qPCR detection of *Thelohania contejeani*, *A. astaci* and WSSv from tissue samples of noble crayfish

Tomas Jinnerot & Anna Aspán

The National Veterinery Insitute

Uppsala. Sweden

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SWEDISH LEGISLATION

It is not allowed to move or restock the wild with crayfish carrying infections with Thelohania or Psorospermium

- health monitoring must be performed before restocking
- including 25 individuals to be analysed for these infectious agents

and

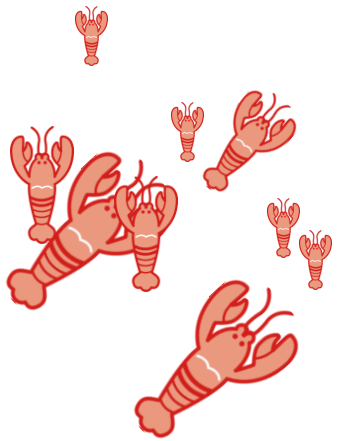
when mortalities are reported in wild crayfish populations

- crayfish plauge
- Thelohania
- Psorospermium

and, from this year

- WSSv

should be analysed for



THELOHANIA IS TIME CONSUMING TO SCREEN FOR BY HISTOLOGY

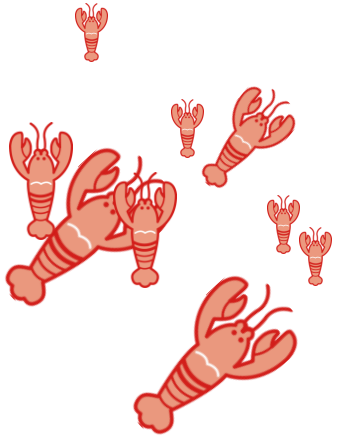
Although easily diagnosed in diseased animals. screening sensitivity in carrier animals is probably low

Foto: Thorbjörn Hongslo/SVA

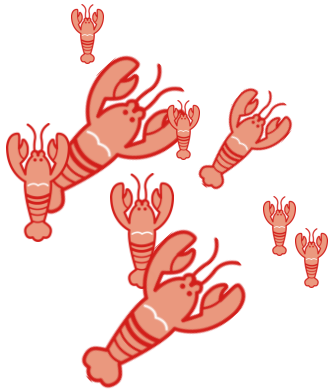
Flodkräfta med vit stjärtmuskulatur på grund av porslinssjuka *(Thelohania contejeani)*.

CHALLENGES……

- Combinations of tissue for best sensitivity also for other pathogens
- Little sequence data available
- Few “true” positive samples available for method validation



We are currently screening for *A astaci* and WSSv by qPCR



Could we use the same DNA sample also for Thelohania?

DNA extraction

- Ca 100mg tissue (ca. 5x5x5 mm)
- Bead-beating
- DNA extraction in robot

Different tissue is recommended for the different disease agents.

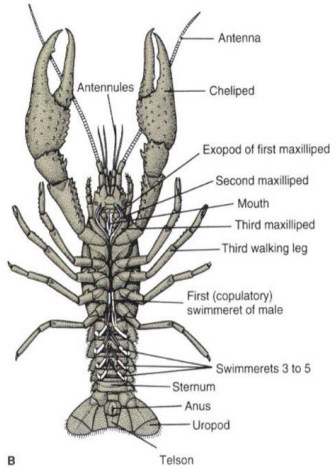
Will we lose sensitivity if combining these different tissues into one DNA extraction?

