

# Chapter 2

## DNA Extraction Protocols for Whole-Genome Sequencing in Marine Organisms

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### Abstract

The marine environment harbors a large proportion of the total biodiversity on this planet, including the majority of the earth's different phyla and classes. Studying the genomes of marine organisms can bring interesting insights into genome evolution. Today, almost all marine organismal groups are understudied with respect to their genomes. One potential reason is that extraction of high-quality DNA in sufficient amounts is challenging for many marine species. This is due to high polysaccharide content, polyphenols and other secondary metabolites that will inhibit downstream DNA library preparations. Consequently, protocols developed for vertebrates and plants do not always perform well for invertebrates and algae. In addition, many marine species have large population sizes and, as a consequence, highly variable genomes. Thus, to facilitate the sequence read assembly process during genome sequencing, it is desirable to obtain enough DNA from a single individual, which is a challenge in many species of invertebrates and algae. Here, we present DNA extraction protocols for seven marine species (four invertebrates, two algae, and a marine yeast), optimized to provide sufficient DNA quality and yield for *de novo* genome sequencing projects.

**Key words** Genomic DNA extraction, Gastropod *Littorina*, Isopod *Idotea*, Barnacle *Balanus*, Brittle star *Amphiura*, Brown alga *Fucus*, Diatom *Skeletonema*, Marine yeast *Debaryomyces*

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### 1 Introduction

With the development of next-generation sequencing (NGS) techniques, we can for the first time get comprehensive genomic information at a reasonable price for marine non-model organisms. However, the success of a genomic project greatly depends on the ability to obtain sufficient amounts of pure and high-molecular weight DNA from the target species. Purity is crucial for the library preparation step; longer insert size Illumina libraries (mate-pair libraries) and Pacific Biosciences (PacBio) sequencing require 5–20 µg of

high-molecular weight DNA. As many marine species have large population sizes and, as a consequence, highly variable genomes [1], whole-genome sequencing projects are greatly facilitated by producing libraries using DNA from one single individual. Thus, optimization of DNA yield per extraction and per individual is a crucial initial task, especially given the small body size of many species. Furthermore, commercially available DNA extraction kits that perform well on vertebrate and plant material often fail to produce good-quality DNA for marine algae and invertebrates due to substances that co-purify with DNA and lead to low DNA yield and/or purity.

In this chapter, we present optimized DNA extraction protocols for seven marine species in the marine genome sequencing project of the Centre for Marine Evolutionary Biology (CeMEB), University of Gothenburg, Sweden [2]: the gastropod mollusk *Littorina saxatilis*, the isopod crustacean *Idotea balthica*, the barnacle crustacean *Balanus improvisus*, the brittle star echinoderm *Amphiura filiformis*, the brown algae *Fucus vesiculosus* (also tested on the closely related species *F. radicans*), the diatom *Skeletonema marinoi*, and the marine yeast *Debaryomyces hansenii*. All seven species are promising systems for studies of adaptation and speciation in the marine environment. However, progress has been impeded in the past by the lack of genomic information, and to remedy this, we undertook an ambitious project to sequence their genomes. (The genome of *D. hansenii* has already been sequenced [3], but our goal here was a comprehensive population genomics re-sequencing effort.) The first challenge was to develop robust and efficient genomic DNA extraction protocols for all these non-model marine species. For each species, the general strategy was to test several methods based on the literature and our previous experience, choose the method providing the highest yield of non-degraded DNA, and then further optimize the protocol to reach the NGS requirements for quantity and quality of DNA. For several of the species, further improvements increasing DNA yield and purity had to be made along the way in response to sequencing failures (see below).

### **1.1 *Littorina saxatilis* (Gastropod Mollusk)**

*Littorina saxatilis* is a marine gastropod mollusk common on the rocky intertidal. Adult shell height is 1.2–25.8 mm; snail size varies between geographic populations and ecotypes [4]. The major challenge in DNA extraction from mollusk tissue is a high content of mucopolysaccharides that tend to co-purify with DNA [5]. In addition, some species including *L. saxatilis* are relatively small, and it is hard to obtain enough DNA from a single individual for a whole-genome sequencing project.

Using commercial kits such as the DNeasy Blood & Tissue Kit (Qiagen), DNeasy Plant Mini Kit (Qiagen), and E.Z.N.A. Mollusc DNA Kit (Omega Bio-tek) for *L. saxatilis* provides DNA suitable for routine PCR amplification of microsatellites, nuclear introns,

and mitochondrial gene fragments, but the total yield ( $\leq 1 \mu\text{g}$ ), concentration ( $\leq 10 \text{ ng}/\mu\text{L}$ ), and absorbance ratios are not compatible with NGS applications.

The protocol described here uses CTAB (cetyltrimethyl ammonium bromide) buffer, which binds proteins and polysaccharides at high salt concentration in combination with Proteinase K, which digests proteins. The DNA is extracted using chloroform: isoamyl alcohol, based on a method earlier suggested for mollusks [5], which has been modified to maximize the yield and improve the purity and integrity of the DNA and to include an RNase A treatment. Further, tissue homogenization in liquid nitrogen has been replaced by homogenization in the digestion buffer, since using liquid nitrogen with small tissues samples is difficult and may lead to loss and/or thawing of material.

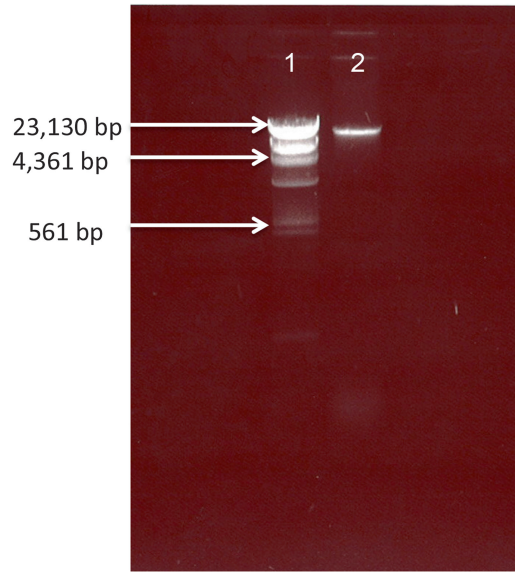
We have successfully used this extraction method to prepare *Littorina* genomic DNA samples for comparative genomics hybridization [6], restriction site associated DNA (RAD) genome scans [7], and to obtain enough material for *L. saxatilis* de novo genome sequencing from one single specimen [2], where we combined sequencing of short- and long-insert Illumina libraries with PacBio sequencing.

One extraction typically gives  $\geq 2 \mu\text{g}$  of genomic DNA at a concentration of  $70\text{--}300 \text{ ng}/\mu\text{L}$ . The DNA has a high-molecular weight (Fig. 1) and typical absorbance ratios are  $1.95\text{--}1.99$  at  $260/280 \text{ nm}$  and  $2.0\text{--}2.22$  at  $260/230 \text{ nm}$ . For de novo genome sequencing, we were able to obtain  $70 \mu\text{g}$  of genomic DNA from a single individual by dividing tissues into 12 separate extractions.

## **1.2 *Idotea balthica* (Isopod Crustacean)**

*Idotea balthica* is a marine isopod living on seaweeds in shallow waters. The average body length of adult animals is  $20\text{--}30 \text{ mm}$ . DNA extraction from arthropods is often difficult, especially from species with body pigmentation [8]. Different DNA extraction protocols have been tested mainly for insect species, e.g., [9]. In our work with *Idotea* species, DNA of a quality suitable for PCR amplification of nuclear and mitochondrial fragments is usually obtained using the Chelex method or the DNeasy Blood and Tissue Kit (Qiagen). However, both methods provide yields ( $< 1 \mu\text{g}$ ) and concentrations ( $< 10 \text{ ng}/\mu\text{L}$ ) below NGS requirements. Here we suggest a protocol based on multiple phenol-chloroform extractions as this gives an approximately 50 times higher yield and high-molecular weight DNA. It includes two extractions with phenol:chloroform:isoamyl alcohol followed by two extractions with chloroform:isoamyl alcohol. In the development of this protocol, we also tried to use fewer extraction steps but this gave lower DNA purity (as estimated by spectrophotometric  $280/260$  ratios) without resulting in higher DNA yield.

DNA samples extracted with this protocol were successfully used to produce 2b-RAD [10] libraries. However, there were

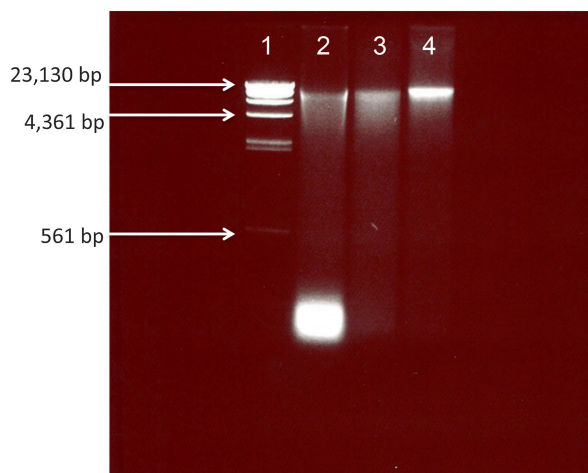


**Fig. 1** Integrity of *Littorina saxatilis* DNA extracted by the CTAB method. Electrophoresis was performed in a 0.8% agarose gel and 1× TAE buffer. DNA was stained with GelRed. *Lane 1*: Lambda DNA/HindIII Marker; *Lane 2*: 200 ng of *L. saxatilis* genomic DNA

problems with the Illumina TruSeq DNA library preparation that may be due to contaminants in the DNA preparations. To remedy this, after phenol-chloroform extraction, the DNA was additionally cleaned using Genomic DNA Clean & Concentrator-10 Kit (Zymo Research). This step removes the low-molecular weight smear (*see* Fig. 2) and contaminants. Using this two-step protocol and dividing the tissue from a large specimen into 24 extractions, we were able to obtain 41 µg of genomic DNA from a single individual that was used in our de novo genome sequencing project.

