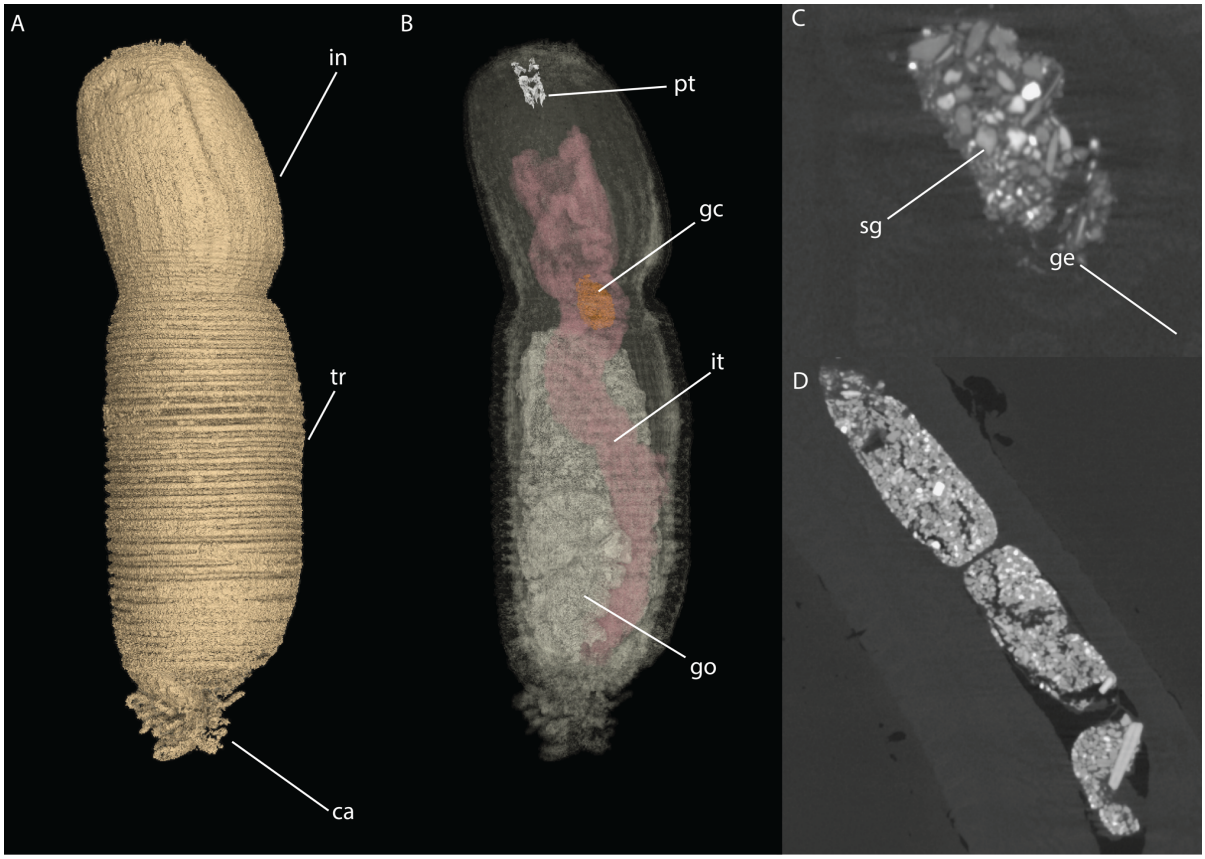

Figure 5: 3D renderings of *P. caudatus* structures. A, External structure of worm with introvert (in), trunk (tr) and caudal appendage (ca); B, Internal structures with pharyngal teeth (pt), gut contents (gc), intestinal tract (it) and gonads (go); C, Gut content, we can see sediment grains (sg) and the structures of gut epithelium (ge); D, Gut content of another specimen.

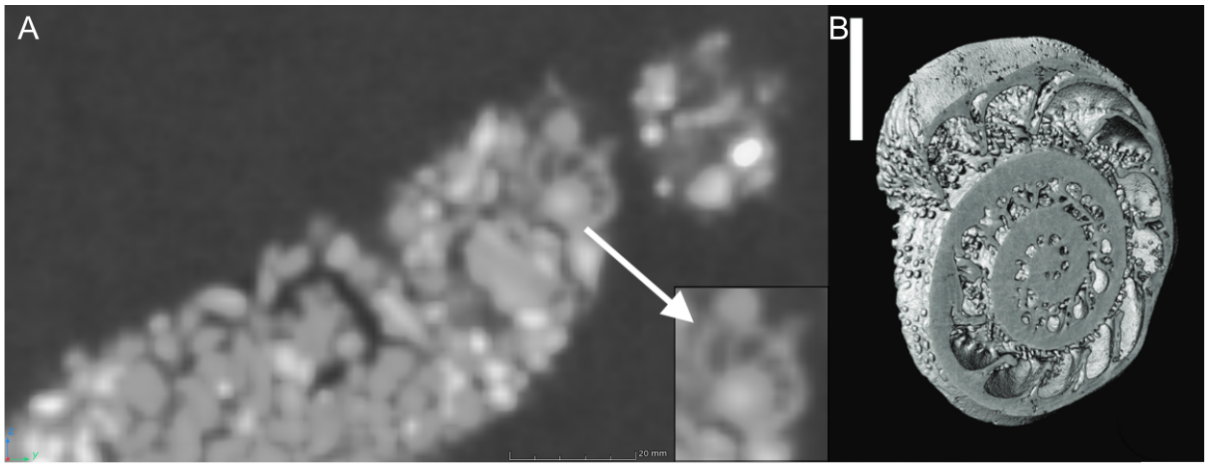

Figure 6: Foraminifera. A, Photograph of gut contents from X-ray image, revealing the structure of a Foraminifera; B, 3D rendering of a Foraminifera from micro-CT investigations, image is sourced from (Heřmanová et al., 2020, Benefits and limits of X-ray micro-computed tomography for visualization of colonization and bioerosion of shelled organism, Palaeontologia Electronica, 23(2), p. 11.) Copyright 2020 Paleontological Society. Licensed under Attribution-NonCommercial-ShareAlike 4.0 International (CC BY-NC-SA 4.0). Adapted from Figure 7B, where it was added to my figure and the placement of letter B was changed.

