Journal of Visualized Experiments

A converging strategy for the generation of a virtually sequenced cDNA library from unreferenced Pacific oysters

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59462R1
Full Title:	A converging strategy for the generation of a virtually sequenced cDNA library from unreferenced Pacific oysters
Keywords:	Pacific oyster; Crassostrea gigas; Magallana gigas; cDNA library; UDP-xylose biosynthesis; shellfish glycobiology
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Additional Information:	
Question	**Response**
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February 19th, 2019

Dear Dr. Nguyen:

Please find the revised version of our manuscript "***A converging strategy for the generation of a virtually sequenced cDNA library from unreferenced Pacific oysters."*** for consideration in the ***Journal of Visualized Experiments***" attached. We are thankful to the reviewers for their comments regarding our original submission and for helping us to improve our manuscript significantly.

After careful analysis of the reviewers' comments, we have revised our manuscript accordingly. In addition to the manuscript file, a point-by-point response to the reviewers' comments and the comments from the editorial office was also uploaded.

Yours Sincerely,

Josef Voglmeir

Professor Josef Voglmeir, Ph.D.
Director of the Glycomics and Glycan Bioengineering Research Center (GGBRC)
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TITLE:

A Converging Strategy for the Generation of a Virtually Sequenced cDNA Library from Unreferenced Pacific Oysters

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KEYWORDS:

Pacific oyster; *Crassostrea gigas*; *Magallana gigas*; cDNA library; UDP-xylose biosynthesis; shellfish glycobiology.

SUMMARY:

We describe a strategy for how to use RNA samples from unreferenced Pacific oyster specimens, and evaluate the genetic material by comparison with publicly available genome data to generate a virtually sequenced cDNA library.

ABSTRACT:

The access to biological material of reference species, which were used previously in key experiments such as in the development of novel cell lines or genome sequencing projects, are often difficult to provide for further studies or third parties due to the consumptive nature of the samples. Although now widely distributed over the Pacific coasts of Asia, Australia and North America, individual Pacific oyster specimens are genetically quite diverse and are therefore not directly suitable as the starting material for gene libraries. In this article, we demonstrate the use of unreferenced Pacific oyster specimens obtained from regional seafood markets to generate cDNA libraries. These libraries were then compared to the publicly available oyster genome, and the closest related library was selected using the mitochondrial

reference genes Cytochrome C Oxidase subunit I (COX1) and NADH Dehydrogenase (ND). The suitability of the generated cDNA library is also demonstrated by cloning and expression of two genes encoding the enzymes UDP-glucuronic acid dehydrogenase (UGD) and UDP-xylose synthase (UXS), which are responsible for the biosynthesis of UDP-xylose from UDP-glucose.

INTRODUCTION:

The acquisition of living referenced biological material may be challenging due to long delivery times, entrepreneurial reasoning, or country-specific customs regulations. As an alternative, the required biological material may be also collected from phenotypically identical specimens. However, these samples may vary significantly at the level of genotype, and therefore comparisons with digitally-stored reference genomes of the same species are often rendered difficult or even futile due to incompatibility of the newly-sourced material with existing DNA amplification methods. Sequencing highly conserved genes of individual samples is a widely used and powerful tool for identifying species[1], such as conserved mitochondrial genes that are frequently used as reference genes for the quality assessment of cDNA libraries[2-6]. The underlying rationale for the herein presented method is that high conservation of mitochondrial gene sequences in individual anonymous oyster samples compared to the corresponding sequences of the reference genome indicates that other genes may also show a low level of divergence, given the generally faster rate of mitochondrial DNA evolution relative to nuclear DNA[7], allowing the amplification and isolation of a wide range of scientifically and industrially relevant genes by simply using publicly available sequencing data as a reference.

The overall goal of the herein described method is to present an optimized workflow for generating a virtually sequenced oyster cDNA library which can be used as template DNA for the cloning of oyster genes. In virtual sequencing, de novo genome sequencing is circumvented; instead, a known, digitally-stored reference sequence is used directly to utilize or design primers for production of cDNAs that will eventually comprise a library (or be added to a pre-existing one). The aim is to produce a convergent cDNA library, meaning that similarities between the generated cDNA sequences and the reference sequence can be ranked from low to high divergence. A key advantage of using Cytochrome C Oxidase subunit 1 (COX1) and NADH Dehydrogenase (ND) as reference genes is that even highly geographically disjunct oyster specimens can be profiled due to the high conservation of these mitochondrial genes. Having proven the approach with these well-established markers, we then demonstrate its application to two enzyme candidates which are involved in sugar nucleotide biosynthesis and may be of industrial relevance[8-10]. The biotechnological potential of the Pacific oyster is still unexplored. Thus, we believe that this converging method for preparing a virtually sequenced cDNA library will also be appropriate for non-specialist researchers who want to generate cDNA from this relevant biological material.

PROTOCOL:

NOTE: A schematic overview is shown in **Figure 1**.

1. Sample collection

1.1. Obtain oyster specimens. Keep oysters on ice during the postharvest period, the transport and prior to laboratory use and process within 4-7 days after purchase.

NOTE: For this protocol, oysters were purchased from Zhong Cai Wholesale Market in Nanjing (originating from Ningde, Fujian, China and Lianyungang, Jiangsu, China), Haijie Aquatic Product Company in Qingdao (originating from Qingdao, Shandong, China), Jucheng Aquatic Product Company in Yantai (originating from Yantai, Shandong, China) and Jinxiu Aquatic Product Company in Qingdao (originating from Qingdao, Shandong, China)).

2. RNA isolation by guanidinium thiocyanate-phenol extraction

2.1. Preparation of oyster tissue sample

2.1.1. Cut out approximately 100 mg of homogeneous soft tissue from the approximate geometric center of each oyster specimen with a sterilized scalpel, and transfer the samples into liquid nitrogen.

2.1.2. Euthanize the remaining part of the oysters by freezing at -80 °C for 1 h and discard as biological waste.

2.1.3. Grind the flash-frozen oyster tissue into a fine powder in a mortar (200 mL) filled with 50 mL of liquid nitrogen.

2.1.4. Weigh out 75 mg of each specimen's frozen tissue into a sterile 1.5 mL centrifuge tube and mix with 1 mL of the guanidinium thiocyanate-phenol extraction reagent. Centrifuge the sample at 14,000 x *g* at 4 °C for 15 min.

2.2. Transfer the supernatant to a new 1.5 mL centrifuge tube, add 200 µL of chloroform and mix thoroughly by using a vortex mixer for 10-15 s until the mixture turns milky-white.

2.3. Centrifuge at 14,000 x *g* at 4 °C for 15 min, and transfer the upper aqueous layer carefully with a 200 µL pipette without disturbing the interphase into a new 1.5 mL centrifuge tube.

2.4. Add 500 µL of isopropyl alcohol and mix the samples gently by inversion, then leave the samples for 20 min on ice. Centrifuge at 14,000 x *g* at 4 °C for 8 min and remove the supernatant.

