Chapter 7

Collecting and Culturing Kamptozoans for Regenerative Studies

Achim Meyer, Julia Merkel, and Bernhard Lieb

Abstract

Kamptozoa, also known as Entoprocta, are small aquatic filter-feeders that belong to the Lophotrochozoan superphylum, which also contains other acoelomate phyla including Annelida, Nemertea, and Mollusca. The study of Kamptozoa is thus of great interest to understand the early Lophotrochozoan evolution. Moreover, many kamptozoans have been shown to possess great regeneration capacities, including whole-body regeneration. In addition, and in particular for colonial cosmopolitan species such as *Barentsia benedeni*, kamptozoans are highly suitable as laboratory model organisms because of their simple culture, low space requirements, transparency and rapid life cycle. This chapter provides a brief introduction into field collection, culturing techniques for both the animals as well as the algae required for their feeding, fixation, staining, and sequencing.

Key words Entoprocta, Kamptozoa husbandry, *Cryptomonas baltica*, Filtration feeding

1 Introduction

Kamptozoans are aquatic acoelomate filter-feeding invertebrates. These animals have a typically minute body, called zooid, ranging from 0.1 mm (Loxosomatidae) to 15 mm (Barentsiidae), composed of a stalk part containing the stolon, and a head part called calyx that contains the other organs (Fig. 1). The calyx of kamptozoans is crowned by solid tentacles, which covering cilia generate a flow of water that brings food particles into the atrium of the animal for feeding. Although most of the approximately 180 described kamptozoan species are found in the marine environment (25 *Loxosoma* spp., 118 *Loxosomella* spp., 50 species from the families Loxosomatidae, Pedicellinidae, and Barentsiidae [1, 2]), some species such as the cosmopolitan *Barentsia benedeni* can retreat into brackish water and two species are found exclusively in freshwater (*Urnatella gracilis* [3], and *Loxosomatoides sirindhornae* [4]). All Kamptozoa bear ovoviviparous trochophore larvae

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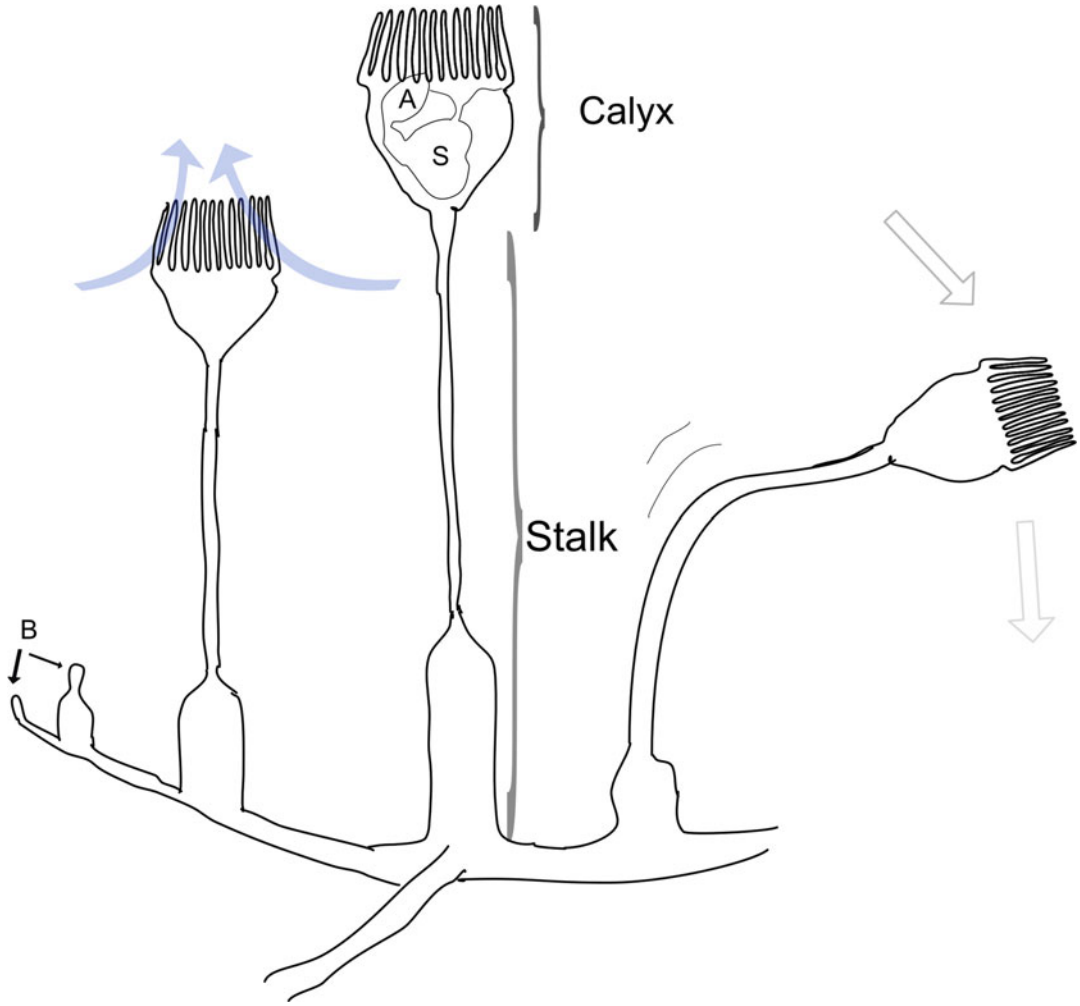