### 1.3 *Skeletonema marinoi* (Diatom)

*Skeletonema marinoi* is a microscopic chain-forming unicellular phytoplankton species. Cell valve diameter is 5–12 µm wide. Cell walls are made of silica and have a complex pore structure [11]. Silica is commonly utilized in commercial extraction kits to retain DNA. Thus, stringent conditions are needed to separate the DNA from the silica walls. The amount of genomic DNA required by any sequencing platform for whole-genome sequencing exceeds the amount available in a single cell (in the range of femtograms); thus, it is necessary to produce monoclonal cultures. We grow our strains in batch culture with F/2 medium supplemented with silica [12]. Culturing of microalgae, however, has several knock-on effects. First, there is a bias due to selection for culturable strains. Secondly, strains in laboratory cultures undergo physiological changes due to adaptation and selection of specific

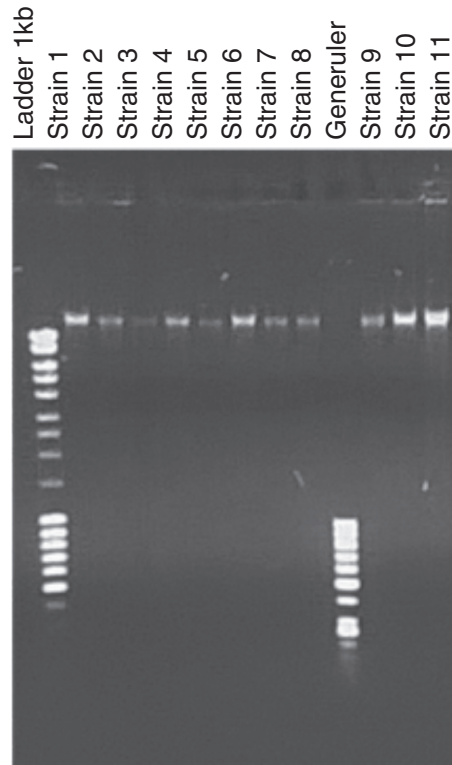


**Fig. 2** Integrity of *Idotea balthica* DNA extracted by the CTAB:SDS method. Electrophoresis was performed on a 0.8% agarose gel and 1× TAE buffer. DNA was stained with GelRed. *Lane 1*: Lambda DNA/HindIII Marker; *Lane 2*: 200 ng of *I. balthica* genomic DNA before the column purification; *Lane 3*: 200 ng of *I. balthica* genomic DNA purified with DNA Clean & Concentrator Kit; *Lane 4*: 200 ng of *I. balthica* genomic DNA purified with Genomic DNA Clean & Concentrator-10 Kit

cell lines and genetic changes due to mutations [13]. Thirdly, cultures also have the disadvantage of acquiring contaminants (e.g., bacteria or fungi), and foreign DNA is extracted together with DNA from the target species. To establish the de novo genome of *Skeletonema marinoi*, we treated the reference strain with antibiotics, as reported in [14, 15] before the DNA extraction. However, even after the antibiotic treatment, some bacterial contamination still remained.

For diatoms, CTAB extractions provide suitable genomic DNA for fingerprinting and fragment amplifications [16]. For de novo genome amplification, we modified the protocol to improve the quality and quantity and obtained an absorbance ratio of 1.8–2.0 at 260/280 nm, a unique high-molecular weight band of genomic DNA (Fig. 3), and a final amount of 10–30 µg of genomic DNA per billion antibiotic-treated cells. The best DNA was obtained when the culture was harvested by mild centrifugation in the exponential growth phase, the samples processed fast to avoid degradation, and the extracted DNA treated with RNase A.

This DNA extraction procedure has been used to prepare mate-pair and paired-end Illumina libraries and for PacBio sequencing. Additionally, the same DNA extraction protocol has been used for re-sequencing of non-axenic environmental isolates.



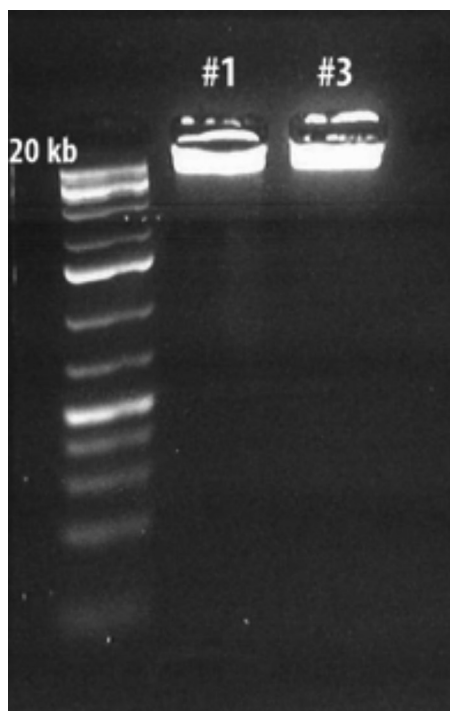
**Fig. 3** Integrity of DNA extracted from 11 strains of *Skeletonema marinoi*. Electrophoresis was performed on a 0.7 % agarose gel and 1× TAE buffer; DNA was stained with Ethidium bromide

#### 1.4 *Amphiura filiformis* (Brittle Star Echinoderm)

*Amphiura filiformis* is a small brittle star that lives on mud and sand bottoms. It has long arms and a small central disk (maximum diameter is 10 mm). Ophiuroids are highly calcified species with very little soft tissue. The viscera inside the disk are the exception, where internal organs of digestion and reproduction are located, along with a high amount of bacteria [17]. Initial DNA extraction on this species aimed to use a single adult male individual, reducing polymorphisms due to high variation among individuals. Using DNAzol (Invitrogen) for extraction of DNA from arms yielded just enough DNA for the project, but the resulting DNA preparation was colored brown, even after several ethanol washes, and the subsequent Illumina library preparation failed. Other protocols such as digestion with CTAB or SDS buffers and the NucleoSpin DNA extraction kit (Macherey-Nagel) did not provide suitable DNA for NGS applications.

Following the protocol routinely used for genomic DNA extraction from echinoderm sperm of many different species including that of the sea urchin *Strongylocentrotus purpuratus* [18], DNA was successfully extracted from *A. filiformis* sperm. This procedure was originally developed for fresh sperm recovered undiluted from spawning sea urchins. It can also be used for lyophilized sperm samples after grinding in dry ice, or frozen sperm.





**Fig. 4** Integrity of DNA extracted from *Amphiura filiformis* sperm. Electrophoresis was performed on a 0.8% agarose gel and 1× TAE buffer. DNA was stained with GelRed. The *first lane* shows a 20 kb size DNA marker and the *second and third lanes* show two samples of *A. filiformis* genomic DNA

This protocol provided around 130 µg of genomic DNA at a concentration of 500–600 ng/µL and absorbance ratios 1.89 and 2.3 at 260/280 and 260/230 nm, respectively. The DNA had a high-molecular weight, as required by NGS service providers (Fig. 4).

### **1.5 *Fucus vesiculosus* (and *F. radicans*) Brown Macroalgae**

*Fucus* seaweeds belong to the taxonomic order Fucales, which includes some of the most common littoral seaweeds. As with other organisms, one of the main challenges to obtain high-quality DNA from brown algae is to remove compounds constituting their cell walls and tissue. Alginates and fucose-containing sulfated polysaccharides are the main cell wall polymers in Fucales [19]. It has been shown that the amount of polysaccharides is directly correlated to the species' position on the shore, suggesting that high contents may confer an adaptive advantage to species frequently exposed to immersion [20]. The removal of these high amounts of polysaccharides is essential when attempting to extract high-quality DNA from these algae. Furthermore, additional polyphenolic compounds also complicate the extraction of pure and intact

DNA. The overproduction of polysaccharides is likely accompanied by the reinforcement of polyphenol-alginate complexes that consolidate further the cell wall architecture [19]. In high amounts, these phenolic compounds get co-washed during most DNA extraction procedures resulting in low-quality extractions and most commercial procedures only partially remove these compounds. Concomitantly, using large amounts of tissue also accumulates large amounts of “contaminant” chemical compounds that decrease the DNA yield per extraction.

Here, we present a combination of approaches that initially remove high contents of polyphenolic compounds using a solvent (acetone), followed by a CTAB-based extraction buffer solution containing chemical complexors of the “contaminant” metabolites (polyvinylpyrrolidone (PVPP) [21, 22], diethyldithiocarbamic acid (DIECA) [23], and a strong reductant of polysaccharides ( $\beta$ -mercaptoethanol). The method requires freshly harvested algal tissue or lyophilized tissue preserved in silica gel for no more than 2 months to avoid degradation of DNA. The DNA is extracted with the aid of the commercial extraction kit NucleoSpin Plant II (Macherey-Nagel) to effectively separate the contaminant proteins. However, it requires an extra cleanup step using DNA Clean & Concentrator (Zymo Research) after the DNA has been extracted to remove co-washed polysaccharides that become a viscous solution in the extract. Using this kit efficiently removes large amounts of undesired polysaccharides.

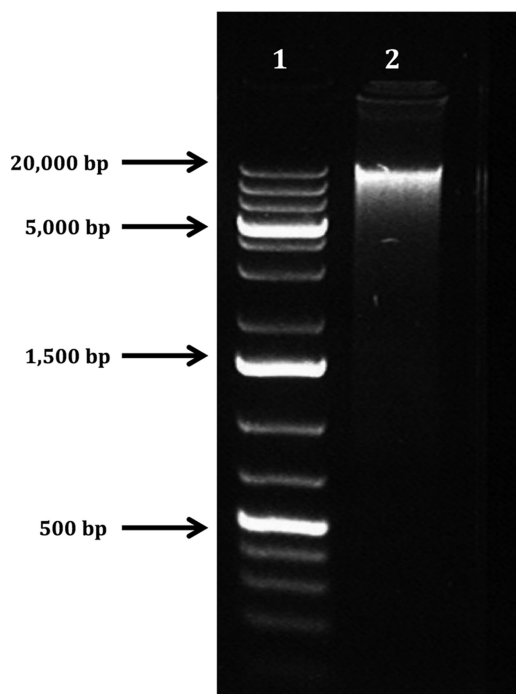
Given the accumulation of contaminating chemicals when large amounts of tissue are extracted at one time, the acquisition of high DNA yields and concentrations required for de novo genome sequencing is achieved by performing several extractions with low amounts of tissue in each. The typical amount of DNA obtained in one extraction is 2.5  $\mu$ g, with high-molecular weight (Fig. 5) and absorbance ratios 1.9 and 1.8 at 260/280 and 260/230 nm, respectively.