2.5. Resuspend each of the pellets in 1 mL of 75% EtOH, and centrifuge at 14,000 x *g* at 4 °C for 5 min. Remove all of the supernatant.

2.6. Repeat step 2.5 once. Dry the pellets for 6 min at room temperature. Do not dry for longer; otherwise, it may be difficult to dissolve the RNA pellet in the next step.

2.7. Dissolve the dried RNA pellet in 25 µL of DEPC (diethylpyrocarbonate)-treated water and keep the tube on ice. Use RNA samples within 24 h.

3. cDNA library generation by reverse transcription

3.1. For each RNA sample, prepare a reaction mixture using the a commercial reverse transcription system using a 10 µL pipette: Add 4 µL of $MgCl_2$ solution, 2 µL of 10x Reaction Buffer, 2 µL of dNTP solution, 0.5 µL of RNase inhibitor, 0.7 µL of AMV Reverse Transcriptase, 0.5 µL of Oligo(dT) 15 primer, 1 µL of the extracted RNA sample and 9.3 µL of H_2O into a 300 µL PCR tube.

3.2. Incubate mixture in a PCR Thermocycler for 60 min at 42 °C, and then increase the temperature to 95 °C for 5 min.

3.3. Store the generated cDNA library for up to 12 months at -20 °C.

4. Mitochondrial gene amplification and purification

4.1. Prepare PCR mixture using a 10 µL pipette. Add 0.25 µL (1.25 U) of high-fidelity DNA polymerase, 2 µL of dNTP solution (2.5 mM of each dNTP), 0.5 µL of the COX1 or ND forward primer (100 µM), 0.5 µL of the corresponding COX1 or ND reverse primer (100 µM), 1 µL of the cDNA library, 5 µL of 5x buffer solution and 16 µL of distilled H_2O into a 300 µL PCR tube.

4.2. Perform the PCR amplification using the following parameters: After an initial denaturation step at 95 °C (duration 5 min), 35 PCR reaction cycles consisting of an annealing step at 55 °C (30 s), elongation step at 72 °C (2 min), and denaturation step at 95 °C (30 s), perform one finalizing elongation step for 5 min at 72 °C.

4.3. Use 5 µL of the PCR product to verify by agarose gel electrophoresis the quality of the obtained PCR product. Observe the amplified COX1 or ND gene as a single band at either 759 or 748 base pairs, respectively.

4.4. Purify the rest of the PCR product with a PCR clean-up kit.

4.4.1. Add 100 µL of solution 'PCR-A' (DNA binding buffer which contains high concentrations of chaotropic salts[11]) to the sample. Vortex briefly to mix the contents.

4.4.2. Place the purification column into a 2 mL centrifuge tube. Pipette the reaction mixture of 4.4.1. into the column. Centrifuge at 14,000 x *g* for 1 min at room temperature.

4.4.3. Discard the filtrate from the centrifuge tube. Return the column to the 2 mL centrifuge tube, add 700 µL of solution 'W2' into the column and centrifuge at 14,000 x *g* for 1 min at room temperature ('W2' is a washing solution which contains high concentrations of ethanol for the removal of residual chaotropic salts from the purification column).

4.4.4. Discard the filtrate and return the column to the 2 mL centrifuge tube. Add 400 µL of solution 'W2' to the column and centrifuge at 14,000 x *g* for 1 min at room temperature.

4.4.5. Pre-heat 1 mL of deionized water to 65 °C in a metal block heater. Transfer the column into a new 1.5 mL centrifuge tube. Pipette 25 µL of the 65 °C hot pre-heated deionized water to the center of the white column membrane. Let the membrane soak for 1 min at room temperature.

4.4.6. Centrifuge at 14,000 x *g* for 1 min at room temperature and discard the column.

4.5. Store the purified PCR product for up to 12 months at -20 °C.

5. Mitochondrial gene sequencing and comparison

5.1. Send the purified PCR samples from step 4.5 for Sanger sequencing using the relevant COX1 or ND forward primers as sequencing primers. Optionally, the COX1 or ND reverse primers can also be used for bidirectional sequencing.

5.2. After retrieving the sequencing results, compare the sequences with the genome sequence of the Pacific oyster reference strain (NCBI Taxonomy ID: 29159) using the NCBI Nucleotide BLAST online tool (blast.ncbi.nlm.nih.gov) (**Figure 2** and **Figure 3**).

6. Applying the cDNA library for cloning the genes MgUGD and MgUXS

6.1. Amplify and purify the MgUGD and MgUXS genes using the respective forward and reverse primers of MgUGD and MgUXS by PCR following steps 4.1. to 4.4.

6.2. Transfer 2 µL of digestion buffer (10x concentrated) and 6 µL of deionized water into a 1.5 mL centrifuge tube. Add 10 µL of the purified MgUGD or MgUXS PCR products together with the restriction endonucleases Nde I and Xho I (1 µL each, 20 U). Incubate at 37 °C for 3 h.

6.3. Prepare the predigested pET-30a vector: Transfer 500 ng of the pET-30a vector into a new 1.5 mL centrifuge tube and top up the volume to 16 µL using deionized water. Add 2 µL of digestion buffer (10x concentrated) together with the restriction endonucleases Nde I and Xho I (1 µL each, 20 U).

6.3.1. After incubating the mixture at 37 °C for 3 h, add 1 µL of alkaline phosphatase (1 U) and incubate at 37 °C for an additional hour. Inactivate the alkaline phosphatase by heating at 75 °C for 10 min in a pre-heated metal block heater.

6.4. Transfer 4 µL of the digested MgUGD or MgUXS DNA products into fresh 1.5 mL centrifuge tubes and add 4 µL of the digested pET-30a vector. Add 1 µL of Ligation Buffer (10x), 1 µL of T4 ligase (3 U) and incubate the reaction mixture at 22 °C for 3 h.

6.5. Transform electrocompetent *E. coli* Mach1 Competent Cells by electroporation using the ligation products. Spread the transformed cells on LB agar plates containing 50 µg/mL kanamycin. Incubate cells at 37 °C for 16 h.

6.6. Verify colonies for the desired insertion by Sanger sequencing using the plasmid-specific T7 promoter and terminator primers. Prepare plasmids from the validated bacterial clones.

7. Expression and activity tests of MgUGD and MgUXS

7.1. Transform *E. coli* BL21 (DE3) Competent Cells with plasmids bearing the MgUGD and MgUXS genes and spread the transformed cells on LB agar plates containing 50 µg/mL kanamycin. Incubate cells at 37 °C for 16 h.

7.2. Cultivate a single colony in 5 mL LB medium with 50 µg/mL kanamycin overnight. Transfer the culture into 400 mL LB medium and continuously shake at 200 rpm at a temperature of 37 °C until the optical density at a wavelength of 600 nm (OD_{600}) reaches an absorption of approximately 0.5.

7.2.1. Reduce the incubation temperature to 20 °C and add 400 µL of isopropyl β-D-thiogalactopyranoside (concentration 1 M). Induce the expression of the recombinant proteins for 3 h.

