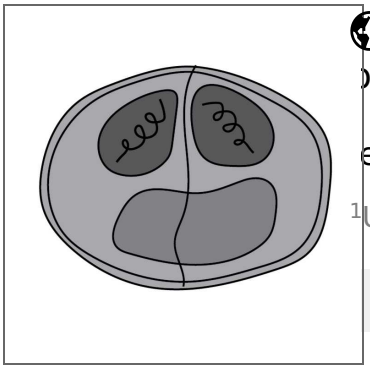

18S rDNA amplification of Kudoa musculoliquefaciens in broadbill swordfish

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Kudoa in swordfish

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OPEN ACCESS

ABSTRACT

Protocol for extracting and purifying genomic DNA from *Kudoa musculoliquefaciens* infecting broadbill swordfish *Xiphias gladius,* harvested off Eastern Australia. Used in Bolin et al. 2021 *Parasitol. Res*. (link) and Bolin et al. 2023 *in review.*

IMAGE ATTRIBUTION

Jessica Bolin

GUIDELINES

- Kudoid gDNA and PCR reagents must be kept on ice or at 4˚C throughout the protocol.
- Best results are obtained with fresh swordfish, or swordfish that has been snap-frozen and stored at -20˚C.
- Avoid repeated freezing and thawing of samples.
- If using frozen tissue, equilibrate the sample to room temperature before commencing protocol.
- All centrifugation steps are carried out at room temperature (15–28˚C).

Protocol Citation: Jessica A Bolin 2023. 18S rDNA amplification of Kudoa musculoliquefaciens in broadbill swordfish. **protocols.io** https://protocols.io/view/18s-rdna-amplification-of-kudoa-musculoliquefacien-cmr8u59w

MANUSCRIPT CITATION: Bolin et al. 2023 In review.

Bolin, J.A., Cummins, S.F., Mitu, S.A. et al. First report of Kudoa thunni and Kudoa musculoliquefaciens affecting the quality of commercially harvested yellowfin tuna and broadbill swordfish in Eastern Australia. Parasitol Res 120, 2493–2503 (2021). https://doi.org/10.1007/s00436-021-07206-8

Protocol status: Working

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MATERIALS

DNA extraction

- 100% molecular-grade ethanol
- 1X PBS
- Sterile scalpel
- Sterile scalpel blades (#10)
- Sterile agar plates
- Fine forceps + scissors
- Microfuge tubes
- Microfuge tube rack
- Pipette tips (10, 20 and 200µl) and corresponding pipettes
- Kim-wipes
- QIAGEN DNA extraction kit (blood and tissue)
- Esky + ice
- Spectrophotometer and Nanodrop 3000 software
- Heat block
- Vortex
- Centrifuge
- 4˚C fridge or -20˚C freezer (if storing gDNA for more than 24 hrs)

PCR and electrophoresis:

- PCR strip tubes and caps
- Microfuge tubes
- Kudoid gDNA and F+R primers
- Pipette tips (10, 20, 200µl)
- RedTAQ Polymerase and PCR reagent water
- PCR UV preparation cabinet
- Esky/tray and ice
- Milli-Q water
- Thermocycler
- 250mL Schott jar
- 0.6X TBE buffer
- Agarose powder
- Scales
- Microwave
- Ethidium bromide (CAUTION: TOXIC)
- 1Kb DNA ladder
- Gel tanks
- Electrophoresis powers supply
- Gel-Doc UV transilluminator
- Microwave
- Electronic scales

SAFETY WARNINGS

Ethidium Bromide Solution, 10ml **Promega Catalog #H5041** is toxic and needs to be handled with care. Always wear gloves, store in a dark cabinet, work with it in a contained area, and do not inhale/spill onto self.

ETHICS STATEMENT

Animal ethics waiver granted by UniSC Research Ethics Committee (ANE1938) in August 2019.

BEFORE START INSTRUCTIONS

- Sanitise work area and equipment with 70% ethanol.
- Preheat heat block to 56˚C.
- If using the DNA extraction kit for the first time, add the specified amount of 100% ethanol to the AW1 and AW2 buffers (will be on the protocol sheet and buffer container). Shake thoroughly after adding ethanol.
- Prepare DNeasy Mini Spin Columns by placing them in associated 2mL collection tubes

Extraction and purification of genomic DNA

1 Cut ~15mg of swordfish muscle tissue into small pieces, and place in a microtube.

2 Add 180µl of ATL buffer to the sample.

3 Add 20µl of proteinase K and vortex for 10 sec. 10s

4 Incubate at 56˚C in the heat block, until the sample is completely lysed (generally takes ~2 hours). Throughout lysis, vortex the sample for 10 sec every 30 minutes. 2h

Expected result

The lysed solution should appear yellowish and viscous (i.e., not gelatinous). If gelatinous, increase incubation time by 30 min and vortex accordingly.

5 After ~2 hours of lysis, vortex the sample for 15 sec. 15s

6 Add 200µl of AL buffer and 100% ethanol to the sample.

7 Vortex for 10 sec. 10s

8 Centrifuge 8000 rpm, Room temperature, 00:01:00 1m

9 Transfer the supernatant into the DNeasy Mini Spin Column + collection tube

10 Centrifuge 8000 rpm, Room temperature, 00:01:00 1m

11 Ensure that the supernatant has fallen through the spin column membrane. If not, this means the sample has not lysed properly and has clogged the spin column membrane. If this happens, either:
(i) Repeat Step 10
(ii) Start protocol from scratch and increase the incubation time.