Fig. 1 Sketch of a colonial kamptozoan. A: Anus, B: Budding zooids, S: Stomach. Blue arrows indicate the aspiration current and filtration mode

which are similar to the mollusk and polychaete larvae. Additionally, all known species propagate by budding.

Kamptozoa, also currently known as Entoprocta, initial classifications (e.g., van Beneden 1845 [5]) described colonial species such as *Pedicellina cernua* as an ingroup of the "moss animals," or bryozoans, also currently known as Ectoprocta. Contradiction was firstly raised by Nitsche 1869 [6] pointing out some fundamental differences between these two groups of animals. One of the most readily observable difference is the location of the anus, which is position inside the atrium in Kamptozoa and outside of it in Bryozoa. Consequently, Nitsche suggested the names Entoprocta and Ectoprocta, and these names are still well accepted. However, the mode of filtration generated by cilia on the tentacles is opposed between Entoprocta and Ectoprocta, thus the name-giving

position of the anus is inevitably a consequence of this divergent filtration feeding pattern. Related to the fundamentally divergent adult body plan is also the reorganization of the alimentary system during metamorphosis of the kamptozoan trochophora, when the gut becomes U-shaped, the mouth opening breaks through at a new position and the anus finally settles down within the crown of solid tentacles of the adult zooid [7]. The alternative name Kamptozoa was dropped by Cori 1936 [7] from the Greek κμπτώ = bent and refers to the curved stolon which is observed as reaction to external stimuli. Thus, kamptozoa describes a feature which is related to the unique behavior of the disturbed living animal that facilitates their identification both in the field as well as below the stereomicroscope. Because Kamptozoa provides a friendlier semantic for a researcher focusing on these taxa, we tend to favor the use of this more recent nomenclature.

Phylogenetically, Kamptozoa belong to the Lophotrochozoan superphylum, which contains other phyla including Annelida, Nemertea, and Mollusca. Kamptozoa are thus of great interest to understand the early Lophotrochozoan evolution. Bleidorn [8] summarizes the current knowledge about Lophotrochozoan systematics including the description of characters which define Mollusca as the sister group of Kamptozoa. The internal kamptozoan phylogeny (Fig. 2) agrees on the distinction of Solitaria (130 species) and Coloniales (50 species) [9, 10]. Many colonial species and one solitary species, *Loxosomella antarctica*, have been shown to be capable of whole-body regeneration [11–13]. Whole-body regeneration can take place both under natural conditions (e.g., low salinity leads to the dropping off the calyx during winter [14]) or after zooid loss due to predation of the calyx [15]).

Understanding the regenerative pattern of Kamptozoa could shed light on general developmental processes. Their pronounced regeneration capacity together with the transparent and minute body size makes Kamptozoa an ideal organism for research on whole-body regeneration using modern laboratory procedures such as advanced labeling and staining methods (Fig. 3).

In this chapter, we present protocols for the isolation, culture and feeding of the colonial species *Barentsia benedeni*. In addition, we provide protocols for the monitoring of WBR as well as for the fixation, staining, and DNA extraction of whole zooids. These protocols can easily be adopted for the study of other colonial species, as it works well for *B. elongata*, and will enable short-term (several weeks) work with most solitary species.