7.3. Harvest cells by centrifugation at 4,500 x *g* for 15 min at 4 °C. Suspend pellets in 10 mL of lysis buffer (100 mM NaCl, 50 mM Tris/HCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl-fluoride (PMSF), pH 8.0).

7.4. Disrupt cells by sonication for 20 min (40 on/off cycles with 20 µm amplitude for 15 s at 4 °C). Centrifuge at 14,000 x *g* at 4°C for 20 min and collect the supernatant for the activity test.

7.5. Perform the activity assay of MgUGD by incubating 2 µL of the cell lysate with 2 µL of UDP-glucose (10 mM), 4 µL of NAD^+ (10mM), 4 µL of $MgCl_2$ (10 mM), 2 µL of Tris/HCl buffer (500 mM, pH 7.5), and 6 µL of deionized H_2O in a new 1.5 mL centrifuge tube and incubate at 37 °C for 30 min.

7.6. Perform the activity assay of MgUXS by incubating 2 µL of the cell lysate with 2 µL of UDP-glucuronic acid (10 mM), 2 µL of Tris/HCl buffer (500 mM, pH 7.5), and 14 µL of deionized H_2O in a new 1.5 mL centrifuge tube and incubate at 37 °C for 30 min.

7.7. Quench the reactions in step 7.5 and 7.6 by adding 20 µL of methanol and 40 µL of chloroform to each mixture. After vortexing the sample mixtures, centrifuge at 14,000 x *g* for 6 min at 4 °C and collect the upper aqueous layer of each tube.

7.8. Analyze the reaction products using MALDI-TOF mass spectrometry in negative ionization

mode in the m/z range from 500-700. Mix 1 µL of sample mixture with 1 µL of 2,5-dihydroxybenzoic acid sample matrix (1% w/V in 50% aqueous acetonitrile). Observe the expected m/z values at 579 and 535 for UDP-glucuronic acid and UDP-xylose, respectively (**Figure 4**).

REPRESENTATIVE RESULTS:

Figure 1 shows a schematic overview of the described preparation method of the convergent cDNA library derived from Pacific oyster individuals. **Figure 2** shows the sequences of the COX1 and ND genes of a distantly related oyster specimen with high divergence from the COX1 and ND gene sequences of the reference material. **Figure 3** shows the sequences of the COX1 and ND genes of a closely related oyster specimen with low divergence from the COX1 and ND gene sequences of the reference material. **Figure 4** shows the successful application of the cDNA library to clone the industrially relevant genes MgUGD and MgUXS.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic overview of the described analysis method for molecular identification of Pacific oyster specimen using COX1 and ND as reference genes.

Figure 2: Sequence alignment of the COX1 and ND gene sequences of a highly-divergent specimen compared with the COX1 and ND gene sequences from the reference Pacific oyster strain.

Figure 3: Sequence alignment of the COX1 and ND gene sequences of a closely related specimen compared with the COX1 and ND gene sequences from the reference Pacific oyster strain.

Figure 4: Schematic overview of molecular cloning, recombinant expression and detection of the reaction products of MgUGD and MgUXS.

DISCUSSION:

The presented protocol allows the genetic identification of unreferenced oyster specimens with similar phenotype from regional seafood markets by comparison of the COX1 and ND genes with a publicly available oyster DNA genome database. The significance of this method lies in its simplicity, as only a single PCR reaction is needed for the evaluation of the virtual cDNA library. The two conserved mitochondrial COX1 and ND genes were amplified from a cDNA library which was generated by reverse transcription of RNA extracts from each oyster. The method of RNA isolation (step 2.1) was simplified by directly grinding the oyster tissue in liquid nitrogen. After sequencing the COX1 and ND genes of each specimen, sequence alignments revealed that some samples show high similarity to the reference strain. The closest relative showed complete identity of both the COX1 and the ND gene sequences.

The most critical steps of this procedure are the RNA extraction step; in order to minimize RNA degradation, it is essential to reduce the time between harvesting the oyster tissue and the RNA extraction.

Successful cloning was recently exemplified by cloning the oyster UGE gene[12] and herein by cloning the MgUGD and MgUXS genes[13], which validated the practicality of the generated cDNA library, allowing cloning of any number of genes of interest without the need for cumbersome cloning strategies using degenerated primers. This method of molecular identification by amplification of the COX1 and ND genes to generate virtually sequenced cDNA libraries may also be used in future applications for other biological materials that do not have physical samples of referenced genomes available.

ACKNOWLEDGEMENTS:

This work was supported in part by the Natural Science Foundation of China (grant numbers 31471703, A0201300537 and 31671854 to J.V. and L.L., grant number 31470435 to G.Y.), and the 100 Foreign Talents Plan (grant number JSB2014012 to J.V.).