12 Discard flow-through and collection tube. Place spin column into a new 2mL collection tube

13 Add 500µL AW1 buffer

14 Centrifuge 8000 rpm, Room temperature, 00:01:00 1m

15 Discard flow-through and collection tube. Place spin column into a new 2mL collection tube.

16 Add 500µL AW2 buffer.

17 Centrifuge 14000 rpm, Room temperature, 00:03:00 3m

18 Ensure that the supernatant has fallen through the spin column membrane and there is no residual buffer/gDNA + the membrane is completely dry. This ensures that no residual buffer will be carried over during the following elution.

Expected result

The spin column membrane must be completely dry.

19 Discard flow-through and collection tube. Place spin column into a new 1.5mL microtube.

20 *Slowly and carefully* add 200µL AE buffer to the centre of the spin column.

21 Incubate at room temperature for 1 min. 1m

22 Centrifuge 8000 rpm, Room temperature, 00:01:00 for final gDNA elution. 1m

23 Store purified DNA sample in 4˚C/-20˚C, or proceed to next section.

Quantify DNA concentration, purity and evidence of degradat...

24 Open Nanodrop 2000 software -> click nucleic acid/DNA -> wait for software to automatically calibrate

25 Open the spectrophotometer probe and place 1µL of AE buffer onto the probe. Close the lid.

26 Click the buffer icon. Spectrophotometer will commence measuring the buffer.

Expected result

The reading should be 0 (i.e., blank screen)

27 Open the spectrophotometer probe, wipe away the AE buffer, and place 1µL of gDNA onto the hole. Close the lid.

28 Click the measure icon. The concentration and purity readings will display on the screen.

Expected result

The expected gDNA concentration should be ~100ng/µL, however this will depend on the 'quality' of the sample (i.e., time spent on the longline, storage methods etc).

The 260/280 ratio should be ~2 (1.8–2.2 is OK). Abnormal 260/280 ratios usually indicate that a sample is contaminated by a residual reagent used in the extraction protocol.

29 Close the Nanodrop software.

30 Make up a 0.8X agarose gel.

30.1 Add 0.8g of agarose power and 0.6X 100µL TBE buffer into a schott jar. Shake to mix.

30.2 Microwave until agarose is completely dissolved.

30.3 Allow jar to cool to room temperature, and place 1 drop of Ethidium Bromide into jar. Immediately close the lid and swirl gently.

Safety information

Ethidium bromide is toxic. Handle with care.

30.4 Pour gel into a gel tray. Add a gel comb, and wait until the gel has completely cooled/solidified (~30-60 min). 1h

31 Mix 11µL of gDNA with 1µL of gel loading dye.

32 Remove the gel comb from the tray. Insert gel tray into gel tank and submerge with 0.6X TBE.

33 Add 3µL of 1Kb DNA ladder to a lane, and the pre-mixed gDNA + dye to another lane.

34 Run the gel at 100V for 1–2 hours. Stop the voltage when the bands have nearly run off the gel. 1h

35 Visualise gel in the UV trans-illuminator.

Expected result

If gDNA isn't degraded, there should be a tight band of high molecular weight in the lane.

If gDNA is degraded, there will be a smear down the entire gel lane. Note that using degraded gDNA in the thermocycler has worked (i.e., can still detect Kudoid gDNA using PCR).

36 Store gDNA sample in 4˚C/-20˚C, or proceed to next section.

PCR

37 Create a mastermix for a 25µL PCR reaction in a microtube, by combining the following reagents:

- 12.5µL of Taq ReadyMix
- 0.5µL of forward primer
- 0.5µL of reverse primer
- 1µL of gDNA
- 10.5µL of PCR pure water (or Ultrapure water)

Note

All PCR preparation must be done in a PCR prep UV cabinet, to avoid contamination.

38 Add the mastermix into a PCR strip tube. Slowly pipette the mixture up and down to mix thoroughly. Repeat for as many samples as required.

39 Store samples at 4˚C or proceed to next step.

40 Set thermocycler parameters to:

- Initialization: 95˚C, 5 min
- Denaturation: 95˚C, 30 sec
- Annealing: 54.4˚C, 30 sec
- Elongation/extension: 72˚C, 1 min
- Repeat steps denaturation to elongation 35 times
- Final elongation: 72˚C, 5 min
- Hold: 10˚C (optional)

41 Insert strip tubes into the thermocycler. Ensure tubes are spread evenly to balance the lid. Apply plastic/gel cover over the gel caps.

42 Close the lid and run the PCR. Will take ~2 hours. 2h

43 Store sample in 4˚C/-20˚C, or proceed to next section.

Electrophoresis 40m

44 Make up 2X agarose gel

44.1 Mix 2g agarose powder and 100mL 0.6X TBE buffer in a schott jar. Shake to mix.

44.2 Microwave until powder has completely dissolved.

> **Safety information**
>
> Be careful not to let the solution boil over into the microwave.

44.3 Let gel cool to room temperature. Add 1 drop of Ethidium Bromide

44.4 Pour gel into a gel tray and add gel comb.

44.5 When gel has completely set, remove gel comb.

45 Insert gel tray into gel tank. Submerge in 0.6X TBE buffer.

46 Load 3µL of 1KB DNA ladder into a well. Load ~10µL of PCR reaction sample into other wells.

47 Close tank lid and set voltage to 120V. Run gel for 30–40 min. 40m

48 Visualise gel on UV trans-illuminator.

Expected result

Positive Kudoid samples are indicated by a tight band corresponding to the ~300bp (i.e., 306bp) molecular weight band of the DNA ladder. Negative samples will have the absence of a band.