3.4 DNA barcoding

3.4.1 Phylogeography

Some individuals acquired from BOLD database do not have available coordinates, hence some *P. caudatus* without coloring in the phylogeny. From the output specimens 1, 4-5 and 2, 6-7 have the exact same sequence (Figure 7), sharing the same haplotype.

All individuals from Sea of Okhotsk are found in their own main clade, and another main clade have individuals exclusively from the Barents Sea (Russia), with strong support under Bayesian criteria. Specimens 1, 2 and 4-8 are in a clade together with other individuals from the Barents Sea as well as some individuals from Dennys Bay (USA) and the Chukchi Sea (between USA and Russia). The fourth clade is the most heterogenous group with individuals from a mix of locations, see Figure 7A, and specimens 3 and 9 are the only ones in this clade from the Barents Sea.

In Figure 7B, the same colors as in Kolbasova et al. (2023) have been used on the main clades for a different approach on the phylogeny and putting my sequences in their broader framework. Their study consists of five distinct clades: The North Atlantic-Arctic-Pacific 1 (NAAP1), North Atlantic-Arctic-Pacific 2 (NAAP2), Arctic 1 (A1), Arctic 2 (A2) and a clade from the Sea of Okhotsk, where all clades but one (NAAP1) had strong support under

Bayesian criteria. In this study with the sequences acquired from BOLD, the sequences making the A1 clade are not present, but individuals from A2 form a clade (Figure 7B). With the individuals I have included in my data set, NAAP2 include individuals from the North (Belgium) and Barents Sea (Tromsø), which are locations not included in the NAAP2 clade from Kolbasova et al. (2023). Their A2 clade consists of individuals from the White and Barents, Laptev and East Siberian Seas, while in this phylogeny, the clade A2 only have individuals from the Barents Sea (Dalnie Zelentsy).

