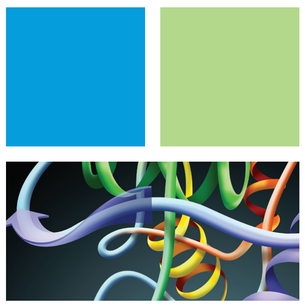

High-throughput direct DNA analysis from intact fish eggs by real-time PCR – Fish

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Introduction, methods and conclusions

Abstract

Current protocols to analyze DNA from fish eggs require multi-step DNA extraction prior to analysis; this limits throughput and increases the sample-to-results time. To overcome these limitations, we developed a workflow that can quantitatively analyze the DNA in a single intact fish egg directly by real-time PCR. Commercial Mallotus Villosus (Capelin) eggs were washed, added individually to wells of a PCR plate and analyzed using an inhibitor-tolerant real-time SYBR Green qPCR supermix and target-specific primers. We find that mitochondrial cytochrome b (CytB) DNA can be amplified efficiently and specifically from a single egg with over 95% success. These findings demonstrate that sample preparation can be bypassed entirely for analysis of fish egg DNA if inhibitor-tolerant real-time PCR reagents are used. This rapid, easy and inexpensive method of fish egg analysis can benefit aquaculture breeding and stock identification. It is also plausible that this methodology can extend to other fields that can benefit from direct analysis of DNA from crude samples.

Materials

Commercial Mallotus Villosus (Capelin) eggs, also called masago, were purchased from a local supermarket (99 Ranch) and stored at 4°C. Capelin eggs were washed two times with PBS in a 50 mL conical tube. Eggs were pelleted by gravity (no centrifugation) and the wash liquid was removed by decanting followed by pipetting. Real-time PCR was performed using SsoAdvanced Universal Inhibitor-Tolerant SYBR Green Supermix (Bio-Rad Laboratories) on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories). Primers (Integrated DNA Technologies) were used at a final concentration of 250 nM each in a final volume of 20 µL per well. Amplified DNA was analyzed on a 3% Mini ReadyAgarose Gel, TBE (Bio-Rad Laboratories) using a Mini Ready Sub-Cell GT electrophoresis system (Bio-Rad Laboratories).

Real time PCR

Individual Capelin eggs were added directly to a 96-well qPCR plate using forceps. As a positive control, DNA was purified from Capelin eggs and diluted to 10 ng/µL; 1 µL of purified DNA was added to positive control wells. Negative control wells contained 1 µL of TE.

20 µL of a mastermix containing 1x SsoAdvanced Universal Inhibitor-Tolerant SYBR Green Supermix and PCR primers (250 nM each) was added to each well and the plate was analyzed by real-time PCR. Cycling conditions were: 98°C for 2 min; 40 cycles of (98°C for 5 sec, 60°C for 30 sec, plate read); melt curve analysis 65°C to 98°C.

Primer design

Capelin mitochondrial cytochrome b DNA sequence was obtained from the below sources. Primers were designed using Primer3Plus

LOCUS FJ010882 966 bp DNA linear VRT 19-APR-2011
DEFINITION Mallotus villosus villosus isolate MvvK-05 cytochrome b (cytb) gene, partial cds; mitochondrial.

REFERENCE 1 (bases 1 to 966)
AUTHORS Skurikhina,L.A., Kukhlevskiy,A.D., Oleinik,A.G. and Kovpak,N.E.
TITLE Phylogenetic Analysis of Smelts (Osmeridae) Based on the Variation of Cytochrome b Gene
JOURNAL Russ. J. Genet. 46 (1), 69-80 (2010)

REFERENCE 2 (bases 1 to 966)
AUTHORS Kukhlevskiy,A.D., Skurikhina,L.A., Kovpak,N.E. and Brykov,V.A.
TITLE Direct Submission
JOURNAL Submitted (12-AUG-2008) Laboratory of Genetics, A.V. Zhirmunsky Institute of Marine Biology Far East Branch of Russian Academy of Sciences, Palchevsky st., 17, Vladivostok, Primorye 690041,

Capelin CytB DNA sequence:

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catttcaatctggtgaaactttggctctcttcttgggctttgcctcattattcaaattcttacgggcctatt
tctggccatacattacactgccgagactgccacagcattctcatccgtagtacacctatgtcgtgacgtaaa
ttatggctgactaatccggaacatgcatgctaacggagcatctttcttctttatttgcatttaccttcatat
cggccgaggccttactacggctctttccttttataaagagacttgaaacgtcggcgtagtccttctcctact
agttatgatgactgcctttgtaggctatgtcctcccttgagggcagatgtctttttgaggagcaacagtaat
tacaaacctcctctcggcggtcccttacatgggtttggacctcgtcctatggttatgaggaggtttttcggt
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agccctattcgcccccaacttgttaggagaccccgacaacttcacggcggccaaccccctagtgactcctcc
acacattaagcctgagtgatacttcctgtttgcatacgctattctacgttccatccccaataaactcggtgg
tgtattagccctcctattctctatccttgtcctcatgctcgttccctttcttcatacctccaagcagcgagg
acttacatttcgaccccttcacacaattcctcttctgagccttggtcgcagatgtcgtcatcctgacctgaat
cgggggaatgccagtagaacacccatttat
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Capelin CytB PCR Primers:

```
CytB-F: cgctttccacttcatccttc
CytB-R: taggccgacagggttgttag
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Capelin CytB amplicon, 97bp:

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cgctttccacttcatccttcctttcattatcgctgcggcaaccgttgtgcacttacttttccttcatcaaac
aggctctaacaaccctgtcggccta
```