### **1.6 *Balanus improvisus* (Barnacle Crustacean)**

*Balanus improvisus* is a relatively small acorn barnacle [24] of only 5–12 mg of tissue in dry weight per individual [25]. It is thus challenging to get high amounts of good DNA from one single individual (*see Note 1*). Due to high genetic variation (roughly 4% sequence divergence between two alleles within one single individual, use of a single individual is highly recommended for whole-genome sequencing in order to optimize the final genome assembly process. Ideally we aim for 10–20  $\mu$ g of DNA from one individual to enable the production of several small and large fragment libraries for sequencing.

In order to optimize the amount and quality of genomic DNA preparations from adult barnacles, we initially tested several methods: the E.Z.N.A. Mollusc DNA Kit (Omega Bio-tek), the CTAB method [5], the DNazol kit (Life Technologies), and the E.Z.N.A. Blood DNA Mini Kit (Omega Bio-tek). The methods

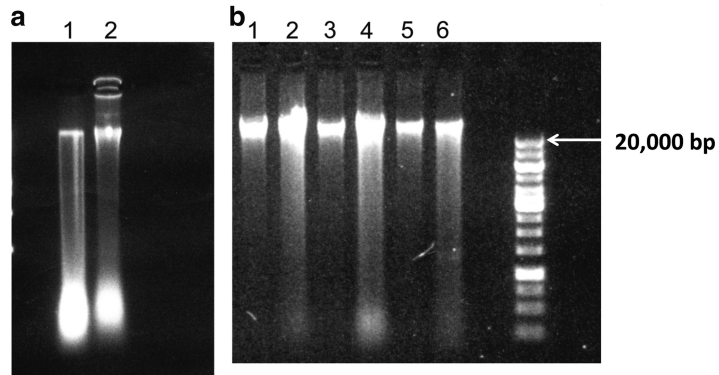




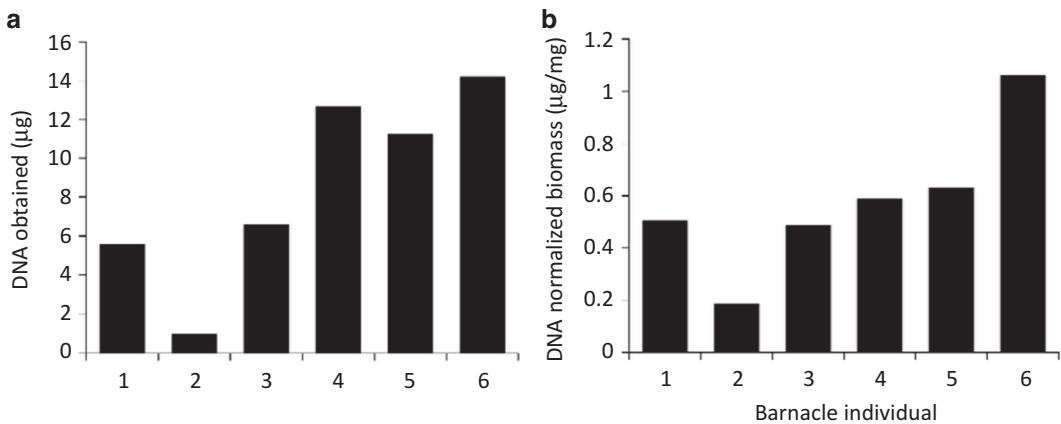
**Fig. 5** Integrity of *Fucus vesiculosus* DNA. Electrophoresis was performed on a 0.8 % agarose gel and 1× TAE buffer. DNA was stained with GelRed. *Lane 1*: 1 kb ladder showing the 20 kb DNA size marker; *Lane 2*: 200 ng of *F. vesiculosus* genomic DNA

were independently tested at least twice, each time using 2–3 individuals. We found that the E.Z.N.A. Mollusc DNA Kit and the CTAB method resulted in either degraded DNA or a weak high-molecular weight DNA band together with an abundant low-molecular weight band that remained even after RNase treatment (Fig. 6a and *see Note 2*). DNAzol gave good DNA, but the DNA was very hard to dissolve in water or TE buffer, which is usually required by sequencing facilities/companies.

The E.Z.N.A. Blood DNA Mini Kit, however, gave good amounts of DNA with high integrity and purity (Fig. 6b). With this DNA kit, we obtained  $8 \pm 5$  µg DNA per individual, but the individual variation was relatively large with a coefficient of variation (CV) of 58 % (Fig. 7a). We tested if this variation is the outcome of variable size (tissue weight) of the individual barnacles; however, even after normalizing for the variation in wet weight, we still see large individual differences in the amount of DNA obtained (CV ≈ 49 %) (Fig. 7b). Thus, it is clear that tissue weight is not the only factor determining the amount of DNA per individual obtained. Preparing DNA from several barnacles and selecting the best preparation for sequencing is therefore recommended to ensure DNA of both high quantity and quality.



**Fig. 6** Integrity of *Balanus improvisus* DNA extracted using two methods. (a) DNA extracted from two different adult *B. improvisus* individuals using the CTAB method. Only a small amount of the DNA appears as a high-molecular weight band and there is a large band of low-molecular weight material. B: DNA extracted from three different *B. improvisus* individuals, two elution steps each, using the E.Z.N.A. Blood DNA Mini Kit. Lanes 1, 2: Individual 1, first elution and second elution; Lanes 3, 4: Individual 2, first elution and second elution; Lanes 5, 6: Individual 3, first elution and second elution. Last lane: 20 Kb DNA size marker. Most of the DNA appears to be  $\geq 20$  Kb

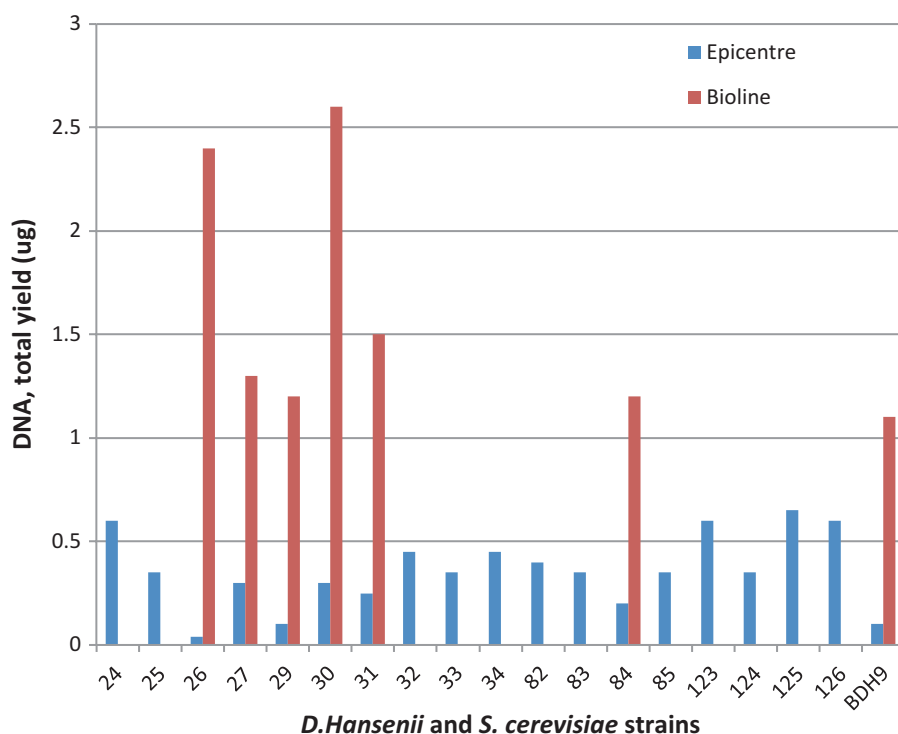


**Fig. 7** Variable yields for genomic DNA preparations of adult *B. improvisus* individuals. The E.Z.N.A. Blood DNA Kit was used to prepare DNA from six different *B. improvisus* individuals. (a) The amount of DNA in µg obtained from each individual. (b) The amount of DNA obtained from each individual is normalized against the wet weight of the individual (wet weight per individual was in the range of 5–22 mg)

### 1.7 *Debaryomyces hanssenii* (Marine Yeast)

*Debaryomyces hanssenii* is a marine yeast that occurs globally with extreme tolerance to salt and dehydration stress. It is slightly halophilic and grows better in seawater compared to freshwater. Studies of *D. hanssenii* will be important in our understanding of the evolution of osmoregulation in marine fungi. The genome sequence of *D. hanssenii* has already been established and published for the type strain CBC767 [3]. In addition, genome contigs from an alternative strain, MTCC 234, were recently published [26]. However, in these genome publications, the method for DNA extraction has not been specified.