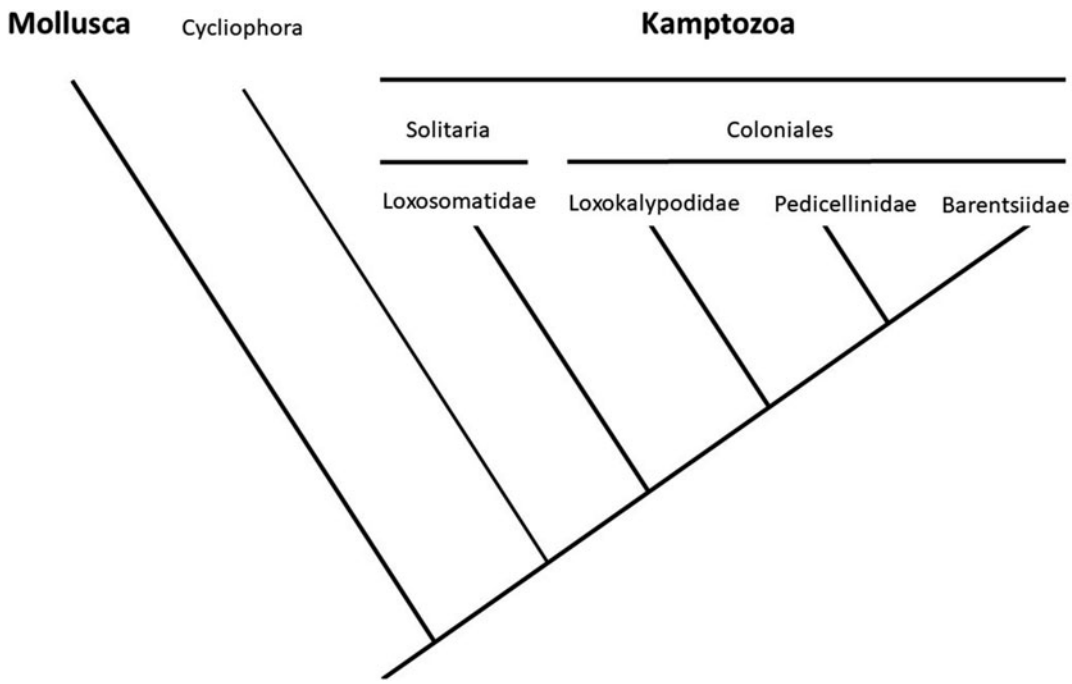

Fig. 2 Phylogenetic relationships of recent kamptozoan families, modified after [8, 9]

2 Material

2.1 Barentsia benedeni *Collection and Culture*

1. Long fine-meshed fishing net.
2. Wide-mouth Kautex jars (e.g., 500 mL).
3. Stereomicroscope.
4. Orbital shaker for kamptozoan culture bowls.
5. Covered glass culture vessel: glass bowl (e.g., Ø 12.5 cm; H: 5.8 cm; 0.36 L) with petri dish as lid and a small piece of cut-in air tubing ø 2/6 mm on the edge of the bowl to provide passive aeration (Fig. 4).
6. Filtered natural seawater (FNSW): natural seawater, 0.22 μm sterile-filtered (*see* **Note 1**). Store in a cool and dark place with air access for up to 1 year (e.g., in a fridge with a cotton plug).
7. Relaxing medium [16]: 79 mM (7.5 g/L) $MgCl_2$ in double-distilled water (ddH_2O). Mix 1:1 with FNSW.

2.2 Live Cryptophyta Algae Stock

Kamptozoans can feed on a variety of live Cryptophyta algae (*see* **Note 2**).

1. 250 mL wide-mouth Erlenmeyer flasks with cotton wool plugs.
2. Luer-lock syringes with sterile 0.22 μm filter unit.