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

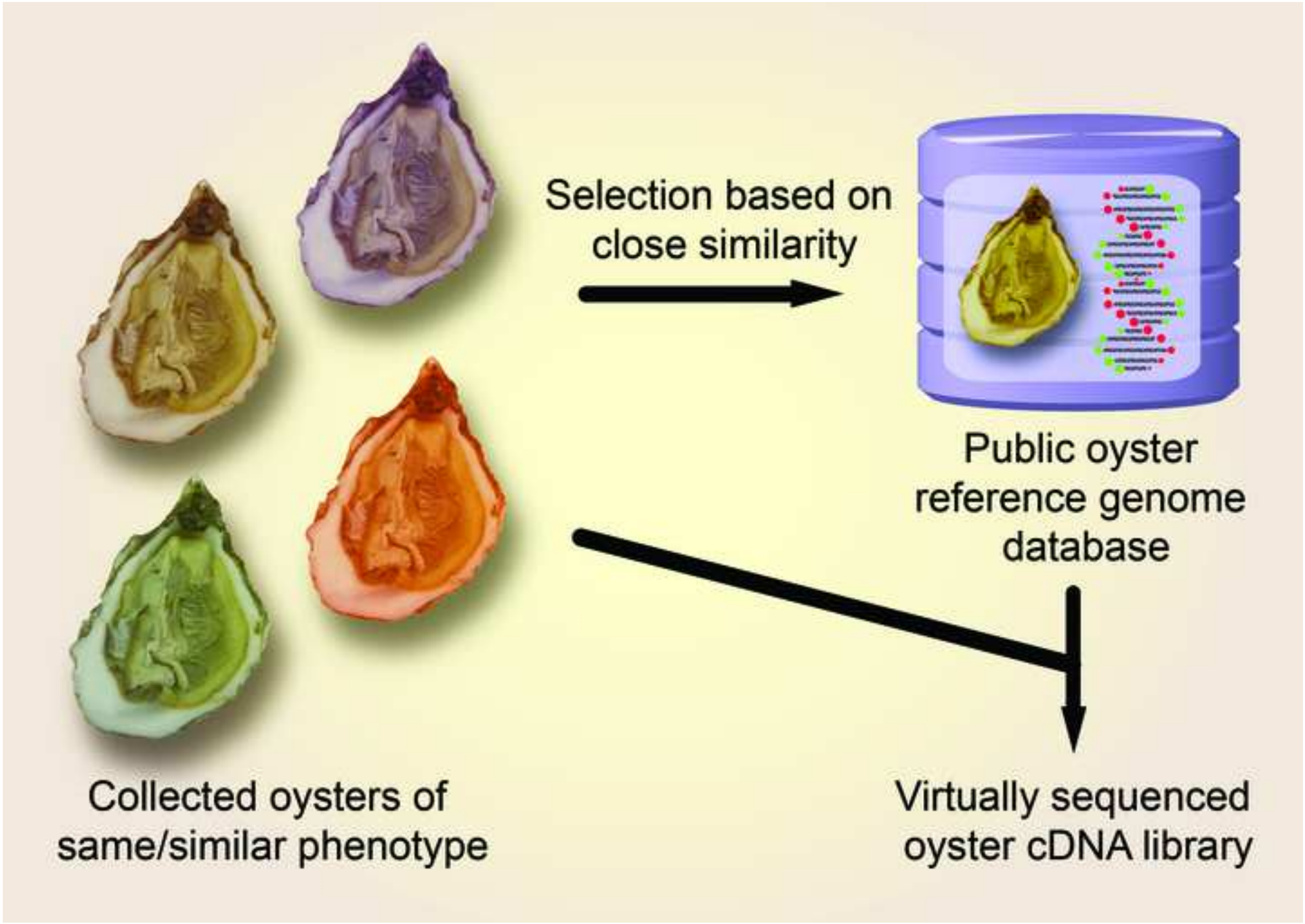
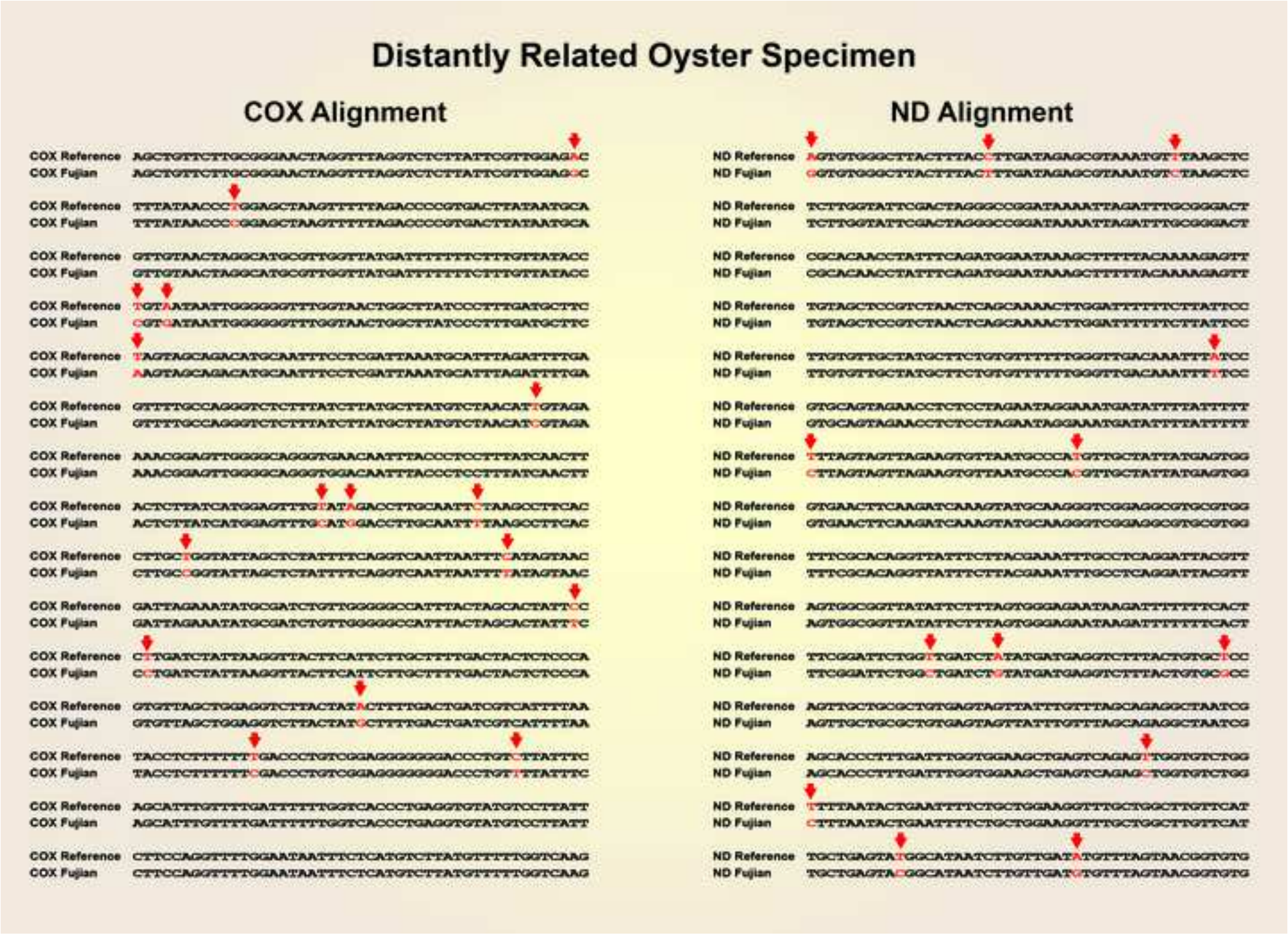