*D. hansenii* is quite distantly related to the common yeast model species *Saccharomyces cerevisiae*. Most of the methodologies developed and applied to the model species work in *D. hansenii*; however, the marine yeast might require slight but important alterations to some of the commonly used yeast protocols. We initiated our population genomics project on *D. hansenii* by applying routine protocols extensively used for extracting DNA from *S. cerevisiae* (breakage of cells with glass beads and extraction with phenol:chloroform). However, we found that the amount of DNA, its quality, and purity were not sufficient when these standard protocols were applied to *D. hansenii*. Instead we found that more and better quality DNA was obtained from *D. hansenii* if some commercial kits were used. In order to test different protocols for efficiency and robustness on different strains, we extracted DNA from 17 different *D. hansenii* isolates (obtained from various geographical locations as well as sources) and two *S. cerevisiae* isolates (as controls). We found that the MasterPure Yeast DNA Purification Kit (Epicentre) yielded good DNA from 12 of the *D. hansenii* isolates (as well as from the two *S. cerevisiae* controls); however, the method provided rather low amounts for seven of the *D. hansenii* strains (Fig. 8). We then tested



**Fig. 8** DNA yield per strain in *Debaryomyces hansenii* and in *Saccharomyces cerevisiae* control. Blue bars show the amount of DNA in µg extracted using the MasterPure Yeast DNA Purification Kit (Epicentre) from 17 strain isolates of *D. hansenii* (numbers 24–124, and BDH9 [a transformation competent laboratory strain]) and from two strains of *S. cerevisiae* (numbers 125 and 126). Strains that yielded a rather low amount of DNA were also extracted using the ISOLATE II Genomic DNA Kit (Bioline) (red bars). For each extraction, 600 million cells were used

the ISOLATE II Genomic DNA Kit (Bioline), which is based on generating spheroplasts using the enzyme zymolyase in order to optimize the amount of DNA extracted. In our hands the Bioline kit yielded substantially higher amounts of DNA for all strains tested (Fig. 8) and would generally be recommended for extraction of DNA from a wide array of *D. hansenii* strains. The 260/280 nm ratio was sufficient using both kits (the recommended ratio is above 1.8), with the kit from Bioline providing somewhat higher purity (average 260/280 nm =  $2.1 \pm 0.06$  [standard deviation]). The quality of the DNA obtained using both methods was high with low levels of degradation, and both yielded high-molecular weight DNA that resulted in good sequence reads.

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## 2 Materials

Equipment and consumables required for all protocols:

1. 1.5 mL microcentrifuge tubes.
2. Centrifuge for 1.5 mL microcentrifuge tubes.
3. Vortex mixer.
4. Pipettes and filter tips of 100–1000, 2–200, and 0.1–1  $\mu$ L.
5. Heat block or incubator.
6. Nanodrop.
7. Qubit (*see* **Note 3**).

Specific materials required for each protocol are given below.

### 2.1 *Littorina saxatilis*

1. CTAB buffer: 2% w/v cetyltrimethyl ammonium bromide (CTAB), 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl pH 8.0, 0.2% v/v  $\beta$ -mercaptoethanol. For 100 mL, weigh 2 g of CTAB and 8.2 g of NaCl; add 70 mL of double-distilled water, 10 mL of 1 M Tris-HCl pH 8.0, and 8 mL of 0.25 M EDTA. Let the salts dissolve under magnetic stirring and bring the volume up to 100 mL with double-distilled water. The solution can be stored at room temperature for 6 months (CTAB will precipitate in the refrigerator). Before the DNA extraction, add 2  $\mu$ L of  $\beta$ -mercaptoethanol per 1 mL of CTAB buffer. This solution can be stored at room temperature for no more than a week (*see* **Note 4** for working with  $\beta$ -mercaptoethanol).
2. TE buffer: 10 mM Tris-HCl pH = 8.0, 1 mM EDTA. To prepare 50 mL, mix 0.5 mL of 1 M Tris pH 8.0 with 0.1 mL of 0.5 M EDTA pH 8.0 and adjust to 50 mL with distilled water. Use a sterile syringe and a 0.2  $\mu$ m non-pyrogenic sterile filter to filter the solution. It is recommended to store the solution at 4 °C for short-term and at lower temperature for long-term storage.

For dissolving DNA, use 0.1× TE buffer (diluted 1:10 with double-distilled water). Alternatively, use nuclease-free water.

3. Proteinase K (20 mg/mL).
4. RNase A (100 mg/mL).
5. Chloroform:isoamyl alcohol 24:1 (CIA).
6. Isopropanol (2-propanol).
7. 70 % ethanol.
8. Phase Lock Gel Heavy 2 mL columns (PGL columns; 5 Prime).
9. Safe-Lock tubes 1.5 mL (Eppendorf).
10. Fume hood.
11. Incubator at 60 °C with a shaking platform (e.g., Heidolph 1000).
12. Mixer Mill (e.g., Retsch MM301 with stainless steel balls).
13. Rotator (e.g., Stuart SB3).
14. Centrifuge for 1.5 mL tubes with cooling capacity (4 °C) and maximum speed  $\geq 18,000 \times g$ .
15. Tissue samples can be collected from live snails or snails fixed in 96–99 % ethanol. For fixation, snails are left at –80 °C overnight, and then the shells are carefully crushed (facilitating the penetration of ethanol but avoiding damage to the tissues). The tissues are then placed in tubes filled with ethanol and stored at –20 °C. It is important to use a relatively large volume of ethanol (e.g., at least 20× the tissue volume) and to check the samples within one week after fixation. If the liquid has a yellow color (often the case when the digestive gland was damaged or when fixing large individuals), the ethanol should be changed. After that, the samples can be stored at –20 to –80 °C for a long period of time; we did not observe any sign of DNA degradation after 3 years of storage.

## 2.2 *Idotea balthica*

1. The SDS/CTAB buffer is a 1:1 mixture of CTAB and SDS (sodium dodecyl sulfate) buffers (*see* **Note 5**). For the recipe for CTAB buffer, *see* Subheading 2.1.

SDS buffer: 0.7 % w/v SDS, 10 mM NaCl, 10 mM Tris–HCl pH 8.0. For 300 mL buffer, add 2.1 g of SDS, 3 mL of 1 M Tris–HCl pH 8.0, and 6 mL of 0.5 M NaCl to 280 mL of double-distilled water. Let the SDS dissolve under magnetic stirring and bring the volume up to 300 mL with distilled water. The solution can be stored at room temperature for months. The day before the extraction, mix equal volumes of CTAB and SDS buffers and add 2 µL of β-mercaptoethanol per 1 mL of mixture (*see* **Note 4** for working with β-mercaptoethanol). Incubate the mixture at 60 °C overnight to dissolve the precipitate.

2. Proteinase K (20 mg/mL).

3. Protease (7.5 AU, Qiagen).
4. RNase A (100 mg/mL).
5. Phenol:chloroform:isoamyl alcohol 25:24:1 (PCIA).
6. Chloroform:isoamyl alcohol 24:1 (CIA).
7. Isopropanol (2-Propanol).
8. Glycogen (5 mg/mL).
9. 70% ethanol, 0.1× TE buffer (*see* Subheading 2.1) or nuclease-free water to dissolve the DNA.
10. Genomic DNA Clean & Concentrator-10 Kit (Zymo Research).
11. Phase Lock Gel Heavy 2 mL columns (PGL columns; 5 Prime).
12. Safe-Lock tubes 1.5 mL (Eppendorf).
13. Fume hood.
14. Incubator for 60 °C with a shaking platform (e.g., Heidolph 1000).
15. Mixer Mill (e.g., Retsch MM301 with stainless steel balls).
16. Rotator (e.g., Stuart SB3).
17. Centrifuge for 1.5 mL tubes with cooling capacity (4 °C) and maximum speed  $\geq 18,000 \times g$ .
18. Tissue samples can be collected from living animals or animals stored in 96–99% ethanol. For fixation, isopods are decapitated, placed in the tubes with ethanol, and stored at 4 to –20 °C. To prevent DNA degradation and improve purity of DNA, it is important to change the ethanol twice, first 1 day and then 1 week after fixation.

### **2.3 *Skeletonema marinoi***

1. CTAB buffer: 2% w/v CTAB, 1.41 M NaCl, 200 mM Tris–HCl pH 8.0, 50 mM EDTA. To prepare 50 mL of the buffer, mix 1 g of CTAB, 10 mL of 1 M Tris–HCl pH 8.0, 5 mL of 0.5 M EDTA, and 4.09 g of NaCl and add distilled water up to 50 mL. Dissolve the chemicals using a hot plate set at 65 °C and a magnetic stirrer. For sterilization, leave the solution overnight under UV radiation. Store the solution at room temperature. Precipitates may form at the bottom of the solution with aging; if so, stir the solution. If the precipitation is too dense, or the solution has become yellow, make a fresh stock.
2. 1 M Tris–HCl pH 8.0: add 121.1 g of Tris to 700 mL of distilled water. After dissolving, adjust to 900 mL with distilled water. Adjust the pH to 8.0 with concentrated HCl (ca. 50 mL) and bring the final volume of the solution to 1 L with distilled water.
3. 0.5 M EDTA pH 8.0: add 186.12 g of EDTA to 750 mL of distilled water. Gradually add 20 g of NaOH and adjust the pH to 8.0. EDTA is not soluble until the solution reaches pH 8.0. Bring the final volume to 1 L with distilled water.



4. Chloroform:isoamyl alcohol solution 24:1 (CIA).
5.  $\beta$ -mercaptoethanol.
6. RNase A (100 mg/mL).
7. Isopropanol.
8. Ice cold ethanol (75 % final concentration diluted in double-distilled water).
9. 0.1 $\times$  TE buffer (*see* Subheading 2.1) for DNA re-suspension.
10. 50 mL Falcon tubes.
11. 1.5 mL Safe-Lock tubes (Eppendorf).
12. Fume hood.
13. Centrifuge for 50 mL Falcon tubes.
14. 1.5 mL tube centrifuge with cooling capacity (4 °C).
15. Samples: after culturing, it is possible to harvest the phytoplankton cells on a membrane filter, or using multiple-centrifugation steps (recommended).
16. A plate reader (e.g., Varioskan TM Flash Multimode Reader, Thermo Scientific) to measure chlorophyll A fluorescence, used as a proxy for the abundance of cells in culture before extraction (*see* **Note 6**).

## **2.4 *Amphiura filiformis***

1. Sperm isolation buffer: 20 mM NaCl, 50 mM Tris pH 8.0, 20 mM EDTA. Dissolve the salts with magnetic stirring and bring the volume to 100 ml with distilled water. Filter sterilize and store at room temperature.
2. STE buffer: 40 mM NaCl, 10 mM Tris pH 8.0, 1 mM EDTA. Dissolve the salts with magnetic stirring and bring the volume to 100 ml with distilled water. Filter sterilize and store at room temperature.
3. 20 % SDS (w/v in distilled water).
4. Phenol:chloroform:isoamyl alcohol 25:24:1 (PCIA).
5. Chloroform:isoamyl alcohol 24:1 (CIA).
6. RNase A 100  $\mu$ g/mL.
7. Proteinase K (20 mg/mL).
8. 3 M sodium acetate.
9. 100, 95, and 75 % ethanol.
10. 0.1 $\times$  TE buffer or nuclease-free water to dissolve DNA.
11. Filtered sea water.
12. DNA Clean & Concentrator Kit (Zymo Research).
13. 5 mL Falcon tubes.
14. Fume hood.
15. Rotator (e.g., Stuart SB3).