Agarose gel electrophoresis

Following real-time PCR, samples corresponding to (1) amplified positive control DNA or (2) amplified Capelin egg DNA were analyzed on a 3% agarose gel. A 100 bp DNA ladder was also analyzed to estimate amplicon size.

Conclusions

1. Mitochondrial CytB can be amplified from washed capelin eggs with no further sample prep.

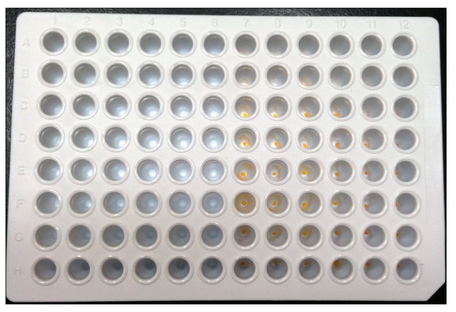
2. This workflow demonstrates the utility of an inhibitor-tolerant real-time supermix.

3. Such rapid, easy and inexpensive fish egg DNA analysis can benefit aquaculture breeding and stock identification.

4. This inhibitor-tolerant real-time supermix may be useful in other fields that can benefit from direct analysis of DNA from crude samples.

Illustrated workflow and results

Wash eggs, add to real-time PCR plate

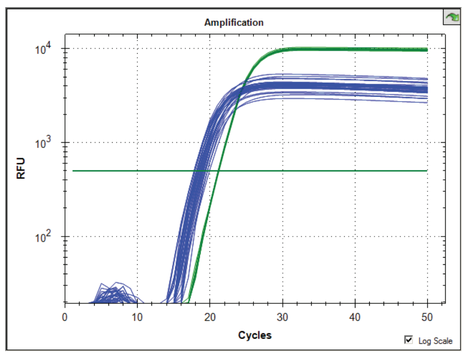


Individual Capelin eggs were added to wells of a qPCR plate (right half). Purified Capelin DNA (positive control) or TE (negative control) was added to wells on the left half of the plate

Real time PCR using SsoAdvanced Universal inhibitor-Tolerant SYBR Green Supermix

Amplification traces

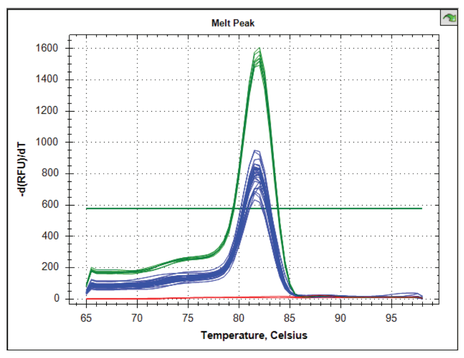
Purified DNA (8 out of 8 positive)
Whole egg (31 out of 32 positive)
Negative control (0 out of 8 positive)



DNA is amplified from single Capelin eggs over 95% of the time. We see amplification of the positive control (purifed caplelin DNA), but no amplification of the negative control.

Melt curve analysis

Purified DNA (8 out of 8 positive)
Whole egg (31 out of 32 positive)
Negative control (0 out of 8 positive)

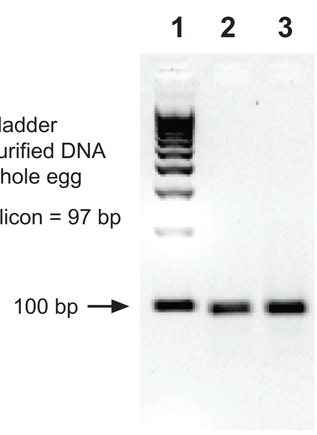


The DNA melt profile of the Capelin egg amplicon is similar to the positive control amplicon. This implies that the amplicons are identical.

Agarose gel electrophoresis

Lane 1: 100 bp ladder
Lane 2: CytB, purified DNA
Lane 3: CytB, whole egg

Expected size of CytB amplicon = 97 bp



Agarose gel electrophoresis shows that amplified DNA from the positive control sample and Capelin eggs are the expected size of the CytB amplicon (97 bp). This implies that CytB is being amplified from the Capelin eggs.

We conclude that by using inhibitor tolerant materials, DNA in single fish eggs can be analyzed by real-time PCR without sample prep.