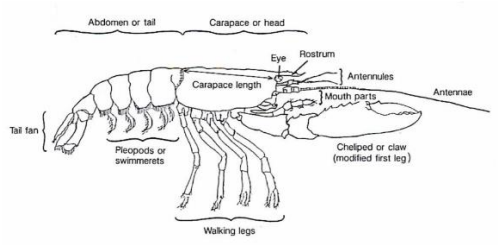
WSSv

Carapace soft tissue

pleopods

ventral abdomen skin

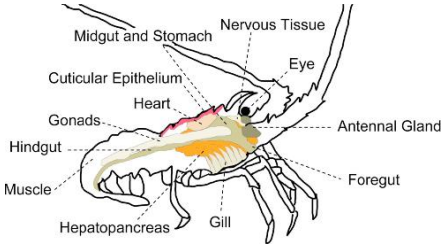




Psorospermium

Carapace soft tissue

Heart tissue

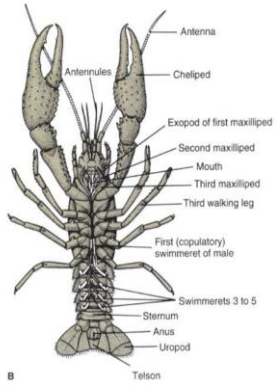


A astasic

Carapace soft tissue

ventral abdomen skin &

& muscel tissue



Thelohania

Muscle tissue from abdomen, back and tail

Composite sample

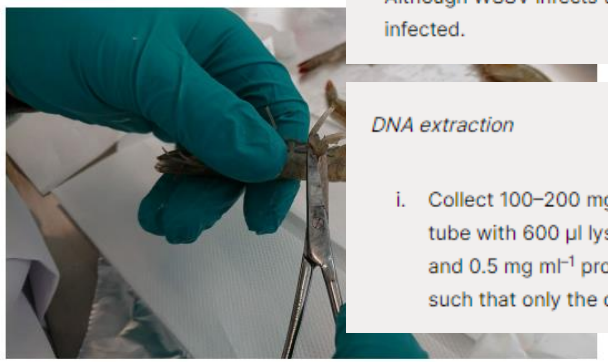
- Ventral abdomen skin
- Carapace soft tissue
- Pleopods
- Muscle tissue
- Piece of melanized shell – if precent

Choise of tissue – WSSv as an example

Samples of integumental epidermis, either dissected or contained within pleopods, or gills of the test animal must be fixed in 80 – 90% ethanol prior to the preparation of samples for PCR. Other samples,

EURL FOR FISH AND CRUSTACEAN DISEASES

DIAGNOSTIC METHODS AND PROCEDURES FOR THE SURVEIL… DETECTION… WITH WHITE… VIRU…



2.2.4. Target organs and infected tissue

The major target tissues of WSSV are of ectodermal and mesodermal embryonic origin, especially the cuticular epithelium and subcuticular connective tissues (Momoyama et al., 1994; Wu et al., 2013). Although WSSV infects the underlying connective tissue in the crustacean hepatopancreas and midgut, the tubular epithelial cells of these two organs are of endodermal origin, and they do not become infected.

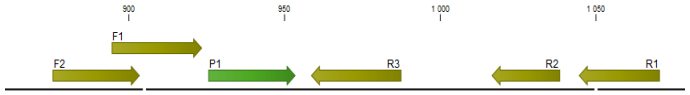
DNA extraction

i. Collect 100–200 mg shrimp tissue (pleopod of live juvenile to subadult shrimp, postlarvae 11 upwards [PL11 up] with removed heads, or whole PL10, or use 100 µl haemolymph) in a 1.5 ml microfuge tube with 600 µl lysis solution (100 mM NaCl, 10 mM Tris/HCl, pH 8, 25 mM EDTA [ethylene diamine tetra-acetic acid], 0.5% SLS [sodium N-laurylsarcosinate] or 2% SDS [sodium dodecyl sulphate], and 0.5 mg ml^{-1} proteinase K added just before use). For non-destructive screening, pleopods can be removed using red-hot forceps. For this procedure, the animal should be wrapped in a wet towel such that only the organ to be excised is left exposed.

CHAPTER 2.2.8.