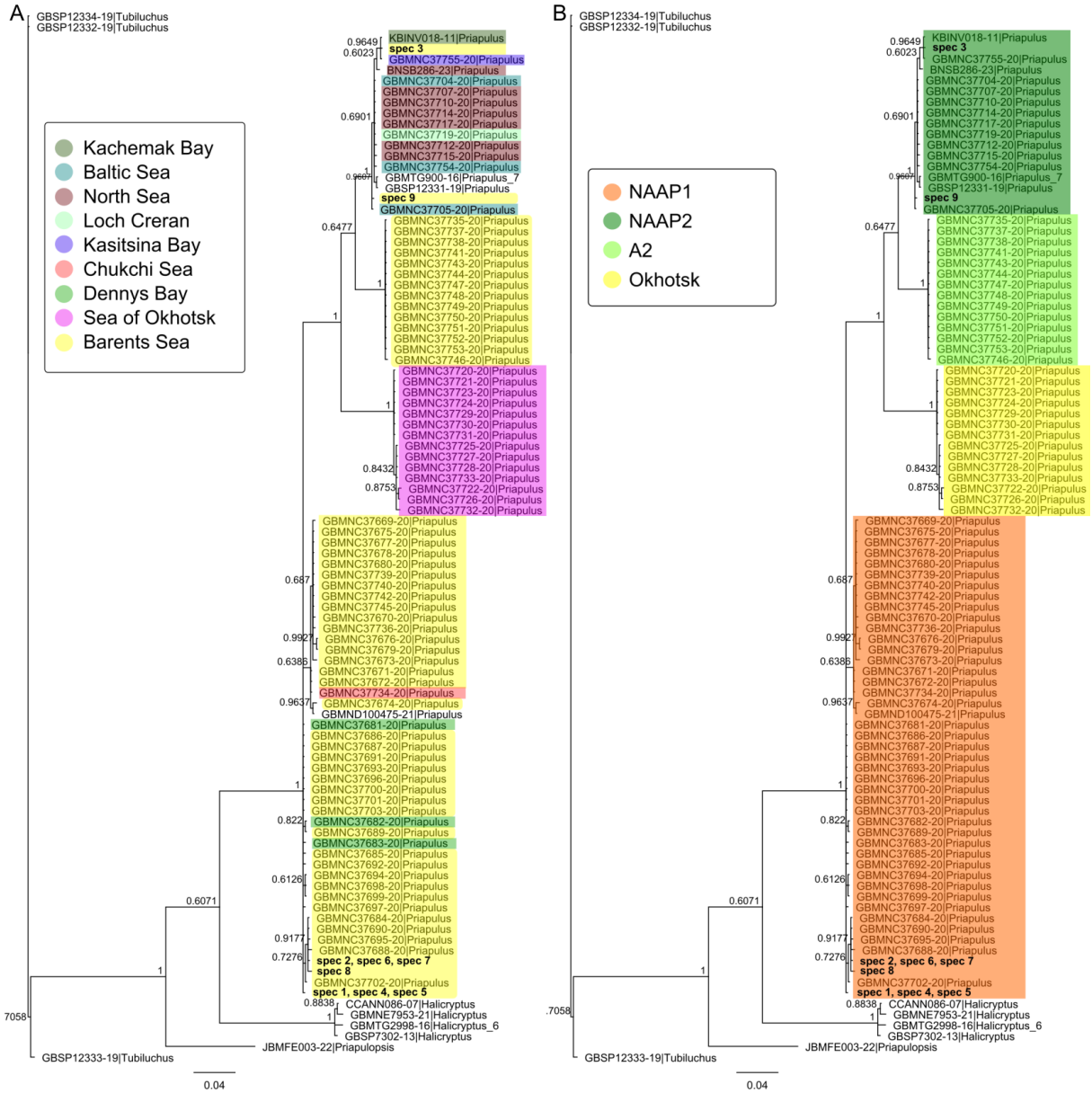

Figure 7: Bayesian tree with specimens 1-9 collected in Tromsø for this study marked with bold. Numbers above nodes are posterior probabilities. A, Colors for locations were given based on the map from Protection of Arctic Marine Environments (PAME, 2016); B, The phylogenic tree with the same coloring of the clades as in Kolbasova et al. (2023), showing four distinct clades: NAAP1, NAAP2, A2 and Okhotsk.

3.4.2 Morphological investigations

Prior to the investigations of scalids the specimens were dissected for DNA extraction, and specimens 4-8 were therefore not in good enough shape to be investigated. Table 3 gives an overview of how many scalids per series were counted in specimens 1-3 and 9. The start of a series was determined as one big scalid followed by a scalid with approximately the same size, and the end with one small scalid. This determination was made based on the description by Adrianov and Malakhov (1996) and Kolbasova et al. (2023).

Table 3: Overview of how many scalids per series in the specimens investigated. Specimens 4-8 are missing because they were not in a shape sufficient for seeing series of scalids, because of the dissection.

Specimen	Number of scalids
1	7-8
2	5-6
3	5-6
9	-

In specimen 1 (Figure 8A) it was difficult to determine how many scalids there were in one series because some of the series did not have a clear indication of where they started or ended, and it could be counted to 11 scalids in what appeared to be one series. In most of the series eight scalids were counted, and in other it looked like six. The series in specimen 2 (Figure 8B) was also difficult to determine as there were no clear empty space between them or a clear size difference in the scalids, but the series possible to count had 5-6 scalids. On specimen 3 (Figure 8C) it was possible to count 5-6 scalids per series, where a noticeable pattern of six scalids in series toward posterior end of introvert and five scalids closer to the anterior end of introvert could be seen. Specimen 9 were quite big, but it was impossible to determine any series because of the state of the introvert, as can be seen in Figure 8D.

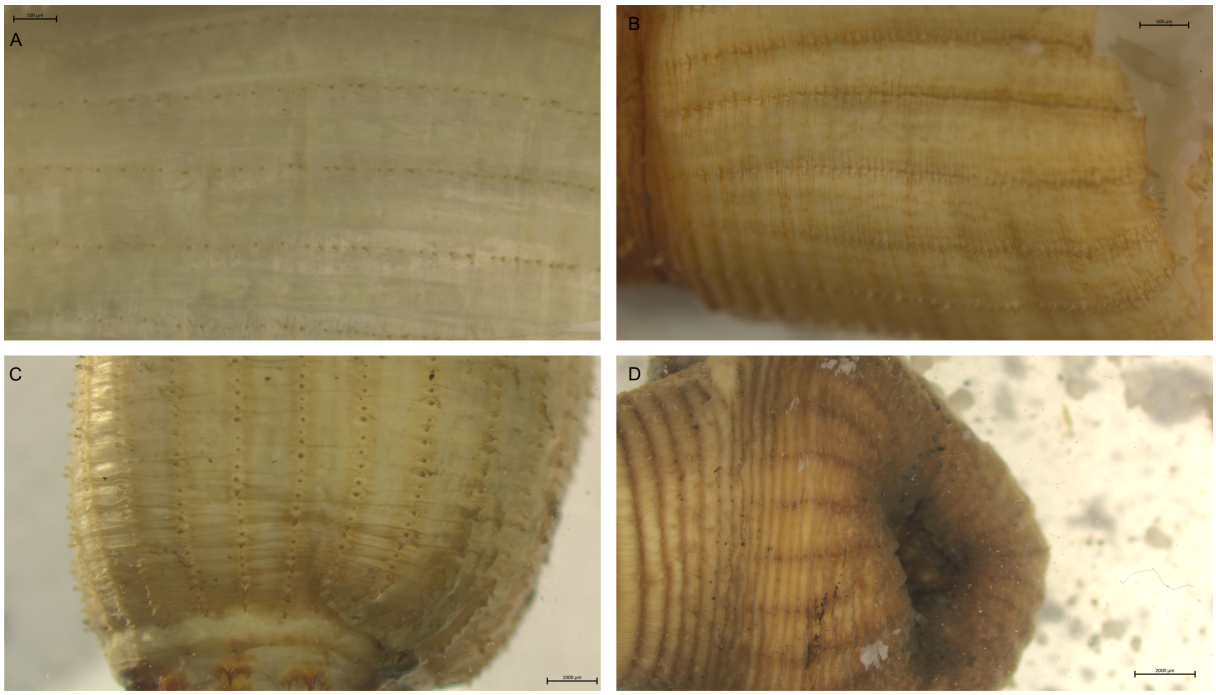

Figure 8: Specimen introverts covered with rows of scalids. A, Specimen 1; B, Specimen 2; C, Specimen 3; D, Specimen 9, impossible to count series of scalids because of the state of the specimen.