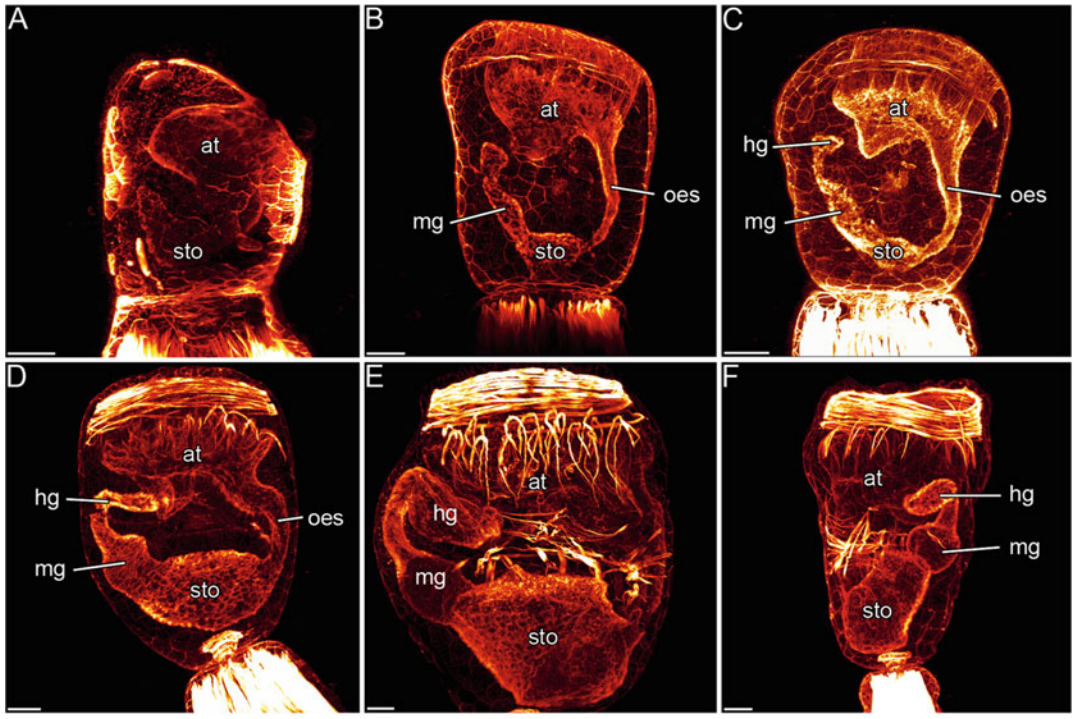

Fig. 3 Confocal micrographs of regeneration stages of *P. cernua* (thesis J. Merkel 2014). Scale bars 20 μm. (**a–e**) lateral view. (**f**) Laterofrontal view. (**a**) First regeneration stage. (**b**) Second regeneration stage. (**c**) Third regeneration stage. (**d**) Fourth regeneration stage. (**e, f**) Fifth regeneration stage. Indicated organs are atrium (at), stomach (sto), esophagus (oes), midgut (mg), and hindgut (hg). Micrographs were gained with a Leica SP5 II confocal microscope

Fig. 4 Culturing bowl with tools for cleaning. Note the green cut-in air tubing

3. Light cabinet with fluorescence bulb: one warm white 18 W T8 fluorescence bulb, 20 cm distance between the light source and the algae is required.
4. 50× Guillard's (F/2) medium [17]: 44.1 mM $NaNO_3$, 1.81 mM NaH_2PO_4, 5.30 mM Na_2SiO_3, 0.585 mM $FeCl_3$, 0.585 mM ethylenediaminetetraacetic acid disodium salt dihydrate (Na_2EDTA), 45.5 μM $MnCl_2$, 3.82 μM $ZnSO_4$, 2.10 μM $CoCl_2$, 1.96 μM $CuSO_4$, 1.30 μM Na_2MoO_4, 14.8 μM thiamine hydrochloride(vitamin B1), 0.102 μM biotin (vitamin H), 0.0184 μM cyanocobalamin (vitamin B12). Store at −20 °C for up to 2 years.