Figure 2



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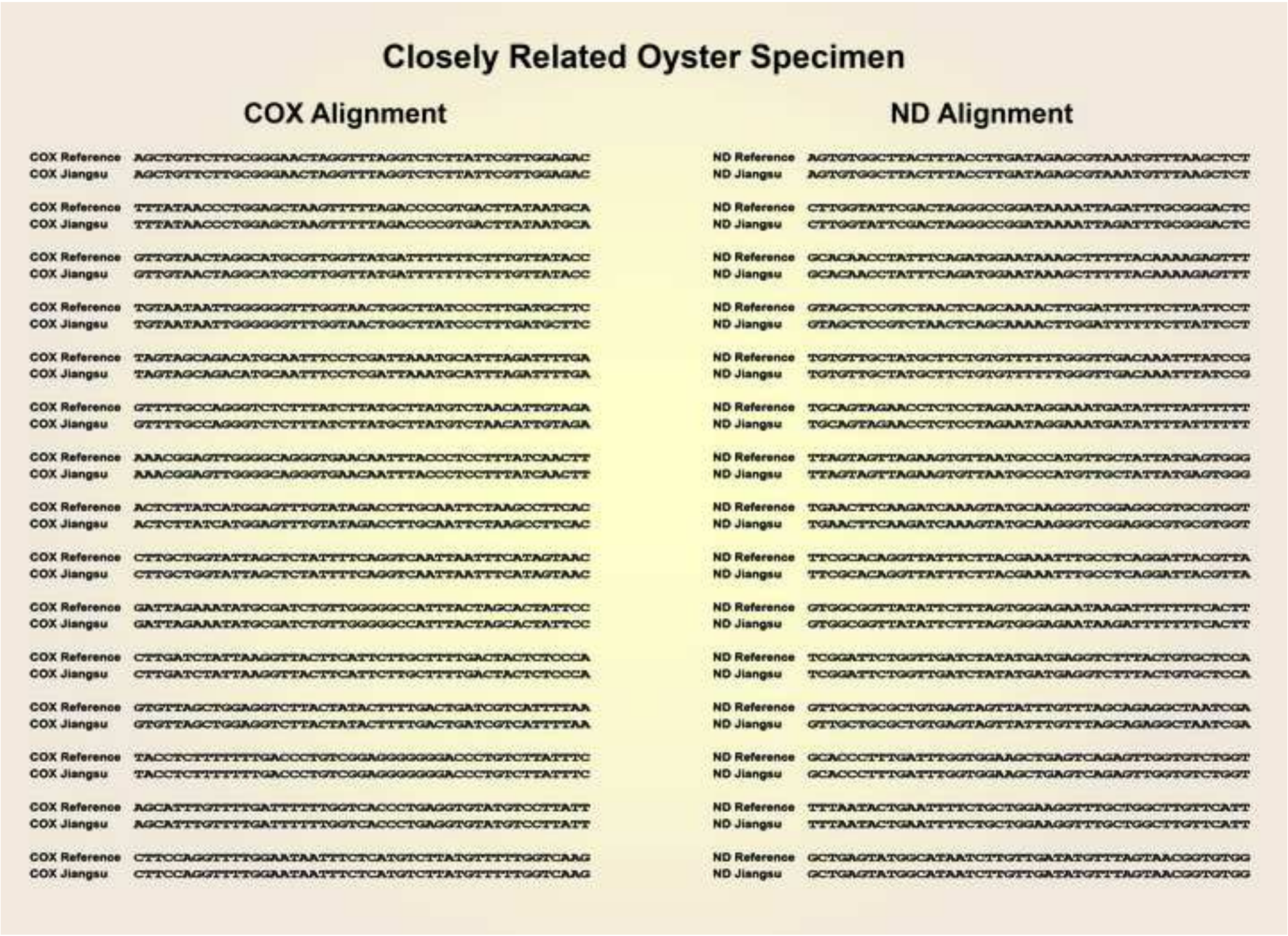