4 Discussion

4.1 Investigation of gut contents

Due to DNA-samples that were too diluted for processing the small amount of DNA extracted, the run of my data failed to provide COI sequences of prey species, and further investigations on the feeding behavior of *Priapulus caudatus* were not feasible during my thesis project due to time constraints. *P. caudatus* shares its habitat with multiple species, competing for available resources. As a group of animals that have a broad diet ranging from other animals to decaying organic matter, being a poor competitor as a predator but a good competitor in the sense that it can tolerate big changes in environmental conditions as well as extended periods without food consumption, it is thought to play a crucial role as a regulator of other fauna (Shirley, 1990; van der Land, 1970; Aarnio et al., 1998). Given that Shirley (1990) collected samples from a similar sub-Arctic area, it was anticipated that my results would align with theirs to some extent.

When some worms were maintained in a tray, it was attempted to feed them portions of an annelid found in the sediment collected from the study site in Tromsdalen. The worms exhibited responses when the annelid was presented to them, but none of them ingested it

successfully. One theory was that their failure to consume the annelid may have been due to their positioning on top of the sediment, but even a worm that had burrowed into the sediment did not consume it despite exhibiting a response to its presence, and Trott (2017) successively fed individuals kept without sediment. This observation, together with the X-ray microtomography (XMT) of gut contents, as more thoroughly discussed in section 4.1.2, suggests that *P. caudatus* primarily eats detritus, and feed opportunistically on other invertebrates present in the system.

4.1.1 DNA metabarcoding

Despite the dissection of gut contents and DNA extraction of nine collected specimens of *P. caudatus* of various shapes and sizes, I did not recover certain prey sequences using the metabarcoding approach. Still, DNA metabarcoding remains a valuable tool that has proven to be effective in other invertebrate diet studies and should be explored further within this animal group. The lack of success in obtaining prey sequences may be due to too little DNA present to successfully amplify, as the worms were quite small with little gut content (Table 2), together with the high amount of consumer DNA present in the digestive tracts relative to how much other material is in the gut. Urban et al. (2022) also suggested the latter point as a potential limitation within metabarcoding.

By direct observation of feeding behavior, proved the predatory ability of *P. caudatus* by observing the ingestion of a prey, and Trott (1998) concluded with it being an omnivorous animal only ingesting species common to its habitat. Because of earlier reports of *P. caudatus* being herbivores, deposit-feeders, and carnivores, he proposed that feeding behavior is influenced by nutritional requirements, and the quantity and quality of available food may explain the diet variations within the species (Trott, 1998).

As mentioned in section 1.4, microscopical investigations has its limitations. Prey items with chitin or calcareous skeletons takes time to digest and is easier to detect and identify, providing biased information of the present animal phyla (Urban et al., 2022; Wang et al., 2022). Compared to traditional morphological methods, DNA metabarcoding has in several studies proven to be more efficient in determining diets within invertebrates, with the ability of covering a broader range of prey taxa (Rodríguez-Barreras et al., 2020; Steyaert et al., 2020; Watts et al., 2019). In a diet investigation of the Arctic shrimp *Pandalus borealis*,

Urban et al. (2022) discovered a diet composition through DNA metabarcoding contrasting to previous reports based on SCA. This molecular-based method revealed soft-bodied prey which had not yet been detected in *P. borealis* through microscopy, providing valuable insights into the trophic ecology in Arctic food webs.

Implementing DNA metabarcoding to investigate gut contents in *P. caudatus* could lead to new discoveries in their feeding behaviors and provide a deeper understanding of their ecological role in the benthic ecosystem. Considering the varied depths and geographic distributions of *P. caudatus*, alongside the identified dietary variations between populations (Shirley, 1990; Trott, 1998), DNA metabarcoding presents an opportunity to deepen our understanding of their ecology. Urban et al. (2022) observed differences between shelf and fjord specimens, further emphasizing the potential of DNA metabarcoding within dietary studies of *P. caudatus*. Although prey sequences were not recovered using the metabarcoding approach, the most plausible explanation may be the limited presence of prey tissue within the guts. This observation aligns with previous findings indicating that *P. caudatus* primarily consumes detritus or sediment with long periods of time between eating, with occasional predatory behavior. A new run should be conducted where the samples should be concentrated maximally prior to being processed for DNA metabarcoding in the laboratory.

4.1.2 X-ray microtomography

In theory, XMT is well suited for investigating gut contents in invertebrates. This method provides detailed representations of a sample's internal structures and composition, allowing for the extraction of 3D renderings for visual analysis (Schoeman et al., 2016). In other methods such as microscopy, samples must be dissected to access the intestine, leaving the sample inaccessible for further studies. XMT on the other hand, eliminates the need for sample dissection to access the intestine. In cases where only one or a few specimens are available, XMT should be considered as it allows for investigation without disturbance of the specimen, preserving it for potential future investigations (Metscher, 2009). This is not a concern for *P. caudatus* but may be useful when studying rare museum specimens where the original specimen should be preserved. Bochaton et al. (2015) successively provided the first data on the feeding behavior of an endangered lizard, providing crucial information for potential conservation.

In this study, the gut contents of the two specimens were identified as sand, detritus and Foraminifera. Observations revealed varying amounts of content in the two specimens (Figure 5C and D), but similarly for both is that there is a high presence of sediment. Foraminifera could be spotted when scrolling through the X-rays but extracting the voxels necessary for creating a 3D rendering proved challenging. This could be because the contrast in the specimens were not quite good, making the small structures especially difficult to extract, and it took a lot of time making the 3D renderings in Figure 5. Iodine was used for coloring to enhance contrast, suggesting the consideration of alternative coloring methods. Metscher (2009) discussed staining methods of animal tissues and mentioned iodine and phosphotungstic acid (PTA) as the most useful contrast stains. PTA proved successful in their stained organisms, from insects to soft-bodied animals, and could be considered when investigating *P. caudatus*. Another reason for the difficulty in seeing details in the gut contents could be that the resolution of the X-rays was too low, as XMT has proven successful in studying e.g. internal cellular microstructures and structural changes during baking (Olakanmi et al., 2023).