2.3 Fixation for F-Actin Staining

1. 0.1 M PBS (phosphate-buffered saline): 1.4 M NaCl, 26.8 mM KCl, 80.9 mM Na_2HPO_4 in H_2O, adjust to pH 7.4 using HCl or NaOH. Can be stored at room temperature for at least 1 year but should be discarded if turbid.
2. Fixative solution: 4% (v/v) paraformaldehyde (PFA) in 0.1 M phosphate buffered saline. Long-term storage (>2 weeks) in plastic containers at −20 °C and short term at 4 °C.
3. PBT: 0.2% (v/v) Triton X-100 in 0.1 M PBS. If stored at 4 °C in the dark it is usable for months.
4. Conjugated phalloidin: dissolve (usually comes as a powder; e.g., Alexa Fluor 488 phalloidin; Invitrogen, BODYPI R6G phalloidin, Oregon Green 514 phalloidin, BODIPY FL phallacidin) in methanol to yield a final concentration of 200 units/mL. This stock solution is stable for at least 1 year when stored at −20 °C.
5. Staining solution (1:20 diluted): 50 μL conjugated phalloidin, 950 μL PBT. Store at 4 °C in the dark for up to 1 week.

2.4 RNA Extraction and Target Gene Amplification

1. Mortar and pestle.
2. Sterile filter tips.
3. TRIzol™ Reagent (Invitrogen).
4. Chloroform.
5. 2-Propanol.
6. Ice-cold (−20 °C) 75% ethanol.
7. Liquid nitrogen.
8. Coprecipitant (e.g., Vivid Violet®, Roboklon; pellet paint®, Novagen; glycogen).
9. DEPC water: 1 mL diethyl pyrocarbonate (DEPC) in 1000 mL water. Mix in a screw-cap glass bottle. Incubate for ~2 h at room temperature in a fume hood with occasional swirling. Autoclave.

10. Reverse Transcriptase Kit (e.g., Super Script III, Invitrogen) which includes reaction buffer, 25 mM $MgCl_2$, DTT, and the reverse transcriptase enzyme.
11. RNase inhibitor.
12. 10 mM anchored oligoT primer oligodT-T7I. (GAGAGAG GATCCAAGTACTAATACGACTCACTATAGGGAGAT$_{25}$V) and 10 mM anchor primer oligodT-T7II (GAGAGAGGATC CAAGTACTAATACGACTCACTATAGG).
13. 10 mM gene-specific forward primer.
14. Taq polymerase master mix (e.g., 2× OptiTaq PCR Master Mix, Roboklon).
15. TAE buffer: 40 mM Tris, 20 mM acetic acid, 1 mM EDTA.
16. 1% (w/v) agarose (for molecular biology) in TAE buffer.

3 Methods

3.1 Collection and Identification

There are pronounced species-specific settlement preferences such as epizoic growth on sponges, bryozoans, polychaetes, and sipunculids or attachment to macroalgae, and for a few species also inanimate surfaces such as rocks or shells. Known substrate preferences will guide the collection of animals in the field. For example, the cosmopolitan colonial freshwater Kamptozoa *Urnatella gracilis* prefers shells of *Dreissena polymorpha* in the area around Berlin [3] and the solitary kamptozoan *Loxosomella murmanica* live epizoically on the sipunculan *Phascolion strombus* which inhabits empty shells of the gastropod *Turritella* sp. and the scaphopod *Antalis* sp.. Such shells can be dredged from about 30 m depth from muddy and rocky bottom in the North Sea [18]. We here present our protocol for the collection of *Barentsia benedeni* colonies.

1. Identify sheltered microhabitats suitable for the presence of *Barentsia benedeni*. Successful collection sites are at the bottom of wooden pier pilings, groynes, and old macroalgae in low-tide pools.
2. Retrieve material attached to the promising substrate either by scrapping it using a fishing net or by hand if within reach.
3. Transfer the sample to a small container filled with seawater.
4. Isolate suspicious samples with whitish material on them to a transport vessel (e.g., Kautex flask).
5. Bring the collected samples back to the lab.
6. Transfer one sample into a large petri dish or tray filled with FNSW.
7. Screen the whitish material under a dissecting microscope.

8. Confirm that identified zooids are kamptozoans by disturbing them using a fine paintbrush and observing their unique bending behavior.
9. Detach the kamptozoans from their substrate using a scalpel blade. Solitary kamptozoans such as *Loxosomella vivipara* immediately glue themselves with their foot on the glass wall of the container and in such cases the relaxing medium must be applied. Wait 1 to 3 min for the animals to relax (*see* **Note 3**).
10. Flush the detached animals using a Pasteur pipette while holding its stolon with curved tweezers to clean them from associated macrofauna such as Gammaridae or other small crustaceans.
11. Pool the cleaned kamptozoans in a container filled with FNSW and continue with either Subheadings 3.3, 3.4, or 3.5, respectively.