Figure 4

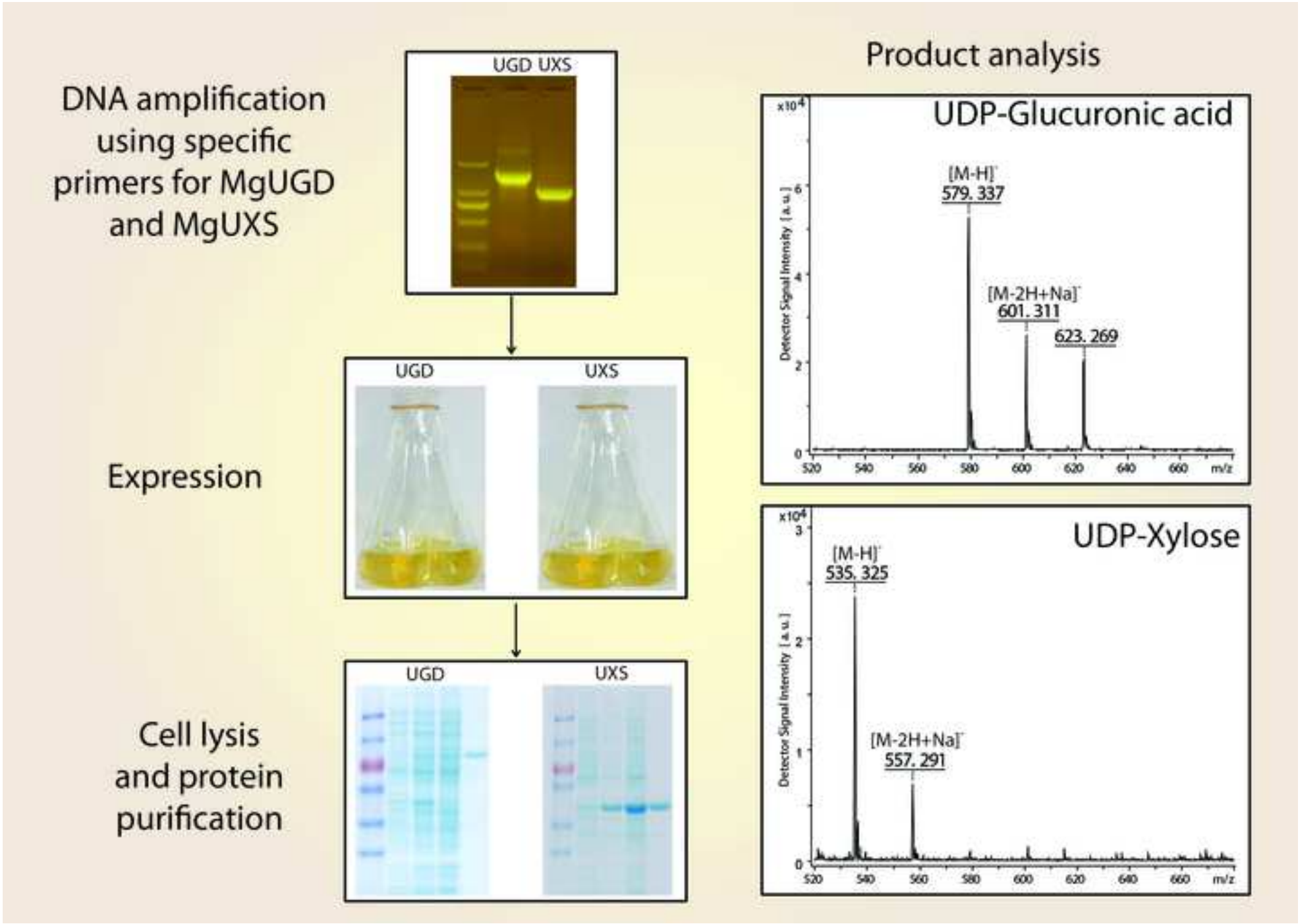

Table of Materials

Click here to access/download;Table of Materials;Table of Materials.xlsx

Name of Material / Equipment	Company	Catalogue Number	Comments/Description
Chemicals:			
1% Triton X-100	Solarbio	9002-93-1	*Alternative distributors possible
2,5-Dihydroxybenzoic acid	Alfa Aesar	490-79-9	*Alternative distributors possible
Acetonitrile	Merck	75-05-8	*Alternative distributors possible
Agarose for molecular biology	Biowest Chemicals	111860	*Alternative distributors possible
Ampicilin	Solarbio	69-52-3	*Alternative distributors possible
Chloroform	Lingfeng, Shanghai	67-66-3	*Alternative distributors possible
DEPC water	Thermo Scientific	R0601	
Ethanol	Jinhuada, Guangzhou	64-17-5	*Alternative distributors possible
Guanidinium thiocyanate-phenol reagent	Invitrogen	15596018	TRIzol reagent
Imidazole	Energy Chemical	288-32-4	*Alternative distributors possible
Isopropyl alcohol	Nanjing Chemical Reagent	67-63-0	*Alternative distributors possible
Isopropyl β-D-thiogalactopyranoside	Solarbio	367-93-1	*Alternative distributors possible
Kanamycin	Solarbio	25389-94-0	*Alternative distributors possible
LB Agar	Thermo Fisher	22700025	*Alternative distributors possible
LB Broth	Thermo Fisher	10855021	*Alternative distributors possible
Methanol	Jinhuada, Guangzhou	67-56-1	*Alternative distributors possible
$MgCl_2$ hexahydrate	Xilong Huagong	7791-18-6	*Alternative distributors possible
NaCl	Xilong Huagong	7647-14-5	*Alternative distributors possible
NAD^+	Duly Biotech	53-84-9	*Alternative distributors possible
Phenyl-methylsulfonyl fluoride	Macklin	329-98-6	*Alternative distributors possible
Tris	Solarbio	77-86-1	*Alternative distributors possible
UDP-glucose	Wuhu Nuowei Chemicals	28053-08-9	*Alternative distributors possible
UDP-glucuronic acid	SIGMA	63700-19-6	*Alternative distributors possible

Tools/Instruments:			
MALDI-TOF mass spectrometer	Bruker	Autoflex	*Alternative distributors possible
Metal block heater	Long Yang Scientific Instruments	Thermoshaker HB20	*Alternative distributors possible
PCR thermocycler	Hema	9600	*Alternative distributors possible

Enzyme and Kits:			
10×Ligation buffer	Thermo Scientific	B69	*Alternative distributors possible
5×PrimeSTAR buffer	Takara	9158A	
Alkaline phosphatase	ThermoFisher FastAP	EF0654	*Alternative distributors possible
COX forward primer	Genscript	**ATGTCAACAAATCATTTAGACATTG**	
COX reverse primer	Genscript	**ACTTGACCAAAAACATAAGACATG**	
Cutsmart Buffer	NEB	B7204S	*Alternative distributors possible
dNTP mix	Invitrogen	18427088	
MgUGD forward primer	Genscript	**ACATATGACCCTGTCCAAGATCTGTTGT**	
MgUGD reverse primer	Genscript	**ACTCGAGACTCTGTGAGGCGGTGGAG**	
MgUXS forward primer	Genscript	**CCATATGGCAGAATCCTCACAATCAC**	
MgUXS reverse primer	Genscript	**ACTCGAGCACATTTTTGAATTTGCAGACGT**	
ND forward primer	Genscript	**ATGAGATGGCAATTATTTTTTAAT**	
ND reverse primer	Genscript	**ATGTATTTTGGAAAAATCTCCAC**	
PCR Cleanup Kit	AxyGen	AP-PCR-250	*Alternative distributors possible
pET-30a(+) vector	Merck Millipore	69909	
PrimeSTAR HS DNA polymerase	Takara	R010Q	
Restriction enzyme Nde I	NEB	R0111	*Alternative distributors possible
Restriction enzyme Xho I	NEB	R0146	*Alternative distributors possible
Reverse Transcription kit	Promega	A3500	
T4 DNA ligase	Thermo Scientific	EL0014	*Alternative distributors possible
T7 promoter primer	Merck Millipore	69348	
T7 terminator primer	Merck Millipore	69337	

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Article Title:	A convergent strategy for the generation of a virtually sequenced cDNA library from unreferenced Pacific oysters.
Signature:	
Date:	November 29 2018

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AUTHORS' RESPONSE TO THE EDITORIAL AND REVIEWERS' COMMENTS

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
2. Please reference at least 10 previous publications.

With the added citations (see specific responses to comments below), there are now a total of ten Citations.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
The authors present a rapid and simplified method for preparing oyster cDNA libraries, that can be used to clone cDNAs of selected genes with industrial applications, such as Mg UXS or MgUSG.

Major Concerns:
None

Minor Concerns:
Abstract:
"The underlying rationale….means that other genes also bear fewer mutations."
Unless this statement has been verified by whole genome sequencing, I'd suggest changing "means" to "indicates". I'd suggest the same change in line # 64.

Authors' response: these changes were made as suggested.

Line # 48: Authors mention Cytochrome C Oxidase - please clarify if a subunit was used (COX 1?)? I suspect a subunit was used as indicated by the length of the PCR product and alignment.

Authors' response: It is indeed COX subunit I. The manuscript has been revised to clearly convey this information.

Line # 88: Please include storage recommendation for sample specimen before RNA extraction. Were oysters stored on room temperature or ice right after sampling? Do authors recommend processing the samples within a specific time frame?

Authors' response: We added the information to the manuscript that the oysters should be kept on ice (to their usual storage in seafood markets and restaurants) prior to RNA extraction (lines 97-99).

Line # 96: It is still not clear what part of the oyster was used for DNA extraction. Please be

more specific. Also, I am wondering why samples were not euthanized first before tissue was obtained.

Authors' response: We did not target any specific organ for tissue removal, but took the tissue samples from the approximate geometric center of the mollusk inside. We did not euthanize the samples prior as we were unsure of what protocol to use that would not damage the material that was important for our purposes. However, we froze the residual part of the oysters immediately after extraction at -80°C for one hour before discarding them. (see lines 109-110).

Line #185, point 5.2:
Is there a reason why authors do not recommend bi-directional sequencing using forward and reverse primers?

Authors' response: Bi-directional sequencing can be certainly used, but having the performance of sequencing readouts of 1000 or more base pairs should suffice for the accurate inference of homologues (the amplified COX1 and ND DNA consist of only 759 and 748 bp, respectively, which is usually within the readout length of unidirectional sequencing). We also added the suggestion that the reverse primer can be also used for bidirectional sequencing in section 5.1. (lines 201-202).

Reviewer #2:
Manuscript Summary:
The paper "A convergent strategy for the generation of a virtually sequenced cDNA library from unreferenced Pacific oysters" describes a protocol which aims to determine the degree of divergence of commercially obtained oysters to that of a reference Pacific oyster and the suitability of corresponding cDNA libraries for downstream molecular applications, such as molecular cloning.