4.2 Phylogeography

I expected that organisms clustered together would exhibit a closer genetic relationship compared to those from other locations. Despite no morphological distinctions among specimens 1-9 (discussed in section 4.3), analyzing sequence variation in segments of the COI gene can facilitate the identification of differences within *P. caudatus*. Given the study's original focus on diet content, the targeted COI fragments were relatively short (~313 bp) due to the challenge of dealing with degraded material in the guts. In contrast, conventional DNA barcoding studies typically use larger fragments of ~650 bp (Berry et al., 2015). Using a larger COI fragment may unveil more profound distinctions in the genome, as multiple specimens of the same species likely share identical genetic codes for the short fragment investigated. DNA barcoding has demonstrated remarkable effectiveness in investigating and identifying cryptic species across a wide range of taxa (Wang et al., 2011). Wang et al. (2011) had great success in identifying species within Aphidina, using longer COI sequences starting from ~590 bp. Laakkonen et al. (2021) suggested the presence of cryptic species of *Priapulus* in the northern hemisphere, further addressed by Kolbasova et al. (2023) who highlighted that between their five main clades the mean K2P distance was 10.03%, which is quite high and corresponds to inter-species distances. In other taxa such as kinorhynchs and marine

nematodes, the interspecific distances are 0-2% and 5%, respectively (Kolbasova et al., 2023). *P. caudatus* represents four different COI clades in the Arctic where they live sympatrically, as individuals found in the Barents, White, Kara and East Siberian Sea appears in all four clades (Kolbasova et al., 2023). The individuals collected in Tromsdalen are found within two of the COI clades (NAAP1 and NAAP2). The fact that they fall in the clades established by Kolbasova et al. (2023), implies that their K2P distances fall into the described range, and there is a potential presence of cryptic species within *P. caudatus* at the study site.

However, it is important to note that the suggestion of the *P. caudatus* individuals from Tromsdalen representing cryptic species should be viewed with caution and is not yet a definitive conclusion. This reservation arises due to the limited sample size of only nine individuals, together with considerations from existing literature. Because of the increased reports of mitochondrial-nuclear discordance (also relevant within *P. caudatus*, reported in Kolbasova et al. (2023)), Hupalo et al. (2022) decided to investigate the COI diversity within freshwater amphipods, underscoring certain constraints associated with estimating species diversity using the COI gene. Specifically, in some invertebrates, mitochondrial genes may not effectively identify species when speciation events are relatively recent or ongoing. Moreover, species with limited dispersal abilities, such as *P. caudatus*, may exhibit high mitochondrial diversity within populations (Hupalo et al., 2022). Divergence in COI is proven to be an effective tool in species identification (Hebert et al., 2003), but the identification of cryptic species should include additional information to COI data (Hupalo et al., 2022), which is not available for this thesis.

Given the small sample size, I would still suggest further investigations of *P. caudatus* on the sampled location in Tromsdalen as the individuals are easily collected, and there is the potential of gaining more knowledge about the ecology and population genetics of this priapulid species. On the COI tree (Figure 7), specimens 3 and 9 are found within a clade exclusively consisting of subtidal genotypes. In addition to COI data, Kolbasova et al. (2023) also investigated the nuclear genes 18S and 28S, and found two differing genotypes, subtidal and intertidal, that correlated with each other, but poorly with COI. In their investigated intertidal populations, all individuals belonged to the intertidal genotype, while the subtidal populations were more heterogenous with representatives from the intertidal genotype (Kolbasova et al., 2023). Priapulids exhibit limited mobility, and the observed heterogeneity

may be attributed to larval migration facilitated by tidal flushing, as subtidal genotypes were absent in the intertidal zone. Kolbasova et al. (2023) also discussed the reasons for nuclear genotypes and COI lineages are not fully a match, highlighting that the most basal NAAP1 clade most likely initially had the intertidal genotype, while the other clades most likely belong to a common ancestor with the subtidal genotype.

4.3 Morphological investigations

Incorporating an investigation of the scalids on the introvert alongside DNA sequence analysis could help identify morphological distinctions among my specimens, particularly specimens 3 and 9, which belong to a different main clade than the rest of my specimens. My results could be compared with those of Kolbasova et al. (2023), who investigated morphological variations in specimens from both intertidal and subtidal genotypes across different main clades. They found no patterns in size, coloration or shape assigned to one genetic type or location, and no differences in scalid series were observed. In addition to investigating the scalids, I tried to find literature describing the synonyms of *P. caudatus*, as the species have had several names since it was discovered (Adrianov & Malakhov, 1996; Paulay, 2024; van der Land, 1970), to look for any morphological hints hidden. All literature describing the synonyms was not possible to access, as many are very old and in different languages.

van der Land (1970) mentions that *P. caudatus* was first described in 1754 under the name *Priapus humanus* by Odhelius, and was later adopted by Linnaeus (Linné & Salvius, 1758). They placed the worm in the same genus as the sea anemone *Priapus equinus* (today *Actinia equina*), hence the name. In 1767 Linnaeus placed *P. humanus* with the sea cucumbers (*Holothuroidea*) within Echinodermata because of the symmetry of the introvert and pharynx and renamed the species to *Holothuria priapus*. In 1816 Lamarck changed the name to *Priapulus caudatus*, and since then the name has been the same, but in which group it belongs to was changed many more times until Priapulida became its own phylum (van der Land, 1970). Two new species of Priapulida from the deep sea were discovered by Sanders and Hessler (1962), one whom they named *Priapulus profundus*, which was later described to be *P. caudatus* and are now considered a synonym (Adrianov & Malakhov, 1996). Their description of the specimens included the series of scalids, which they found to be four

scalids in the first few anterior series, then three, two and one further towards the posterior end of the introvert. Additionally, it was assumed the specimens had to be different species of Priapulida as the region investigated had no previous records of *P. caudatus* (Sanders & Hessler, 1962). I did not manage to extract information on all synonyms, however, most names after *P. caudatus* were assigned due to new individuals found at different depths, such as the deep sea instead of the subtidal, or in parts of the world their presence had not yet been discovered, as seen from the discovery of Sanders and Hessler (1962).