3.2 Food Algae Culture

Wear gloves to avoid contamination.

1. Pour a 200 mL aliquot of the algae stock on a petri dish.
2. Screen for obvious signs of contamination with a microscope.
3. Discontinue contaminated cultures, start with a new stock in **step 1**.
4. Transfer 150 mL NFSW into a 250 mL Erlenmeyer flask with a cotton plug.
5. Heat up for 1 min at 600 W in a microwave. Do not let the water boil or salts will precipitate [17].
6. Add 3 mL of freshly filter-sterilized 50X F/2 medium to the hot seawater (*see* **Note 4**).
7. Mix gently by swirling without wetting the cotton plug.
8. Wait 1 h for the medium to cool down to culturing temperature (RT or lower).
9. Inoculate with up to 50 mL algae stock.
10. Place the culture on an orbital shaker (~120 rpm) in the light cabinet (*see* **Note 5**).
11. Leave the culture to grow for 2 weeks before using it for feeding kamptozoans.
12. Algae cultures can be kept up to 1 month.
13. Renew the culture by starting with **step 1** using the old culture as stock.
14. Keep two 50 mL cultures at lower light, without shaking nor routine opening, for up to 2 months as backups.

3.3 Barentsia benedeni Culture

In our culturing setup, collected colonies will not attach themselves again to the walls of the culture vessel but will become floating ball-like structures.

1. Gently shake colonies within the culturing water using curved tweezers to remove detritus and filamentous algae.
2. Gently flush the colonies with a Pasteur pipette while holding them to remove potential unwanted macrofauna.
3. Transfer the cleaned colony to a tray filled with FNSW.
4. Confirm cleanness and health condition under a stereo microscope.
5. Discard sick or contaminated colonies.
6. Transfer the colony to a clean culturing bowl filled with FNSW (Fig. 4).
7. Place the culture bowl on a rotating shaker with low speed <100 rpm at 16–19 °C and dim indirect light during daytime (12 h/12 h) (*see* **Notes 6** and **7**).
8. Add once a week 5 mL of a dense algae culture with a Pasteur pipette to the *Barentsia* culture vessel (*see* **Notes 8** and **9**).
9. Leave the animals to feed for 24 h.
10. Gently shake colonies within the culturing water using curved tweezers to remove detritus and uneaten algae.
11. Clean the colonies and maintain the culture by restarting at **step 1**.

3.4 Tissue Isolation and Regeneration

1. Transfer a cleaned colony to a container filled with fresh FNSW.
2. Starve the colony for 1 week to fully isolate the zooid (*see* **Note 10**).
3. Clean the colony and the culture vessel twice during the starvation.
4. Amputate the tissue to be isolated using a fine pair of Vannas scissors.
5. Transfer the colony back to a clean culture vessel, without feeding.
6. Monitor the regenerating colony every 48 h.
7. In the case of whole-body regeneration induced by a stalk cut, regeneration will proceed as follows (Fig. 3).
8. 2 days postamputation (dpa): first regeneration stage, atrium and stomach have already formed.
9. 4 dpa: second regeneration stage, the esophagus has elongated, midgut is formed, and atrium is bulged on the anal side.
10. 6 dpa: third regeneration stage, developing hindgut and atrium converge.
11. 8 dpa: fourth regeneration stage, hindgut and atrium are interconnected.
12. 10 dpa: fifth regeneration stage, intestinal tract is again fully developed.

3.5 Fixation and F-Actin Staining

This protocol describes staining against F-actin but can be generalized to other conjugated probes by adapting the composition and incubation time of the staining medium. Thus staining with other primary and secondary antibodies could be applied to study different structures such as nuclei or keratin.

1. Transfer a fresh tissue sample to a 2 mL tube containing 1.5 mL of fixative solution.
2. Incubate for 1 h at room temperature.
3. Rinse the sample for 15 min in 0.1 M PBS.
4. Rinse the sample two more times by repeating **step 3**.
5. Store for up to 1 week at 4 °C in 0.1 M PBS containing 0.1% sodium azide (NaN_3) if subsequent developmental stages need to be collected (optional).
6. If sample was stored in NaN_3 repeat **step 3** and **4**.
7. Replace the solution with 1.5 mL of PBT.
8. Incubate for 1 h at room temperature to permeabilize the cell walls.
9. Replace the solution with 1 mL of staining solution.
10. Incubate for 4 h in the dark at room temperature.
11. Rinse three times in PBS for 15 min.
12. Transfer the sample onto a glass slide.
13. Mount with aqueous mounting medium.
14. Cover with a coverslip.
15. Image on a fluorescence microscope.