Major Concerns:
The authors of the paper suggest the use of Cytochrome C Oxidase (COX) and NADH Dehydrogenase (ND) as molecular markers to study the divergence of commercially purchased oysters of phenotype similar to the Pacific oyster, and comparing these sequences to the Pacific oyster reference genome. The approach undertaken by the authors here is essentially the same as that of DNA barcoding; with the exception that cDNA is used as template instead of genomic DNA. While I appreciate that this may be convenient for researchers looking to perform gene expression studies or any other kind of work requiring cDNA, it is unconventional to utilize cDNA for molecular marker genotyping and there are flaws in doing so. For one, errors could potentially be introduced by the reverse transcriptase enzyme during RT. Also, mitochondrial RNA is not exempt from RNA editing (see Cell paper by Benne et al, 1986), although I will admit I am not familiar if this mechanism has been observed in the Pacific oyster specifically. While both of these events are rare, it appears as if the authors have chosen to use cDNA out of convenience, which reflects poorly given that genomic DNA extraction was an option and would provide more confidence in the results.

The protocol itself is well detailed, but is generic and does not address any issue in particular. It follows the steps of a basic guanidinium thiocyanate-phenol-chloroform RNA extraction, followed by reverse-transcription using an oligo-dT and PCR using COX and ND specific primers. The authors use a high-fidelity Taq polymerase for their PCR reactions, but unfortunately this does not account for errors that the reverse-transcriptase could have potentially introduced. I also feel the generation of a cDNA library suitable for molecular cloning could have been achieved using just about any commercial RT-PCR kit, and do not see how this protocol benefits from a video, especially given the availability of RNA extraction protocols by TRIzol and the ease of access to RT-PCR kits.

Authors' response:
We believe the reviewer sees the similarity with DNA barcoding because of the use of mitochondrial markers, particularly the widely-used COX1. A previous version of this manuscript indeed used a genomic locus (actin), but this ultimately proved problematic given the highly paralogous nature of the oyster genome and these results were discarded on the objections of previous reviewers. On the suggestion of these previous reviewers we shifted to using mitochondrial markers that were well-characterized and, more importantly, present only in single copies. The protocol used for the actin data was the same as that used for the COX1 and ND data here. Further, given the aforementioned paralogous nature of the oyster genome, genomic data extraction and analysis would have proved quite troublesome as we would have been faced with multiple uncertainties about which reference sequence paralogue actually matched our obtained sample sequences and primer sites. Hence, use of cDNAs from these mitochondrial markers was not merely "out of convenience".
Given that the herein presented method tries to identify the closest match of specimens to the reference genome, and given that the same error rate can be expected for all samples during the reverse transcription of the RNA templates, we presume that the most similar specimens are still selectable. Given that almost all PCR and reversed transcriptase methods nowadays rely on kits, we used the standard polymerase in our Lab which happens to be a high-fidelity Taq polymerase. We presume that the protocol would still work with a standard Taq polymerase (which would add some additional mutations to the PCR products), but likely to a lesser extent as one observes from naturally occurring mutations.
The protocol does not only demonstrate the preparation of cDNA samples from oysters, but it also demonstrates how easily these specimens can be evaluated for cloning purposes. Before we started this work, we had big concerns if we could amplify any functional genes in oysters, and the method we described here can give other scientists confidence of trying to isolate genes from natural sources in case no original reference material is available.

The introduction to the paper requires some more work. Some of the statements made in the introduction seem contradictive. For example, the authors write "However, these samples may vary significantly at the level of the genotype, and therefore genomic databases of the reference genomes of the same species are often rendered futile". Why is that? It seems contradictive, as the authors compare the obtained COX and ND cDNA sequences of their unreferenced oysters to the complete genome of the Pacific oyster.

Authors' response:

If the reference sequence is too divergent from the sequence obtained from the newly sourced material, then standard laboratory materials used for DNA amplification (e.g. primers) may not work well or at all. In our particular case, the sequences obtained from our anonymous samples were sufficiently close to the reference sequence that the standard primers and protocols were effective. We have acknowledged this and rephrased the sentence in the revised manuscript as follows:
"However, these samples may vary significantly at the level of genotype, and therefore comparisons with digitally-stored reference genomes of the same species are often rendered difficult or even futile due to incompatibility of the newly-sourced material with existing DNA amplification methods."

Meanwhile, other ideas are not very well explained or explained at all. For example, the abstract mentions the cloning and expression of the UGD and UXS genes, but is neither mentioned nor explained in the introduction, despite being a large part of the protocol. I also suspect that this step could have been successful even the oysters used had not been closely related to the Pacific oyster. If the intent was simply to show that the resulting cDNA is fit for downstream molecular applications such as cloning, it is acceptable, but it should have been made clearer in the paper.

Authors' response: We made some alterations in the introduction to better explain the purpose of our methodology. We do believe that having a cDNA library of a specimen close to the reference genome is of benefit, maybe not if one looks at 2 or 3 genes, than various samples can be screened for amplification, but we cloned more than 60 glycoenzyme candidates and it was very advantageous and convenient to have the reliable selected cDNA template available.

We have added the following to the end of the introduction which addresses the last two of the above points, i.e. that the UGD and UXS genes were included, and that they demonstrate an application of the method outside of well-established markers, to genes of potential industrial relevance:

"Having proven the approach with these well-established markers, we then demonstrate its application to two enzyme candidates which are involved in sugar nucleotide biosynthesis and may be of industrial relevance…"

Figures 2 and 3 show the BLAST alignments of 2 oysters with a reference genome. This includes the ones with the most and least divergence to the reference Pacific oyster genome. However, the remaining data is not shown, and is necessary to understand what constitutes low and high divergence. How do the authors suggest doing this, and how exactly is the cut-off determined? How many nucleotide differences between both genes were deemed acceptable to proceed with downstream application? Phylogeny should have been done and would help support these two figures much better.

Authors' response: We showed low and high divergence from the reference strain as examples, and it is literally that the samples which showed the least number of mutations should be considered to be able to amplify other genes of interest – presume that this is a feasible statement and as we mentioned before should give other researchers the confidence to work with more or less anonymous specimen from a seafood market, or literally any species which can be hard to

identify exactly by the non-expert. We have no objection to providing the remaining data as a supplement if this is suitable and needed.

In its current format, the paper appears to suggest that comparing the cDNA libraries of unreferenced oysters using COX and ND as molecular markers to a reference oyster genome is an appropriate way to determine species similarity or divergence. While practical for some applications, the information obtained from cDNA is simply not as robust as the information retrieved from genomic DNA barcoding and is a poor substitute. I would not suggest this method unless sample availability was extremely low and genomic DNA extraction was not an option. I also fail to understand why the protocol requires a video, and think that the introduction would highly benefit from a re-write. In the event that the paper is revised, I have included some additional comments down below.

Authors' response: The above paragraph summarizes the reviewer's previous points which we have addressed above.

Minor Concerns:
Introduction:
"The underlying rationale for the herein presented method is that high conservation mitochondrial gene sequences in individual oyster samples compared to the mitochondrial gene sequences of the reference genome means that other genes also bear fewer mutation…" - this sentence requires citations.