The presence of rows of scalids covering the introvert, allows for differentiation based on the number and arrangement of scalids in each series (van der Land, 1970). The scalids in each series decrease in size, where the second one is quite similar in size as the first one (Adrianov & Malakhov, 1996; van der Land, 1970). Variation in scalids presence, structural features, the proximity of series, and scalid size distinguish species. However, Kolbasova et al. (2023) did not find any reliable distinctions in morphology from individuals from different COI lineages, and most morphological variations are most likely due to different sizes which is linked to differences in age.

5 Conclusion and future outlook

Considering that most studies on priapulids have been on morphology, taxonomy and systematics, there is a knowledge gap in understanding their role in the marine benthic ecosystem. *P. caudatus* have been reported to be generalists who feed on what is available in their surroundings. The aim of this thesis was to investigate the diet of priapulids found around Tromsø, using DNA metabarcoding and XMT. The metabarcoding approach proved unsuccessful as prey sequences could not be obtained, possibly due to insufficient prey tissue in the guts, and observations from XMT implied the specimens investigated being detritivorous with few other invertebrates present. These results, together with the observation of the priapulids in the tray struggling to feed on an annelid given to them, suggests that the population of *P. caudatus* investigated primarily are detritivorous and opportunistic predators that eat other invertebrates on occasion. XMT has previously proven to be a good method for investigating gut contents in invertebrates and should be explored further for samples of *P. caudatus*, where other methods to enhance the contrast and resolution mentioned in 4.1.2 could be explored.

Sequences from P. caudatus in the Arctic are found within five COI lineages, with a high mean K2P distance of 10.03% that corresponds to inter-species distances. The specimens obtained in Tromsdalen for this thesis were found within two of these clades, implying they are within the same K2P distance. This suggests the potential for cryptic species within *P. caudatus* at the study site. However, there are reasons to be cautious and not make this the definite conclusion. Firstly, K2P distances were not calculated for the specimens from Tromsdalen. Secondly, both my specimens and those collected from BOLD are found in different COI lineages, but live sympatrically within these. Additionally, no significant morphological distinctions were found between individuals that would indicate that they are separate species. Furthermore, Kolbasova et al. (2023) found two different genotypes, intertidal and subtidal, within the nuclear genes 18S and 28S. Two specimens from Tromsdalen were found within a clade exclusively consisting of subtidal genotypes, and I suggest further investigations on both the mitochondrial COI and the nuclear 18S and 28S of the populations around Tromsø to enhance the understanding of the phylogeographic patterns of *P. caudatus*.

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7 Appendix

A1: R-script: map over distribution of *P. caudatus* in Norway and Priapulida in the world

```
# Load necessary libraries for both maps
library(maps)
library(ggplot2)
library(ggOceanMaps)
library(ggspatial)


# Read the data files into R
data <- read_excel("/Users/MarenHansen/OneDrive - UiT Office 365/BIO-3950 PRIAPULIDA/Data/Dataset_GBIF_PCaudatus_21092023.xls")  # Replace "Dataset_GBIF_PCaudatus_21092023.csv" with the actual filename and path
data2 <- read_excel("/Users/MarenHansen/OneDrive - UiT Office 365/BIO-3950 PRIAPULIDA/Data/Priapulida-occurrence-2.xls")


# World map of distribution Priapulida genus
# Load a world map dataset
world <- sf::st_as_sf(map("world", plot = FALSE, fill = TRUE))
# Making a color palette for the points
pal <- c(
  "#FFB400",
  "#C20008",
  "#13AFEF",
  "#8E038E",
  "black",
  "white")
# Create a world map of distribution Priapulida genus
p <- ggplot(data = world) +
  geom_sf(fill = “gray60”, color = NA) +                    # color = NA removes border colors
  xlab("Longitude") + ylab("Latitude") +
  theme(panel.background = element_rect(fill = "white")) +
  geom_point(data = Priapulida_occurrence_2, shape = 21, color = "black", aes(x = decimalLongitude, y = decimalLatitude, fill = genus), size = 2) +
  scale_fill_manual(values = pal) +  # Assigning colors from 'pal' to fill aesthetic
  theme(legend.text = element_text(face = "italic"))
# Print the plot
print(p)
```

```
# Bathymetric map of distribution P. caudatus in Norway

# Create a data frame with longitude and latitude coordinates
coordinates_df <- data.frame(
  Longitude = data$dec.Long,
  Latitude = data$dec.Lat)

coordinates_df$Longitude <- as.numeric(coordinates_df$Longitude)
coordinates_df$Latitude <- as.numeric(coordinates_df$Latitude)

# Create a bathymetric map
basemap(limits = 60, bathymetry = TRUE) # A synonym: basemap(60)

# Set coordinate limits to map and add points from data set
map <- basemap(limits = c(-15, 40, 54, 81), bathymetry = TRUE) +
  geom_point(data=transform_coord(coordinates_df),
             aes(x = Longitude, y = Latitude), colour = "Black",
             fill = "#8E038E", pch = 21, size = 2, alpha = I(0.7))

# Print the plot
print(map)
```

A2: DNA extraction protocol

Quick-Start Protocol July 2022

DNeasy® PowerMax® Soil Kit

The DNeasy PowerMax Soil Kit (cat. no. 12988-10) can be stored at room temperature (15–25°C) until the expiry date printed on the box label.