16. Centrifuge for 5 mL Falcon tubes.
17. Shaking incubator.

## **2.5 *Fucus vesiculosus* and *F. radicans***

1. Extraction buffer: 2 % CTAB, 3 % polyvinylpyrrolidone (PVP-40 or 0.1 % PVPP-“PVP-cross linked”), 1.4 M NaCl, 20 mM EDTA pH 8.0, 100 mM Tris-HCl pH 8.0, 1 %  $\beta$ -mercaptoethanol. For 100 mL buffer, mix 2 g of CTAB, 10 mL of 1 M Tris-HCl pH 8.0, 4 mL of 0.5 M EDTA, 28 mL of 5 M NaCl (or 8.1 g of NaCl), and 3 g of PVP-40 (MW: 40,000). Add double-distilled water to a final volume of 100 mL. Heat with stirring to dissolve the CTAB (be careful not to boil over) and autoclave.
2. 3.5 mM diethyldithiocarbamic acid (DIECA).
3. 100 % acetone.
4. RNase A (10 mg/mL).
5. NucleoSpin Plant II kit (Macherey-Nagel).
6. DNA Clean & Concentrator-25 Kit (Zymo Research).
7. Double-distilled or nuclease-free water to elute DNA.
8. Fume hood.
9. Mixer Mill (e.g., Retsch MM301 with stainless steel balls).
10. Rotator (e.g., Stuart SB3).

## **2.6 *Balanus improvisus***

1. E.Z.N.A. Blood DNA Mini Kit (Omega Bio-tek).
2. RNase A (100 mg/mL).
3. Isopropanol.
4. 100 % ethanol.
5. Microcentrifuge tubes with corresponding pestles (VWR).

## **2.7 *Debaryomyces hansenii***

1. YPD medium: 2 % w/v yeast extract, 1 % w/v peptone, 2 % w/v glucose.
2. 10 mM EDTA (ethylenediaminetetraacetic acid) pH 8.0.
3. MasterPure Yeast DNA Purification Kit (Epicentre).
4. ISOLATE II Genomic DNA Kit (Bioline).

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# **3 Methods**

## **3.1 *Littorina saxatilis***

1. Prepare 1.5 mL Safe-Lock tubes (*see Note 7*) with 500  $\mu$ L of digestion buffer (CTAB- $\beta$ -mercaptoethanol) and 20  $\mu$ L of Proteinase K and place them in the heat block at 60 °C.
2. Dissect tissue samples of no more than 2  $\times$  2  $\times$  2 mm in size (*see Note 8*) and place them directly upon dissection in the tubes with preheated digestion buffer.

3. Vortex the samples well and incubate at 60 °C on a shaking platform set at 150 rpm for 1 h (if an incubator with shaking platform is not available, *see Note 9*).
4. Homogenize the tissues in the Mixer Mill: add two steel balls per sample and shake for 5 min at 30 Hz/s (*see Note 10* for more information on tissue homogenization).
5. Incubate at 60 °C as in **step 3** for 30 min to let the bubbles and foam formed during homogenization disappear and then transfer the homogenate to a fresh tube using a pipette or by placing a magnet at the bottom of the tube to hold the metal balls in place while pouring out the homogenate.
6. Add 5 µL of RNase A (*see Note 11*), vortex, and incubate 1 h at 60 °C as in **step 3**.
7. Cool the samples for 5 min at room temperature. During this time, pre-spin the PGL columns (one for each extraction) for 1 min at 14,000×*g* at room temperature.
8. Add 500 µL of CIA (in the fume hood), close the lids tightly, and mix by inversion in the rotator for 10 min at 40 rpm.
9. Pour the mixture including the white precipitate into a PGL column and centrifuge for 5 min at 14,000×*g* at room temperature to separate the phases (if PGL columns are not available, phase separation can be done directly in the microcentrifuge tubes, *see Note 12*).
10. Carefully pour the upper phase into a new 1.5 mL microcentrifuge tube. If there is a thin gel layer on top of the upper phase, puncture it with a pipette tip to facilitate pouring.
11. Add 500 µL of room temperature isopropanol. Often one can see DNA threads precipitate. Mix slowly by inverting ten times and incubate for 5 min at room temperature.
12. Centrifuge for 30–40 min at maximum speed ( $\geq 18,000\times g$ ) and 4 °C to pellet the DNA. After centrifugation, there will be whitish DNA pellets visible at the bottom of the tubes.
13. Carefully decant or pipette out the liquid.
14. Add 1 mL of room temperature 70% ethanol and wash the pellets by inverting the tubes in the rotator for 5 min at 20 rpm.
15. Centrifuge for 5 min at 14,000×*g* at room temperature.
16. Carefully decant or pipette out the ethanol. It is not necessary to remove all the ethanol, just as much as can be removed without disturbing the DNA pellet. The pellets may float and it is important to make sure they are not lost during the washing steps. Repeat washing **steps 14** and **15** with 500 µL of 70% ethanol.
17. Remove all the ethanol: first decant as much liquid as possible and then spin briefly to collect the drops at the bottom and remove the rest using a 100 µL pipette.

18. Air-dry the pellets in the open tubes at room temperature. Avoid over-drying (*see* **Note 13**).
19. Add 50  $\mu\text{L}$  of 0.1 $\times$  TE buffer or nuclease-free water and let the pellets dissolve overnight at 4  $^{\circ}\text{C}$ .
20. Mix gently by inverting and tapping the tubes (vortexing of the DNA solution may lead to shearing of the DNA), spin briefly to collect the solution at the bottom of the tube, and proceed to quantification.
21. Samples can be stored at  $-20$  to  $-80$   $^{\circ}\text{C}$  for more than one year. (After 3 years, we did not observe any signs of DNA degradation, but multiple thawing-freezing cycles may lead to partial degradation.)

### 3.2 *Idotea balthica*

1. Prepare 1.5 mL Safe-Lock tubes (*see* **Note 7**) with 400  $\mu\text{L}$  of the digestion buffer (CTAB:SDS: $\beta$ -mercaptoethanol), 20  $\mu\text{L}$  of Proteinase K, and 20  $\mu\text{L}$  of Protease. Place them in the heating block at 60  $^{\circ}\text{C}$ .
2. Dissect tissue samples of approximately  $2 \times 2 \times 2$  mm size, avoiding the gut and exoskeleton (*see* **Note 14**), and place them directly upon dissection in the tubes with preheated digestion buffer.
3. Vortex samples well and incubate at 60  $^{\circ}\text{C}$  on the shaking platform for 1 h at 150 rpm (if an incubator with a shaking platform is not available, *see* **Note 9**).
4. Homogenize the tissues in the Mixer Mill: add two steel balls per sample and shake for 5 min at 30 Hz/s (*see* **Note 9** for more information on tissue homogenization).
5. Incubate for 30 min at 60  $^{\circ}\text{C}$  as in **step 3** to let the bubbles and foam formed during homogenization disappear and then remove the metal balls used for homogenization with forceps or a magnet.
6. Add 5  $\mu\text{L}$  of RNase A (*see* **Note 11**), vortex, and incubate for 1 h at 60  $^{\circ}\text{C}$  as in **step 3**.
7. Cool the samples for 5 min at room temperature. During this time, pre-spin the PGL columns (three per extraction) for 1 min at  $14,000 \times g$  at room temperature.
8. Add 400  $\mu\text{L}$  of PCIA, close the lids tightly and mix by inversion in the rotator for 15 min at 40 rpm.
9. Pour the mixture into PGL columns and centrifuge for 5 min at  $14,000 \times g$  at room temperature to separate the phases (if PGL columns are not available, phase separation can be done in microcentrifuge tubes; *see* **Note 15**).
10. Add 400  $\mu\text{L}$  of PCIA into the same PGL column, close the lids tightly and mix by inversion in the rotator for 10 min at 40 rpm.

11. Centrifuge for 5 min at  $14,000\times g$  at room temperature to separate the phases.
12. Pour the upper phase into a new PGL column, add 400  $\mu\text{L}$  of CIA, and mix by inversion as in **step 10**.
13. Centrifuge as in **step 11**.
14. Transfer the upper phase into a new PGL column, add 400  $\mu\text{L}$  of CIA, and mix by inversion as in **step 10**.
15. Centrifuge as in **step 11**.
16. Transfer the upper phase into a new microcentrifuge tube.
17. Add 2  $\mu\text{L}$  of glycogen and 400  $\mu\text{L}$  of room temperature isopropanol. Mix by inverting slowly five times and incubate for 5 min at room temperature.
18. Centrifuge for 45 min at maximum speed ( $\geq 18,000\times g$ ) and  $4^\circ\text{C}$  to pellet the DNA. At the end of centrifugation, there will be whitish DNA pellets visible at the bottom of the tubes.
19. Carefully decant or pipette out the liquid.
20. Add 1 mL of 70% ethanol (room temperature) and wash the pellets by inverting the tubes in the rotator for 5 min at 20 rpm.
21. Centrifuge for 5 min at  $14,000\times g$  at room temperature.
22. Carefully decant or pipette out the ethanol. It is not necessary to remove all the ethanol, just as much as can be removed without disturbing the DNA pellet. The pellets may float and it is important to make sure they are not lost during the washing steps. Repeat washing **steps 19** and **20** with 500  $\mu\text{L}$  of 70% ethanol.
23. Remove all the ethanol: first decant as much liquid as possible and then spin briefly to collect the drops at the bottom and remove the rest using a 100  $\mu\text{L}$  pipette.
24. Air-dry the pellets in the open tubes at room temperature. Avoid over-drying (*see Note 13*).
25. Add 50  $\mu\text{L}$  of  $0.1\times$  TE buffer or nuclease-free water and let the pellets dissolve overnight at  $4^\circ\text{C}$ .
26. Mix gently by inverting and tapping the tubes (vortexing of the DNA solution may lead to shearing of the DNA), centrifuge briefly to collect the solution at the bottom of the tubes, and measure the DNA concentration.
27. Use the Genomic DNA Clean & Concentrator-10 Kit according to the manufacturer's protocol to further clean the samples (*see Note 16* on amount and concentration of DNA in this step).