INFECTION WITH WHITE SPOT SYNDROME VIRUS

Mix	Primer combinations	Amplicon size
TH 1	F1 + R1	174 bp
TH 2	F1 + R2	143 bp
TH 3	F1 + R3	92 bp
TH 4	F2 + R1	193 bp
TH 5	F2 + R2	162 bp
TH 6	F2 + R3	111 bp



Oligo	Sequence	Fluorophore	Target
Thelohania-F2	CATTTTTAGAAGTGAATATGAATGATRT		SSU rDNA
Thelohania-R3	TTTCATATATAACTCATTCAAATTCAAAA		111bp
Thelohania-P1 FAM	TGGTGCATGGCCGTTAACAATACGTGAT	FAM/ZEN/IBFQ	

Probe from IDT
With efficent black hole quenching ZEN™/Iowa Black™ FQ

Real-time PCR

PCR-systemet developed by Tomas Jinnerot. SVA

20x Primer/probe-mix pre-prepared from 100µM oligo solutions

PerfeCta qPCR Toughmix	7.5µl
20× Primer/prob-mix	0.75µl
Nukleasfritt vatten	4.75µl

13µl master mix + 2µl DNA

Temp	Time	Cycles
50°C	10 min	×1
95°C	3 min	×1
95°C	3 sek	×45
60°C	30 sek	

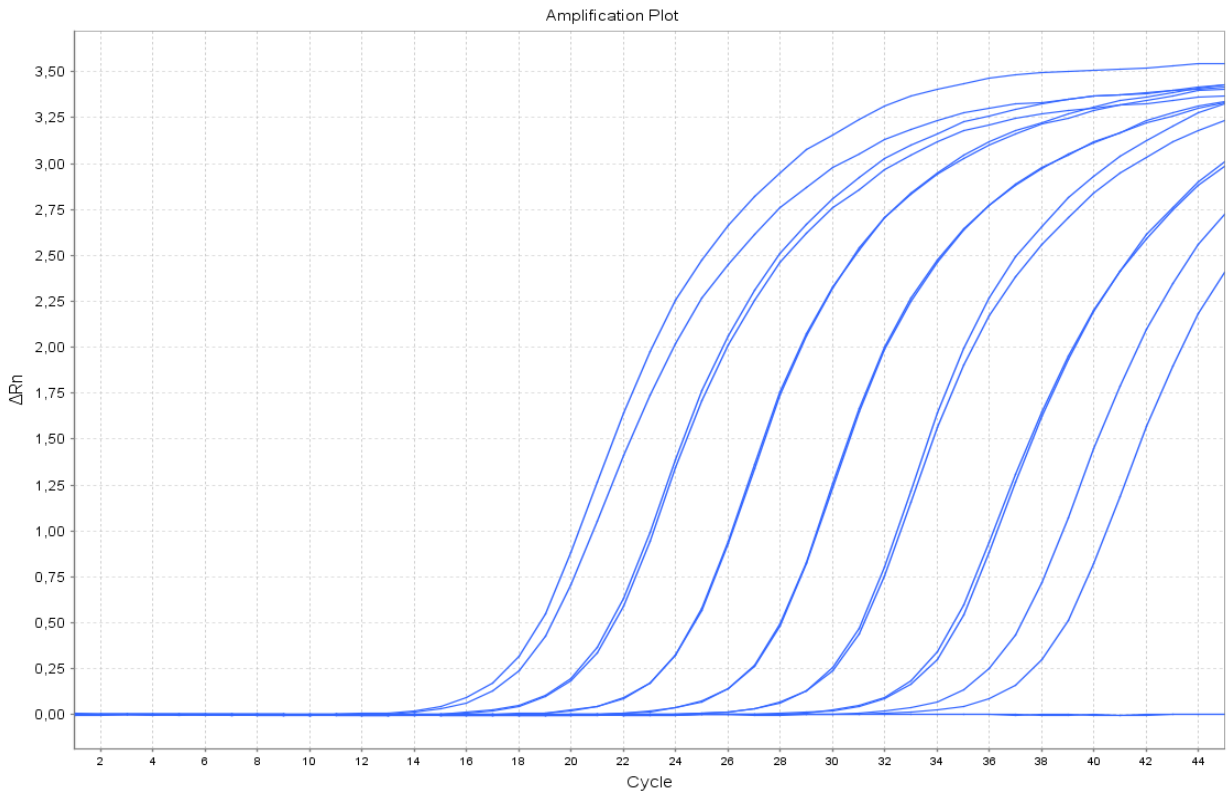
Tabell 1. BLAST primers and probe

Primer BLAST visar att primrarna skulle amplifiera & proben detektera följande sekvenser:	
>MF344630.1	Thelohania contejeani clone 1 18S ribosomal RNA gene and internal transcribed spacer 1. partial sequence
>MF344633.1	Thelohania contejeani clone 2 18S ribosomal RNA gene and internal transcribed spacer 1. partial sequence
>MF344631.1	Thelohania contejeani clone 3 18S ribosomal RNA gene and internal transcribed spacer 1. partial sequence
>MF344632.1	Thelohania contejeani clone 4 18S ribosomal RNA gene and internal transcribed spacer 1. partial sequence