Further information

- *DNeasy PowerMax Soil Kit Handbook*: **www.qiagen.com/HB-2259**
- Safety Data Sheets: **www.qiagen.com/safety**
- Technical assistance: **support.qiagen.com**

Notes before starting

- Shake to mix Solution C4 before use.
- If Solution C1 has precipitated, heat to 60°C until precipitates dissolves.
- Please wear gloves at all times

Procedures

1. Add 15 ml of PowerBead Solution to PowerMax Bead Pro Tube.
2. Add up to 10 g of soil sample in the PowerMax Bead Pro Tube containing PowerBead Solution. Vortex vigorously for 1 min.

   Note: Please refer to the Troubleshooting Guide before deciding on the amount of soil to process.
3. Add 1.2 ml of Solution C1 to the PowerMax Bead Pro Tube and vortex vigorously for 30 s.
4. Place the PowerMax Bead Pro Tube on a vortex adapter (cat. no.13000-V1-50) and vortex for 10 min at the highest speed. Alternatively, place the tube in a shaking water bath set at 65°C and shake at maximum speed for 30 min.
5. Centrifuge at 2500 x *g* for 3 min at room temperature.
6. Transfer supernatant to a clean collection tube (provided).

   Note: The supernatant may still contain some soil particles and color.

7. Add 5 ml of Solution C2. Invert twice to mix. Incubate at 2–8°C for 10 min.
8. Centrifuge at 2500 x *g* for 4 min at room temperature.
9. Avoiding the pellet, transfer the supernatant to a clean collection tube (provided).
10. Add 4 ml of Solution C3 and invert twice to mix. Incubate at 2–8°C for 10 min.
11. Repeat steps 8 and 9 once. Then proceed to step 12.
12. Shake to mix Solution C4. Add 30 ml of Solution C4 to supernatant and invert twice.
13. Fill an MB Spin Column with the solution from step 12.
14. Centrifuge at 2500 x *g* for 2 min at room temperature. Discard the flow-though and add second volume of supernatant to the same spin column and centrifuge again at 2500 x *g* for 2 min at room temperature. Discard the flow-though. Repeat until entire volume has been processed. This will take up to 4 total spins.
15. Add 10 ml of Solution C5 to the MB Spin Column. Centrifuge at 2500 x *g* for 3 min at room temperature. Discard the flow-through.
16. Centrifuge at 2500 x *g* for 5 min at room temperature.
17. Carefully place the MB Spin Column in a new collection tube (provided). Avoid splashing Solution C5 onto the spin filter.
18. Add 5 ml of sterile Solution C6 to the center of MB Spin Column membrane and centrifuge at 2500 x *g* for 3 min at room temperature.
19. Discard MB Spin Column. The DNA is now ready for downstream applications.

A3: Protocol COI Metabarcoding Leray-XT

METABARCODING PRIMERS

We use the Leray-XT primer set (Wangensteen et al. 2017, https://peerj.com/preprints/3429/). This is a highly-degenerated primer pair able to amplify a 313 bp fragment of cytochrome *c* oxidase subunit I (COI) from a wide array of eukaryotic groups, including virtually all metazoans. The sequences (where "I" stands for deoxy-inosine) are:

Forward, **miCOIint-XT**: 5'-GGWACWRGWTGRACWITITAYCCYCC-3'
Reverse, **jgHCO2198**: 5'-TAIACYTCIGGRTGICCRAARAAYCA-3'

DNA AMPLIFICATION

We use a simple 1-step PCR protocol for amplifying the Leray fragment. The metabarcoding primers have an 8-base sample-tag attached (each tag with at least 3 differences out of 8 bases). Also, we add a variable number (2-4) of leading Ns, in order to increase sequence variability to improve Illumina sequencing. Each forward and reverse primer has the same sample-tag attached in both ends. E.g.:

Primer F1: NNaacaagccGGWACWRGWTGRACWITITAYCCYCC
Primer R1: NNNNaacaagccTAIACYTCIGGRTGICCRAARAAYCA

Primer F2: NNNggaatgagGGWACWRGWTGRACWITITAYCCYCC
Primer R2: NNNggaatgagTAIACYTCIGGRTGICCRAARAAYCA

Primer F3: NNNNaattgccgGGWACWRGWTGRACWITITAYCCYCC
Primer R3: NNaattgccgTAIACYTCIGGRTGICCRAARAAYCA

We have 96 such different pairs, so we can multiplex up to 96 samples in one library.

The PCR protocol uses Amplitaq Gold 360 master mix (ThermoFisher)
https://www.thermofisher.com/order/catalog/product/4398886
and bovine serum albumin (BSA)
https://www.thermofisher.com/order/catalog/product/B14?ICID=search-B14

The PCR mix is as follows:

AmpliTaq Gold Master Mix	10.00	µl
BSA 20 µg/µl	0.16	µl
H2O	5.84	µl
Forward primer 5 µM	1	µl
Reverse primer 5 µM	1	µl
DNA Template	2	µl

Note that the primers cannot be added to the PCR master mix for aliquoting (as is common practice for preparing normal PCRs). They have to be added to every individual sample, since every sample will be amplified with a different version of the primer set.