### 3.3 *Skeletonema marinoi*

#### *Collection, storage, and lysis of cells:*

1. Harvest the diatom cells through multiple-centrifugation steps: Initially centrifuge a larger volume of culture in 50 mL

falcon tubes for 10–20 min at  $3900\times g$ . Subsequently, resuspend the pellet into a smaller volume and transfer it to 1.5 mL microcentrifuge tubes. A second centrifugation step for 6 min at  $20,200\times g$  at 4 °C will remove most of the supernatant. A third centrifugation step for 4 min at  $20,200\times g$  at 4 °C will remove the rest of the media. *See Note 17.*

2. To each 1.5 mL microcentrifuge tube, add a master mix composed of 500  $\mu\text{L}$  of CTAB buffer, 4  $\mu\text{L}$  of RNase A, and 12  $\mu\text{L}$  of  $\beta$ -mercaptoethanol. Prepare the master mix containing CTAB and RNase A, mix, and add the  $\beta$ -mercaptoethanol under the fume hood. Add the total volume of 516  $\mu\text{L}$  to each sample and securely close the cap of each tube before homogenization and transfer to the incubator.
3. Incubate the tubes for 1 h at 65 °C with a vortex agitation of a few seconds every 15 min. Check that the pellet is diluted at the start of the incubation.
4. Transfer the tubes on ice for approximately 1 min.

*DNA extraction:*

5. Add 500  $\mu\text{L}$  of CIA under the fume hood and invert the tubes repeatedly until the two solutions are mixed.
6. Centrifuge for 10 min at  $20,200\times g$  at 4 °C. After centrifugation, put the tubes on ice, taking care not to disturb the three layers.
7. Transfer the upper phase to a fresh 1.5 mL microcentrifuge tube. Be careful not to pipette the white intermediate layer, or the greenish oily bottom layer.
8. Check visually that the upper phase is clear and homogeneous (without oily structures). If not, add 500  $\mu\text{L}$  of CIA as in **step 5** and repeat **steps 6–7** with caution, as the three phases are shades of white.

*DNA purification:*

9. Add one volume (400–600  $\mu\text{L}$ ) of ice-cold isopropanol (pre-stored at –20 °C).
10. Invert the tubes several times and incubate the tubes at –20 °C for a minimum of 1 h, but preferably overnight. *Note:* when the concentration of genomic DNA is high, the solution can turn purple-pink and the DNA precipitates, forming milky trails.
11. Centrifuge for 30 min at  $20,200\times g$  at 4 °C. After centrifugation, put the tubes on ice, taking care not to disturb the DNA pellet.
12. Pour the liquid gently without disturbing the pellet. The pellet forms a white spot at the bottom of the tube. The size of the



pellet depends on the amount of DNA extracted and the color on its purity and humidity.

13. Add 400  $\mu\text{L}$  of ice-cold 75 % ethanol (pre-stored at  $-20\text{ }^{\circ}\text{C}$ ).
14. Centrifuge for 15 min at  $20,200\times g$  at  $4\text{ }^{\circ}\text{C}$ . After centrifugation, put the tubes on ice, being careful not to disturb the DNA pellet.
15. Pour the liquid gently without disturbing the pellet.
16. Prepare a clean area with blotting paper under the hood. After pouring, turn each tube upside down to dry at room temperature.

*Resuspension of DNA:*

17. After 1–2 h, the dried pellet is dissolved in 25–50  $\mu\text{L}$  of double-distilled  $\text{H}_2\text{O}$ , TE buffer, or nuclease-free water, *see* **Note 18**.
18. The tubes are gently agitated manually and stored at  $4\text{ }^{\circ}\text{C}$  overnight to dissolve the pellet. DNA can be stored at  $4\text{ }^{\circ}\text{C}$  for up to 1 week, at  $-20\text{ }^{\circ}\text{C}$  for up to 1 month, or at  $-80\text{ }^{\circ}\text{C}$  for a longer period.

### **3.4 *Amphiura filiformis***

*Sperm collection:*

1. Washed animals are spawned individually in around 20 mL of filtered sea water in 50 mL Falcon tubes by transferring them to the light after a night in the dark. Remove the animal when spawning is completed.
2. Filter the sperm through a 100  $\mu\text{m}$  mesh to remove larger pieces of debris.
3. Transfer the solution into a 50 mL tube and centrifuge gently for 10 min at  $1000\times g$ .
4. Remove the supernatant, leaving a small amount of liquid; resuspend in the remaining liquid; and transfer to a 1.5 mL tube. Centrifuge again for 10 min at  $1000\times g$ .
5. Remove all supernatant; store the sample at  $-20\text{ }^{\circ}\text{C}$  or proceed to DNA extraction.

*DNA extraction:*

6. Add 600  $\mu\text{L}$  of sperm isolation buffer and 3  $\mu\text{L}$  of fresh Proteinase K solution to the collected sperm in a 1.5 mL tube and vortex.
7. Add 15  $\mu\text{L}$  of 20% SDS and shake vigorously by hand as sperm lyse (*see* **Note 19**). Pulse spin. If the suspension is too thick to flow easily, then add more buffer, Proteinase K, and SDS solution.
8. Incubate for 1 h at  $65\text{ }^{\circ}\text{C}$ , then at  $37\text{ }^{\circ}\text{C}$  overnight.
9. Add 3  $\mu\text{L}$  of fresh Proteinase K and shake vigorously. (If you added more isolation mix at **step 7**, increase the amount of Proteinase K accordingly.) Incubate for 1 h at  $65\text{ }^{\circ}\text{C}$ .

10. Add an equal volume of PCIA and extract with gentle shaking in an incubator for 5–8 h at 37 °C.
11. Centrifuge for 5 min at maximum speed and transfer the clear aqueous phase to a fresh tube.
12. Add one volume of PCIA and leave overnight at room temperature on the rotator wheel.
13. Centrifuge for 5 min at  $18,000\times g$ . Transfer the clear aqueous phase to a new tube. Repeat this step again if the aqueous phase is not completely clear. Add one volume of CIA and incubate for 1 h at room temperature.
14. Centrifuge for 5 min at  $18,000\times g$ , transfer the clear aqueous phase to a new tube, add one volume of CIA, and incubate for 8 h at room temperature.
15. Centrifuge for 5 min at  $18,000\times g$  and transfer the clear aqueous phase to a new tube. Bring the volume up to 500  $\mu\text{L}$  with STE buffer. Add 1 mL of 100% ethanol. (If you added more isolation mix at **step 7**, increase the volume proportionally.) Mix and precipitate for at least 30 min at room temperature (*see Note 20*).
16. Centrifuge for 15 min at  $18,000\times g$  and decant the supernatant.
17. Add 1 mL of 75% ethanol at room temperature and wash the pellets by inverting the tube by hand several times.
18. Centrifuge for 2 min at maximum speed and room temperature.
19. Decant the ethanol carefully. A small amount can be left in the tube. Be careful not to disturb the pellet or drop it when decanting.
20. Repeat washing **steps 17–19** with 95% ethanol.
21. Dry the pellet at 37 °C in the heating block with the tube open.
22. Resuspend in 100  $\mu\text{L}$  10% TE buffer with 20  $\mu\text{g}/\text{mL}$  RNase A.
23. Incubate 3 h at 37 °C.
24. Add 100  $\mu\text{L}$  of PCIA, shake quickly and vigorously, centrifuge for 5 min at  $18,000\times g$ , and transfer the aqueous phase to a fresh tube.
25. Add 100  $\mu\text{L}$  of CIA, shake quickly and vigorously, centrifuge for 5 min at  $18,000\times g$ , and transfer the aqueous phase to a new tube.
26. Repeat **step 25**.
27. Precipitate the DNA by adding 1/10 volume of 3 M sodium acetate and two volumes of 100% ethanol.
28. Centrifuge for 15 min at  $18,000\times g$  and decant the supernatant.

29. Wash the pellets with 75 % ethanol, then 95 % ethanol, and dry the pellets as in **steps 17–21**.

30. Resuspend the DNA in 100  $\mu$ L of 10 % TE buffer.

*DNA cleanup with the Zymo DNA Clean & Concentrator:*

31. Preheat the nuclease-free water to 65 °C for the final elution (65  $\mu$ L per extraction).

32. Add 100  $\mu$ L of DNA Binding Buffer to each DNA sample. Mix 1 s in a vortex mixer.

33. Transfer the mixture to a Zymo-Spin column in a collection tube.

34. Centrifuge for 30 s at 14,000 $\times g$ . Discard the flow-through.

35. Add 200  $\mu$ L of DNA Wash Buffer to the column. Centrifuge for 30 s at 13,000 $\times g$  and discard the flow-through.

36. Repeat the washing step.

37. Place the Zymo-Spin column into a new 1.5 mL tube. Pipette 29  $\mu$ L of nuclease-free water (preheated at 65 °C) onto the membrane. Incubate the column for 5 min at 65 °C. Centrifuge for 30 s at 13,000 $\times g$  to elute the DNA. Repeat this step with the same volume and elute into the same tube.

### 3.5 *Fucus vesiculosus* and *F. radicans*

*DNA extraction:*

1. Place a small piece of algal tissue (10–15 mg) in a 1.5 mL microcentrifuge tube along with two small stainless steel ball bearings (5 mm) and close the lid. Place it into the Mixer Mill and pulverize the tissue for 1.5 min at 30 Hz/s.

2. To remove polyphenolic compounds, suspend the ground material in 1 mL of 100 % acetone for 10 min in the rotator.

3. Centrifuge the samples for 1 min at 12,800 $\times g$ , pour out the acetone, and discard it.

4. Repeat **steps 2** and **3** and air-dry the samples for 5–10 min (avoid over-drying).

5. Add 500  $\mu$ L of 2 % CTAB extraction buffer to each tube. Use a pipette tip to scrape the tissue pellet off the tube wall.

6. Add 17.5  $\mu$ L of 0.1 M DIECA, 5  $\mu$ L of  $\beta$ -mercaptoethanol under a fume hood, and 10  $\mu$ L of RNase A solution, vortex vigorously, and incubate the mixture for 1 h at 65 °C. Vortex the mixture every 10 min.