Authors' response: We have added the following reference, as we reason that, given the generally faster rate of mitochondrial DNA evolution relative to nuclear DNA, observed low levels of divergence between mtDNA samples imply even lower divergence between nuclear DNA samples from the same individuals:

Delsuc, F., Stanhope M.J., and Douzery, E.J.P. Molecular systematics of armadillos (Xenarthra, Dasypodidae): contribution of maximum likelihood and Bayesian analyses of mitochondrial and nuclear genes. *Molecular Phylogenetics and Evolution*, **28**, 261-275, (2003).

We have rephrased the sentence to convey this point, also making it sound less decisive as recommended by reviewer #3.

"The underlying rationale for the herein presented method is that high conservation of mitochondrial gene sequences in individual anonymous oyster samples compared to the corresponding sequences of the reference genome means indicates that other genes may also show a low level of divergence, given the generally faster rate of mitochondrial DNA evolution relative to nuclear DNA…"

References are also required to justify the use of COX and ND genes.

Authors' response: We have already included the following references:

Wen, J. et al. Species identification of dried shellfish (oyster, clam and mussel) products sold on the Chinese market. *Food Control*, **90**, 199–204, (2018). (for COX1)

Sell, J. and Spirkovski, Z. Mitochondrial DNA differentiation between two forms of trout Salmo letnica, endemic to the Balkan Lake Ohrid, reflects their reproductive isolation. *Molecular Ecology*. **13**, 3633-3644, (2004). (for ND)

justifying previous use of these two particular markers for species (or at least population) identification, even if the latter is in an osteichthyan, as opposed to a bivalve mollusc.

Protocol:
Protocol section 1.1: Change "bought" to "purchased".
Change made.
Protocol section 2: Change title to "RNA isolation by guanidinium thiocyanate-phenol extraction".
Change made.
Protocol section 2.1.4: Change "fresh 1.5 mL centrifuge tube" to "sterile 1.5 mL centrifuge tube".
Change made.
Also, is any kind of homogenization (vortex mixing) required before proceeding to the centrifugation step?
As stated in point 2.4, inversion of the sample tube is enough (line 125).
Protocol section 2.6: Perhaps the pellets would dry quicker under a fume hood?
Protocol section 4.1: "and 0.5 uL of the corresponding COX or ND…". Remove the word "and".
Change made.
Protocol section 4.2: I find the description of their PCR cycling parameters a bit unconventional, as the steps are described as annealing first, followed by extension and finally denaturation. I believe it simply needs re-wording (the steps should be denaturation first, followed by annealing and finally extension).
In the first sentence of section 4.2 we mention an initial denaturation step (line 159), which starts the actual PCR procedure before the cycling protocol. This may be a bit unconventional but we wanted to emphasize that the first denaturation step is longer than the other denaturation steps of the procedure.
Protocol section 6.3: Replace the word "fresh" with "new" or "sterile".
Change made.
Protocol section 6.5: I would suggest replacing the term "transfect" with "transform" and changing the sentence to reflect that it is the bacteria that are being transformed with the vector, not the other way around (i.e. "Electrocompetent E. coli were transformed using the ligation products…").
Changes made.
Protocol section 7.1: See comment from section 6.5.
Changes made.
Figures 2, 3 and 4 were of relatively poor resolution and the associated text was difficult to read.
In the revised version high resolution bitmap and the vector graphics were added to the protocol.

Reviewer #3:
A convergent strategy for the generation of a virtually sequenced cDNA library from

unreferenced Pacific oysters
JoVE59462

Yong M. Lyu, Yu Q. Li, Hui B. Song, Ting Wang, Li Liu, Gabriel Yedid, and Josef Voglmeir

Manuscript Summary:
This paper describes a protocol to select oyster samples with a low nucleotide divergence to publicly available genome data using two mitochondrial genes. The selected oyters will probably offer a higher rate of success when used to, for example, clone genes based on reference sequencing data.

This protocol seems an interesting approach to increase efficiency in studying gene sequences in species with significant variation at the genotype level and genomic data available.

Major Concerns:
- Title: A convergent strategy for the generation of a virtually sequenced cDNA library from unreferenced Pacific oysters
I found the terms "convergent strategy" and "virtually sequenced" to be somewhat unclear. The authors should consider changing the wording or offering a clear definition of these terms.

Authors' response: By "convergent", we meant getting closer to the reference genome i.e. converging on a solution. We have changed the term to "converging" and explained this in the text as follows, as none of the usual synonyms for "converging" accurately convey our intended meaning:
"The aim is to produce a convergent cDNA library, meaning that similarities between the generated cDNA sequences and the reference sequence can be ranked from low to high divergence."
By "virtual sequencing", we mean that no direct sequencing involving extraction of genomic material and processing through standard DNA sequencing protocols (these days almost always done commercially) is performed. We take the reference sequence, already stored as a digital record, and use that to directly utilize or design primers for production of cDNAs that will eventually comprise a library or be added to a pre-existing one. We have added this explanation to the text as follows:
"In virtual sequencing, de novo genome sequencing is circumvented; instead, we take a known, digitally-stored reference sequence and use that directly to utilize or design primers for production of cDNAs that will eventually comprise a library (or be added to a pre-existing one)."

- L64-66 ... samples compared to the mitochondrial gene sequences of the reference genome means that other genes also bear fewer mutations, allowing the amplification and isolation of a wide range of scientifically and industrially relevant genes by simply using publicly available sequencing data as a reference.
Although it might be a somewhat reasonable assumption I would be more cautious when expressing it. Two individuals could share the mitochondrial genome but have divergent nuclear genomes.
Instead of:
"... that other genes also bear fewer mutations,"

you could say something like,
"... that other genes could also show a low divergence level,"

Authors' response: Change made as follows on lines 66-67 of the revised manuscript:
"The underlying rationale for the herein presented method is that high conservation mitochondrial gene sequences in individual oyster samples compared to the mitochondrial gene sequences of the reference genome indicates that other genes may also show a low level of divergence, , allowing the amplification and isolation of a wide range of..."

Minor Concerns:
- L55-56 "The acquisition of living referenced biological material may be challenging due to long delivery times, entrepreneurial reasoning..."
Could you give specific examples of "entrepreneurial reasoning"?

Authors' response: By this, we mean simply that the two sides are not able to come to an agreement regarding provision of the requested material; "entrepreneurial reasoning" is simply a terser wording.

- L117. "Remove the supernatant quantitatively."
What do the authors mean by "quantitatively"?
Authors' response: We changed 'quantitatively' with 'all of the supernatant' for better clarification.

- L157. "PCR-A".
I'm aware that no commercial trademarks and names are allowed in the protocol section, but maybe you could specify the function of this buffer (maybe between parentheses).
Authors' response: Although composition of these kit solutions proprietary, the general purpose of these buffers is known – we therefore added the basic purpose and a reference of this buffer in lines 171-172.

- L164, L168. "solution 'W2'".
See comment above for "solution PCR-A".
Authors' response: We also added the basic purpose of this buffer in lines 181-182.

- References.
Check that genus and species are correctly italicized.
Changes made.

We would like to thank all reviewers and the editor for their helpful comments and suggestions!