The PCR programme is:

95°C	10 min	(needed for denaturing the blocking antibody of Taq polymerase)
94°C	1 min	
45ºC	1 min	x 35 cycles
72°C	1 min	
72°C	5 min	(extension time)

LIBRARY POOLING AND CONCENTRATION

Once all samples are amplified, the success of amplifications may be checked by gel electrophoresis in 1% agarose. Note that the samples must be prepared in a clean room to avoid contaminations. They should never be opened in a common electrophoresis laboratory. We routinely use 2 µl of the PCR products for the electrophoresis. The rest (18 µl per sample, including the blank samples) will be pooled together in a single Eppendorf tube and this pool is then thoroughly homogenized by vortexing.

The pool is then purified using MinElute columns for removing DNA fragments below 70 bp. This step will also concentrate the amplified DNA around 10 times. https://www.qiagen.com/qdm/aw/cup/pcr-purification/

These MinElute columns have a maximum sample volume capacity of 130 µl per sample. So you will probably need to use 10 or 12 of such columns, depending on the total volume of your pool. Follow the protocol in the kit. In the final step, you can elute every column in 12-15 µl of elution buffer. Then pool all the eluates together and homogenize thoroughly by vortexing.

You can measure the DNA concentration in the final pool using a Qubit fluorimeter with the Broad-Range DNA quantification kit. You need a minimum concentration of 75 ng/µl in the final pool for a best performance of the next ligation step.

LIBRARY PREPARATION

For library preparation, we use a PCR-free ligation protocol, the NEXTflex PCR-Free DNA Sequencing Kit from BIOO Scientific: http://www.biooscientific.com/Next-Gen-Sequencing/Illumina-Library-Prep-Kits/NEXTflex-PCR-Free-DNA-Sequencing-Kit

We use 3 µg of DNA (up to 40 µl of the previous pool) as starting material. The instructions for preparing a COI library are exactly the ones described in the kit manual: http://www.biooscientific.com/Portals/0/Manuals/NGS/5142-01-NEXTflex-PCR-Free-DNA-Seq-Kit.pdf

Note this protocol is valid for selecting fragment sizes of 300-400 bp, exactly the right size for the Leray fragment. If you want to use a different metabarcoding marker with a shorter fragment, then you need to change Step B of the protocol (size selection).

With this kit, you will get to ligate your amplicons to the Illumina adapters and a 6-base library tag. The basic kit includes just one such library-tag, which is enough for multiplexing 96 samples with our set of 96 sample-tags. If you wish to multiplex over 96 samples, you could use two or more library tags. For this, you would need to buy an extra box of BIOO barcodes, which come in 6, 12, 24, 48 or 96 versions: http://www.biooscientific.com/Next-Gen-Sequencing/Illumina-Adapters/DNA-Seq/NEXTflex-DNA-Barcodes

You will need to use magnetic beads for some steps of this protocol. The original Agencourt AMPure XP beads are quite expensive, but they are most convenient. http://uk.beckman.com/nucleic-acid-sample-prep/purification-clean-up/pcr-purification?geolocation=gb

LIBRARY CHECKING

We usually analyse the final library using either an Agilent TapeStation or Bioanalyzer, in order to check that the ligation has gone well. If you don't have any of these analyzers available, then you could use just a gel electrophoresis to check the right migration of the fragment. Note that the library fragments are the result of a special Y-shaped adapter ligation and they will not be linear DNA. So they will migrate anomalously in all these analytical methods. The library peak will not appear at the expected size of ~ 510 bp, but it will produce a broad peak of ~ 800 bp. This strange migration behaviour is normal and won't interfere with the MiSeq sequencing.

LIBRARY QUANTIFICATION

In order to load the right concentration of the library in the MiSeq, it is essential to check the exact concentration of the library using a specific qPCR method. This method will use a specific probe for the Illumina adapter sequence, so it allows to quantify exactly which molarity of adapter you will be loading into the MiSeq, which is crucial for not overclustering the Illumina flow-cell.

For this purpose, we use the NEBNext Library Quant Kit from New England Biolabs: https://www.neb.com/products/e7630-nebnext-library-quant-kit-for-illumina

We usually analyse library dilutions of 1:5000, 1:10,000 and/or 1:50,000.

You will need to use a qPCR machine. In Salford, we use the Rotor-Gene Q from QIAGen but, of course, any qPCR machine will work: https://www.qiagen.com/us/search/rotor-gene-q/

LIBRARY DILUTION AND MiSeq LOADING

The final target concentration for the MiSeq loading depends on the version of the kit you want to use for the MiSeq sequencing: v2 or v3 MiSeq sequencing kits. With a v2 kit (2x250 bp), you can get up to 15 M reads, and you will use a sample load with up to 10 pM DNA concentration. With a v3 kit (2x300 bp, though we are using just 2x250 cycles for better error rates) you will get up to 25 M reads, and you will use a sample load with up to 20 pM DNA concentration. We usually target at 9 pM for v2 or at 18 pM for v3, in order to prevent overclustering of the flow-cell.

We will prepare our sample including a 1% of PhiX genomic library, which will be used as internal sequencing control for calculating error rates per cycle. https://www.illumina.com/products/by-type/sequencing-kits/cluster-gen-sequencing-reagents/phix-control-v3.html

The protocol for the final sample denaturation before loading is as follows:

- Prepare a mix of up to 10 µl of your library (or libraries) and PhiX-library (mixed in the right molar proportions and with the right total concentration in function of the final concentration to be targeted) and put it in the bottom of a 2-ml Eppendorf tube.
- Denature with the same volume of 0.2N NaOH during 5 min. During this time, you may vortex once and spin in a centrifuge for recovering the sample in the bottom of the tube.
- Add HT1 hybridization buffer (included with your the MiSeq reagent kit) for a total final volume of 2 ml and vortex thoroughly to stop the denaturation and the hydrolysis.
- Load 600 µl of this denatured sample into the the MiSeq for sequencing.