7. From this step onwards, the NucleoSpin Plant II Kit is used following the manufacturer's instructions. Preheat the nuclease-free water to 65 °C for the final elution (55  $\mu$ L per extraction).

8. Place a NucleoSpin Filter (violet ring) into a new collection tube (2 mL) and load the crude lysate onto the column using wide-bore filter tips. Centrifuge for 2 min at 12,800 $\times g$ , collect the clear flow-through, and discard the Filter. If not all the

liquid has passed through the filter, repeat the centrifugation step. If a pellet is visible in the flow-through, transfer the clear supernatant to a new 1.5 mL tube using wide-bore pipette tips. Alternatively, place the NucleoSpin Filter into a new 1.5 mL tube and pass the pre-cleared supernatant through the filter once more to remove solid particles completely.

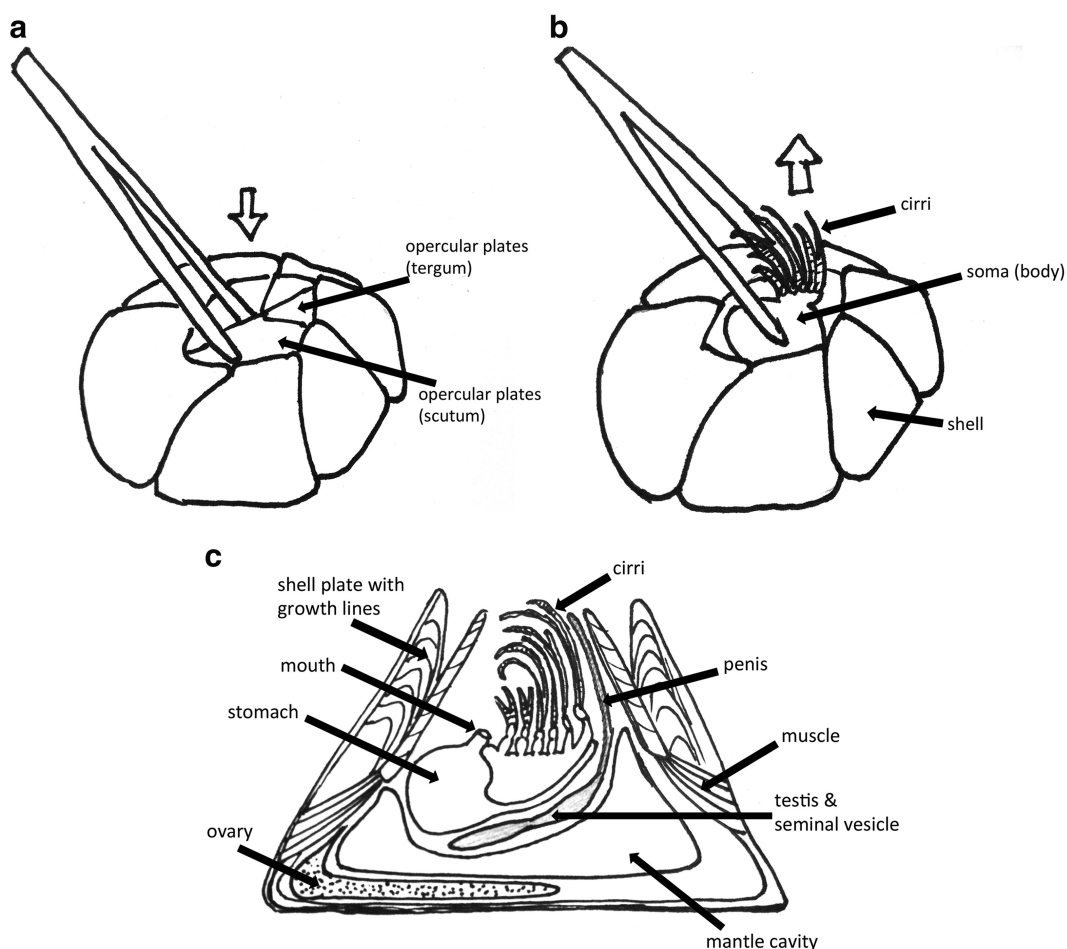
9. Add 450  $\mu\text{L}$  of Buffer PC and mix thoroughly by gentle inversion a few times.
10. Place a NucleoSpin Plant II Column (green ring) into a new Collection tube (2 mL) and load a maximum of 700  $\mu\text{L}$  of the sample. Centrifuge for 1 min at  $12,800\times g$  and discard the flow-through.
11. Add 400  $\mu\text{L}$  of Buffer PW1 to the NucleoSpin Plant II Column. Centrifuge for 1 min at  $12,800\times g$  and discard the flow-through.
12. Add 700  $\mu\text{L}$  of Buffer PW2 to the NucleoSpin Plant II Column. Centrifuge for 1 min at  $12,800\times g$  and discard the flow-through.
13. Add another 200  $\mu\text{L}$  of Buffer PW2 to the NucleoSpin Plant II Column. Centrifuge for 2 min at  $12,800\times g$  and discard the flow-through.
14. Place the NucleoSpin Plant II Column into a new 1.5 mL tube. Pipette 50  $\mu\text{L}$  of nuclease-free water (preheated to 65 °C) onto the membrane. Incubate the NucleoSpin Plant II Column for 5 min at 65 °C. Centrifuge for 1 min at  $12,800\times g$  to elute the DNA.

*DNA cleanup:*

15. From this point, we use the Zymo DNA Clean & Concentrator-25 Kit. Preheat the nuclease-free water to 65 °C for the final elution (65  $\mu\text{L}$  per extraction).
16. Add 100  $\mu\text{L}$  of DNA Binding Buffer to each DNA sample. Mix 1 s in a vortex mixer.
17. Transfer the mixture to a Zymo-Spin column in a collection tube.
18. Centrifuge for 30 s at  $14,000\times g$ . Discard the flow-through.
19. Add 200  $\mu\text{L}$  of DNA Wash Buffer to the column. Centrifuge for 30 s at  $12,800\times g$  and discard the flow-through.
20. Repeat the wash step.
21. Place the Zymo-Spin Column into a new 1.5 mL tube. Pipette 58  $\mu\text{L}$  of nuclease-free water (preheated to 65 °C) onto the membrane. Incubate the NucleoSpin Plant II column for 5 min at 65 °C. Centrifuge for 30 s at  $12,800\times g$  to elute the DNA.

### 3.6 *Balanus improvisus*

1. Select large and short-term starved fresh individuals for DNA extraction (*see* **Note 21**). Clean the barnacle shell with a fine brush to minimize the risk of contamination from other species (e.g., bacteria, algae). Remove the top plates (tergum and scutum) using a pair of tweezers (Fig. 9a, b and *see* **Note 22**). Grab the animal and pull it out of the shell. Mostly, this results in the soma (body) and cirri appearing without the mantle (*see* **Note 23**).
2. Put the soma and cirri from one adult barnacle into 250  $\mu\text{L}$  of ice-cold buffer EB from the E.Z.N.A. Blood DNA Mini Kit in a 1.5 mL microcentrifuge tube. Homogenize with the plastic pestle in roughly five strokes (*see* **Note 24**).



**Fig. 9** Removing the body of an acorn barnacle from its shell. (a) Grab one of the top opercular plates by inserting tweezers gently through the aperture. Pull gently to remove the plate and to expose the animal. (b) Pull out the animal by grabbing the soma part below the cirri. (c) The overall anatomy of acorn barnacles. The mantle and potentially fertilized eggs (in the ovary) stay in the shell cavity when the body is pulled out and are discarded (they are not used for the DNA extraction)

3. Add 25  $\mu\text{L}$  of protease solution OB, 250  $\mu\text{L}$  of buffer BL and 2.5  $\mu\text{L}$  of RNase A. Vortex for 15 s at full speed. Homogenize again with the plastic pestle (2–3 strokes).
4. Incubate the samples for 10 min at 65 °C. Vortex briefly 2–3 times during the incubation.
5. Centrifuge for 3 min at maximum speed.
6. Transfer the supernatant with a pipette into a new microcentrifuge tube.
7. Add 260  $\mu\text{L}$  of 100 % ethanol and vortex for 20 s at maximum speed.
8. For the remaining steps (chromatography, washing, and elution), follow the kit according to manufacturer's instructions.

### 3.7 *Debaryomyces hansenii*

1. Yeasts can be stored at –80 °C in a glycerol solution (20 % v/v) almost indefinitely. Inoculate a loop-full of cells, directly taken from the frozen stock with a heated loop, onto YPD agar plates (*see* **Note 25**) and streak them out for single colony growth. Inoculate the plates for 48 h at 25–30 °C (*see* **Note 26**).
2. Inoculate a loop-full of yeast cells from a single colony on the agar plate into 10 mL of fresh YPD medium in a 50 mL Falcon tube. Incubate overnight at 25–30 °C with shaking. The optical density (600 nm) of the cultures is usually in the range of 2–6 optical units after roughly 17 h of incubation.
3. Harvest the cells by centrifugation for 10 min at 5000  $\times g$ . Wash the pellet with 1 ml of 10 mM EDTA, pH 8.0.
4. For both DNA kits, follow instructions from the manufacturer.
5. For the ISOLATE II Genomic DNA Kit, the enzyme zymolyase is used to produce spheroplasts. The seven strains tested with this protocol displayed somewhat different sensitivity to the zymolyase treatment, and for some of the strains, incubation had to be prolonged compared to the standard recommended time to complete spheroplast formation (*see* **Note 27**).

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## 4 Notes

1. Barnacle species vary considerably in size. *B. improvisus* is a relatively small species and usually does not exceed 20 mm in diameter. The maximum wet weight obtained is about 30 mg. We have taken our individuals from laboratory cultures from the Swedish West Coast (from Tjärnö Marine Biological Station, Strömstad, Sweden, salinity ~30 psu).