3.6 Total RNA Extraction and Target Gene Amplification

Although RNA can be extracted from any piece of tissue, we obtained good yields by pooling together tissue from approximately 40 clonal zooids. The sample volume should not exceed 10% of the volume of the TRIzol used for lysis. You may wash your bench and pipettes with RNase erase solution before work. All steps at room temperature unless otherwise specified. Wear protective gloves and googles when working with liquid nitrogen or deep-frozen devices. Use a fume hood during processing of phenol. Wear disposable gloves while handling organic reagents such as TRIzol (contains phenol) and RNA samples to prevent RNase contamination from the surface of the skin; change gloves frequently, particularly as the protocol progresses from crude extracts to more purified materials.

1. Precool mortar and pestle at −80 °C for several hours or overnight.
2. Fill a 2 mL reaction tube with 1 mL of TRIzol.
3. Dab the isolated tissue onto a paper wipe.

4. Transfer the tissue to a 2 mL tube.
5. Shock-freeze the tube in liquid nitrogen.
6. Store the frozen tissue at −80 °C until needed.
7. Transfer the deep-frozen tissue to the precooled mortar.
8. Grind the frozen tissue to a fine powder. You may carefully add liquid nitrogen using a ladle if signs of thawing occur during homogenization (*see* **Note 11**).
9. Transfer the powdered tissue to the prepared reaction tube with TRIzol using a spatula below a fume hood.
10. Incubate for 5 min.
11. Add 0.2 mL of chloroform.
12. Securely cap the tube and shake hard for 30 s while securing the lid with your thumb.
13. Incubate for 2–3 min.
14. Centrifuge the sample for 15 min at 12,000 rcf at 4 °C. The mixture separates into a lower red phenol–chloroform, an interphase, and a colorless upper aqueous phase containing the RNA.
15. Transfer the upper aqueous phase to a new sterile tube by angling the tube at 45° and avoid touching the interphase. Be generous to sacrifice RNA containing aqueous phase to the benefit of avoiding carry over contaminations from the interphase or the red organic phase.
16. Add 2 μL of coprecipitant to the aqueous phase in the new tube.
17. Add 0.5 mL of isopropanol.
18. Vortex briefly.
19. Incubate for 10 min.
20. Centrifuge for 10 min at 12,000 rcf at 4 °C. Total RNA precipitates as a pink pellet or a white gel-like pellet (if using glycogen) at the bottom of the tube.
21. Discard the supernatant using a pipette.
22. Resuspend the RNA pellet in 1 mL cold 75% ethanol by vortexing and flicking the tube.
23. The tube can be stored up to 1 year at −20 °C.
24. Centrifuge for 5 min at 7500 rcf at 4 °C.
25. Discard the supernatant using a pipette. Avoid touching or detaching the pellet.
26. Let the RNA pellet dry for 5–10 min by opening the lid in a dust free space.