>AM261747.1	Thelohania contejeani 16S rRNA gene (partial). 23S rRNA gene (partial) and ITS1. isolate Tcc3APb
>AF492594.1	Thelohania contejeani isolate TcC3 small subunit ribosomal RNA gene. partial sequence
>AF492593.1	Thelohania contejeani isolate TcC2 small subunit ribosomal RNA gene. partial sequence
Följande sekvens är kort & innehåller endast ena primern & proben. Båda matchar till 100%	
>AF303105.1	Thelohania contejeani small subunit ribosomal RNA gene. partial sequence
BLAST visar att denna sekvens skulle amplifieras av primrarna & detekters av proben. Sekvensen tillhör en icke-klassificerad mikrosporidie*	
>AJ438963.1	Microsporidium sp. JES2002H macronuclear 16S rRNA gene. 23S rRNA gene (partial) and ITS1. isolate T6
Varken Thelohania parastaci. T. montirivulorum eller andra mikrosporidier amplifieras av primrarna med Primer-BLAST	

*AJ438963.1 show high identity with *T. contejeani* (>97%) while the identity with other microspridians is significantly lower (<89%) thus, this sequence is probably also from a *T. contejeani* speciment.

Tabell 2. Ct from dilutions

	Dilution series 1		Dilution series 2	
dilutions	Ct	Ct	Ct	Ct
Un-diluted	16.1	16.7	16.1	16.6
1:10	18.1	17.9	19.0	18.9
$1:10^2$	21.3	21.1	22.2	22.1
$1:10^3$	24.5	24.5	25.5	25.5
$1:10^4$	28.2	27.9	28.6	28.6
$1:10^5$	31.4	31.2	32.0	32.2
$1:10^6$	34.3	34.2	36.2	34.5
$1:10^7$	36.2	37.3	-	-