2. For large fragment libraries that are used in particular for scaffolding and also for single-molecule long-read technologies such as PacBio, it is essential that the DNA has a high-molecular weight.
3. For quality control of the DNA sample, use a Nanodrop or any other spectrophotometric method to get an estimation of the 260/280 and 260/230 nm absorbance ratios of the sample, that indicate the amount of contamination with proteins and other organic compounds. Although spectrophotometric methods give an estimation of the concentration of DNA, it is highly recommended to measure the amount using Qubit (or similar DNA-specific methodology), which gives a more accurate determination of the concentration. In addition, it is important that the samples are run on a gel to estimate the amount in the low-molecular weight (degraded) and high-molecular weight DNA fractions. Ideally for sequencing, the DNA sample should be free of any degradation products and only contain material greater than 10–20 kb.
4.  $\beta$ -Mercaptoethanol has a characteristic unpleasant smell and is very toxic. It is extremely harmful if inhaled or swallowed and may be fatal if absorbed through the skin. While working with  $\beta$ -mercaptoethanol, wear protective gloves and perform all pipetting steps in a fume hood. When the tubes containing  $\beta$ -mercaptoethanol are taken out of the fume hood, their lids should be tightly closed. In the case of liquid spill on the surface of the tubes, wipe thoroughly with tissue, change gloves, and leave the tube in the fume hood until all smell disappears. Gloves, pipette tips, tissues, and plastic ware that have been in contact with  $\beta$ -mercaptoethanol should be disposed of as hazardous waste. Read the material safety data sheet before starting the work.
5. While most protocols use either CTAB or SDS buffer, a mixture of the two is sometimes used for DNA extractions in plants. In our tests, the SDS/CTAB mixture gave a better yield and purity of *Idotea* DNA than either of the buffers alone.
6. The plate reader can be used to rapidly assess the abundance of cells in culture before extraction in order to relate the amount of DNA extracted to the initial abundance of cells. Other alternative techniques are available. The extraction protocol works for small to large amount of *Skeletonema* cells and has been tested on a range from 0.31 to 1.8 billion cells counted before centrifugation using the plate reader. However, the purity of DNA (assessed by 230/260 absorbance ratio) decreases as the density of culture increases.
7. Safe-Lock tubes are recommended at this step. When the samples are mixed with chloroform:isoamyl alcohol, the pressure in the tubes may force the lid to open unexpectedly. If you use regular microcentrifuge tubes, after adding chloroform:isoamyl

alcohol, invert each tube a few times in the fume hood while holding the lid closed and then open the lid to release the pressure and close again tightly.

8. For one standard Illumina library, a single extraction from a piece of *Littorina* foot muscle of this size will be enough (DNA yield  $\geq 2$   $\mu\text{g}$ ). To obtain the maximum amount of DNA from a single individual, perform multiple extractions with the starting amount of tissue recommended in the protocol. Increasing the starting amount of tissue in a single extraction does not increase the yield proportionately and leads to lower DNA purity. Extractions from the digestive gland (hepatopancreas) give high yields but partially degraded DNA. Therefore we recommend carefully dissecting and discarding the hepatopancreas before collecting tissue samples for extraction. All other tissues and body parts (foot, head, mantle, all parts of the reproductive system) can be used for extraction of high-quality DNA. However, we recommend keeping different tissues separate in the extractions and assessing DNA integrity and purity for each extraction before pooling. Shell fragments should be avoided when collecting tissue samples for DNA extraction; they may result in a slightly pink coloration of the DNA solution due to co-purification of pigments (especially when the shell had a dark color).
9. If an incubator with a shaking platform is not available, incubate the sample in the heating block and vortex every 15–20 min.
10. Successful homogenization is crucial for high DNA yields. It is important to incubate the sample at 60 °C before the homogenization step. Normally no tissue pieces are visible after 5 min homogenization in the Mixer Mill. If tissue pieces are still visible, repeat the homogenization step for five more min (we never observed DNA shearing using this tissue homogenization method). If the Mixer Mill is not available, any other method of tissue homogenization can be used, including manual homogenization with pestles for Eppendorf tubes in the fume hood (although this is laborious for the processing of many samples).
11. To perform RNase A treatment directly in the digestion buffer, it is important to use a large amount of RNase A (5  $\mu\text{L}$  of 100 mg/mL stock) and a long incubation time (1 h). On-column RNase A treatment after DNA extraction led to a loss of 50–90 % of the DNA.
12. If PGL columns are not available, phase separation can be done by centrifuging the tubes at **step 8**. After centrifugation, transfer them carefully to the fume hood (avoiding shaking) and collect the upper phase (approx. 500  $\mu\text{L}$ ) by pipetting. Be careful not to touch the white interphase. If the phases are mixed by mistake, repeat the centrifugation step.

13. At this step, it is important to allow the ethanol to evaporate without over-drying the pellet, because it will be difficult to dissolve. Depending on the size of the pellet and on how much ethanol was left in the tube, drying can take from 5 to 40 min. Check the pellets every 5–10 min. When dry, the DNA pellets look transparent; there should not be any ethanol drops left within the tube and no residual ethanol smell either.
14. In *I. balthica*, high-quality DNA can be extracted from the head and the abdominal muscle. Any pieces of the gut and its content should be avoided because it would yield highly degraded DNA. In addition, gut contents will lead to contaminant sequences. Before collecting tissue samples, cut the carapace along the dorsal side (being careful not to disturb the gut), remove the gut, and wash the animal in ethanol. Parts of the exoskeleton and the appendages should be avoided as well; pigments in the exoskeleton co-purify with the DNA and can inhibit downstream reactions such as amplification, ligation, etc.
15. If PGL columns are not available, all PCIA and CIA extraction steps can be performed in Safe-Lock or regular microcentrifuge tubes by centrifugation and pipetting of the upper phase into a new tube each time (being very careful not to disturb the phases). However, with four extraction steps, this will be time-consuming and using the PGLs column is recommended.
16. Column binding capacity in the Genomic DNA Clean & Concentrator-10 Kit is 10 µg. Measure DNA concentration and calculate the total amount of DNA in the samples before using the kit. Multiple extractions from the same individual can be pooled together before cleaning as long as the total amount of DNA is below 10 µg. If the amount of DNA is above 10 µg, split the sample into aliquots for cleaning. The kit can also help to concentrate DNA samples by using a small volume of elution buffer. Depending on DNA integrity, 50–90% of the DNA is recovered after cleaning with the kit (the recovery rate is lower when samples have a large fraction of short degraded fragments). Also, after extractions with phenol, the DNA samples may contain contaminants causing over-estimation of the DNA concentration measured by the spectrophotometer. To avoid over-dilution of samples, it is better to use approximately ½ of the volume of elution buffer calculated from the total DNA amount applied to the cleaning column and dilute the samples afterwards if necessary. A second elution gives much lower DNA concentrations (10–20 ng/µL). If desired, it can be performed in a new tube.
17. During supernatant removal, the pellet will become loose, and therefore, it is important to split the last centrifugations into two steps. Always work under a clean hood. Pelleted cells can be stored at –80 °C. Keeping the cells at –80 °C prior to DNA

extraction facilitates the lysis of the cells. To avoid DNA degradation, we do not recommend repetitive thawing and freezing of the samples. Cells can also be concentrated on membrane filters (pore size 3  $\mu\text{m}$ ) and filters can be stored at  $-80\text{ }^{\circ}\text{C}$ . However, we recommend the use of centrifugation on axenic cultures. We noticed that even gentle filtration breaks the cells with consequential DNA loss.

18. We recommend using nuclease-free water to resuspend the cleaned DNA pellet, because some genomic analyses require the absence of EDTA. The volume added depends on the size of the DNA pellet. For pellets of the size of a pen-tip or smaller, use 25  $\mu\text{L}$ .
19. No automatic vortexing at this step.
20. You can stop the procedure here and keep the solution at  $4\text{ }^{\circ}\text{C}$  overnight and perform the next step on the next day.
21. Individuals used for DNA extraction were starved for at least 2 days. This is to avoid contamination with DNA of digested foods (*Artemia*, microalgae, etc.). We also tested using individuals preserved in ethanol at  $-20\text{ }^{\circ}\text{C}$ . However, this procedure resulted in low DNA quality (degradation is seen more frequently). This is why we recommend using fresh samples.
22. When pulling out the first operculum plate, the whole animal is sometimes attached to it. If not, remove the other operculum plates to have easy access to the animal (Fig. 9a, b).
23. Sometimes parts of the mantle can be attached to the soma and cirri and be hard to remove. The mantle epithelium, ovary tissues, and sometimes fertilized eggs can be found inside the shell (Fig. 9c). To minimize genetic variation, we avoided including the eggs in the DNA preparation. Mantle and ovary tissues were also excluded to avoid the risk of gonad contamination.
24. Sonication can also be used to dissolve the tissues. However, care should be taken not to do this extensively since it will also result in DNA degradation. For this reason, we have abandoned sonication in favor of using the pestle, which is much more gentle to DNA.
25. The marine yeast *D. hansenii* can be grown on a wide variety of media. A frequently used medium is yeast nitrogen base (YNB; DIFCO), which is a synthetic defined medium used for cultivation of several different yeast species. This can be used for either solid (by adding agar) or liquid growth. Another commonly used rich medium is yeast peptone dextrose (YPD), which is less well defined but is used to obtain high biomass.
26. Modelspeciesofyeast, such as *S. cerevisiae* and *Schizosaccharomyces pombe*, are usually grown at  $30\text{ }^{\circ}\text{C}$ . This temperature also works for growth of *D. hansenii*, and since  $30\text{ }^{\circ}\text{C}$  rooms/incubators are found in most standard microbial laboratories, this temperature could be handy to work with. However, *D. hansenii*

grows better at slightly lower temperatures (25–27 °C), which should be preferred in physiological experiments. For the purpose of obtaining enough yeast cells for DNA extraction, either temperature works fine; here we have used 30 °C.

27. The efficiency of spheroplast formation is checked under the microscope as follows: roughly 5 µL of the cell suspension is applied to a microscopy slide and gently covered by a cover slip. A small drop of pure water is applied just at the edge of the cover slip, thereby gently diluting the cell suspension. This leads to a lowering of the concentration of the osmotic stabilizer (sorbitol), whereby proper spheroplasts become osmotically fragile and lyse (cells burst). If this is not observed, the incubation with zymolyase should be extended.

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