27. Resuspend the pellet in 20–50 μL DEPC water and store the RNA solution at −80 °C or proceed to the next step setting up a rtPCR.
28. Thaw the following tubes from the cDNA synthesis kit at room temperature: 10× RT buffer, 25 mM $MgCl_2$ and 0.1 M DTT but keep the RNase inhibitor and the reverse transcriptase at −20 °C until needed (**step 32**).
29. Combine the following in a 0.2- or 0.5-mL tube: 1–4 μL RNA solution, 1 μL oligodT-T7I primer, 1 μL dNTP mix.
30. Fill up to 10 μL using DEPC water.
31. Incubate the tube at 65 °C for 5 min.
32. Prepare the following cDNA Synthesis Mix, adding each component in the indicated order in a 0.2 mL tube on ice: 2 μL 10× RT buffer, 4 μL 25 mM $MgCl_2$, 2 μL 0.1 M DTT, 1 μL RNase inhibitor, 1 μL reverse transcriptase.
33. Place the incubated RNA on ice for at least 1 min.
34. Add the 10 μL cDNA Synthesis Mix to the RNA/primer mix.
35. Incubate 50 min at 50 °C.
36. Terminate the reactions at 85 °C for 5 min.
37. Chill on ice for 1–5 min.
38. cDNA synthesis reaction can be stored at −20 °C or used for PCR immediately.
39. For PCR, add each component in the indicated order in a 0.2 mL tube on ice: 25 μL Master Mix, 1 μL gene specific forward primer, 1 μL oligodT-T7II, 1–4 μL cDNA mix and fill up to 50 μL using sterile water.
40. Amplify the PCR product using the following temperature settings in a thermocycler: Denaturation at 93–95 °C 2 min, 30× (denaturation at 93–95 °C 15 s, annealing at 50–68 °C for 30 s, elongation at 72 °C for 20 s/kb), final extension 7 min. Store at 4–16 °C.
41. Control visually for the presence of a single sharp PCR product band on an agarose gel.
42. Send 50 ng up to 200 ng DNA (e.g., 2 μL of a 1/10 diluted PCR reaction) in a 10 pmol primer solution to a Sanger sequencing company (*see* **Note 12**).

4 Notes

1. In our lab, we use commercially available FNSW which is shipped in 5 L containers. Natural seawater is mandatory for long term culture of the species, but not for the algae. For algal culture, it is thus possible to use artificial seawater instead.

2. Published lab culture of kamptozoans are based on feedings with algae suspensions from strains such as *Isochrysis*, *Monochrysis* [19], and *Cryptomonas* or for freshwater species *Ankistrodesmus*, *Scenedesmus*, and *Chlorella* [3]. In our lab, we have been using successfully *Cryptomonas baltica* for over 5 years. If this specific algae cannot be obtained, we would suggest as an alternative *Pyrenomonas helgolandii*. It can be assumed that this alga is just as suitable as *Cryptomonas* as complete feed, but it might require another medium than F/2 to grow as the advice of the SAG (Culture Collection of Algae at Göttingen University) is to use media including soil extract for this species.
3. Proceed with steps under anesthesia as efficiently as possible. Although $MgCl_2$ anesthesia is reversible, such a treatment is stressful for the animal. Thus, living healthy individuals for culturing purpose are best obtained without or very short $MgCl_2$ incubation times.
4. Although high temperatures might be harmful to the vitamins, we have been introducing the 50× F/2 medium shortly after heating without obvious negative effects. On the contrary, the high temperature appears to be helpful in avoiding contaminations.
5. Algae can alternatively be cultured without shaking but with daily manual swirling of the flasks. If no light cabinet is available, algae can be cultured simply using daylight from a northern window avoiding direct sun and high temperatures (Fig. 5).
6. *B. benedeni* can be kept in brackish water with a salinity of 20 ppt to allow slight evaporation to reduce the risk of losing the animals in times of lower maintenance due to excessive increase in salinity.
7. Animals can be placed on the same shaker as the algae flasks but draw-off should take place in another room to reduce the contamination risk. If a separate room isn't available, clean thoroughly the opening of the algae flask either by flaming or by using ethanol before collecting out of the flask.
8. Increase feeding rate if stronger growth is desired. Increased feeding must be done with more frequent feeding, not higher algae quantity in a single feeding, and needs always be accompanied by subsequent water exchange.
9. There are concentrated algae paste commercially available (e.g., Shellfish Diet 1800, Reed Mariculture). We have no experience using instant algae paste for Kamptozoa, but it might be possible to skip the algae culture part by using such products. Nonliving feed will increase the risk of water deterioration and will require careful water exchange and transfer to clean bowls more often.

Fig. 5 Simple algae culturing unit using natural light without shaker. Bottles are shaken manually once every working day because algae tend to settle on the bottom. *Isochrysis* sp. (left bottle) and *Nannochloropsis* sp. (right bottle)

10. Starvation time might be reduced because it has been shown that Kamptozoa have fast digesting times of a few hours [7].
11. In case of low tissue amount the tissue can be grinded directly in a 2 mL reaction tube using an EPPI-Pistill.
12. In case of heterogenous or low signal reads, PCR cleanup using SAP digestion or agarose gel clean up should be applied to improve the quality of the sequencing reads.

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