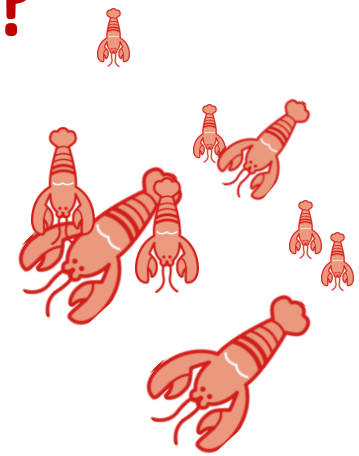


Sample ID	Material	Comments	qPCR results	seq PCR results	Sequencing results	Previous results
2002 nr 57	pleopod	kept at -20 C	-	-	nd	Thelohania sp. positive
2002 nr 57	pleopod	kept at -20 C	-	-	nd	Thelohania sp. positive
2002 nr 57	mage	kept at -20 C	-	-	nd	Thelohania sp. positive
2002 nr 57	lever	kept at -20 C	-	-	nd	Thelohania sp. positive
2008 nr 14	not given	glycerol at RT	-	-	nd	Thelohania sp. positive
2008 nr 14	not given	Davidsons at RT	-	-	nd	Thelohania sp. positive
2008 nr 14	not given	70% EtOH at RT	14.8	+ (260 bp)	T. contejeani	Thelohania sp. positive
2008 nr 14	not given	N-formalin at RT	-	-	nd	Thelohania sp. positive
2011 nr 15	not given	kept at -20 C	-	-	nd	Thelohania negative
2011 nr 8	lever	kept at -20 C	-	-	nd	Thelohania negative
2020 nr 1	Tissue for A.astaci PCR	kept at -20 C	-	-	nd	A. astaci & WSSV negative
2020 nr 2	Tissue for A.astaci PCR	kept at -20 C	-	-	nd	A. astaci & WSSV negative
2020 nr 3	Tissue for A.astaci PCR	kept at -20 C	-	-	nd	A. astaci & WSSV negative
2020 nr 4	Tissue for A.astaci PCR	kept at -20 C	-	-	nd	A. astaci & WSSV negative
2020 nr 5	Tissue for A. astaci PCR	kept at -20 C	37.6	-	nd	A. astaci & WSSV negative
2020 nr 5	Back muscle		40.4	nd	nd	
2020 nr 5	gut		43.3	nd	nd	
2020 nr 5	gut diluted 1:10		-	nd	nd	
2020 nr 5	pleopod		33.5	+ (260 bp)	T. contejeani (pool of PCR products)	
2020 nr 5	claw		33.6	+ (260 bp)		
WSSV ringtest	pleopod	kept at -20 C	-	nd	nd	WSSV+
WSSV ringtest	pleopod	kept at -20 C	-	nd	nd	shrimp SPF
WSSV ringtest	pleopod	kept at -20 C	-	nd	nd	shrimp SPF
WSSV ringtest	pleopod	kept at -20 C	-	nd	nd	WSSV+
WSSV ringtest	pleopod	kept at -20 C	-	nd	nd	shrimp SPF
A. fennicus M6/1 DNA	DNA from culture	kept at -20 C	-	nd	nd	
A. astaci	DNA	kept at -20 C	-	nd	nd	
P. leniusculus	DNA	kept at -20 C	-	nd	nd	
P. neurophilia	DNA from zebrafish	kept at -20 C	-	nd	nd	

Could EURL help us with well charaterized tissue samples?

MTA =
means trouble ahead

samples analysed at EURL		Ct values
392/ITT/18.1	**tissue from *T. contejeani*-infected *A. pallipes***	
undiluted		17.75
diluted 1:10		20.95
diluted 1:100		24.46
negative contol		-
392/ITT/18.2	**tissue from *T. contejeani*-infected *A. pallipes***	
undiluted		18.67
diluted 1:10		22.24
diluted 1:100		25.66
negative control		-



EURL used the primer / probe developed.
but with different chemistry and PCR instrument

Conclusions

- The new qPCR was shown to be specific to *Thelohania contejeani* both bioinformatically and experimentally.
- no cross-reactivity with other crayfish pathogens such as *Ahpanomyces astaci*. WSSv. *Psorospermium* or with DNA from signal- or noble crayfish was found
- no cross-reactivity with *Pseudoloma neurophilia,* a different microsporidium.

The availability of positive reference material was very sparse

but

- samples identified by real-time PCR could be verified by conventional PCR and sequencing.
- known positive samples from *Austropotamobius pallipes* were tested positive at EURL

- The efficiency of the PCR system was within acceptable range
- The method meets our validation criteria
- It will be used and further evaluated during 2021

F. Quaglio et al.: Knowl. Managt. Aquatic Ecosyst. (2011) 401, 27

Figure 2
A. pallipes *infected with* T. contejeani *with typical signs of "porcelain disease".*

Future collaborations??

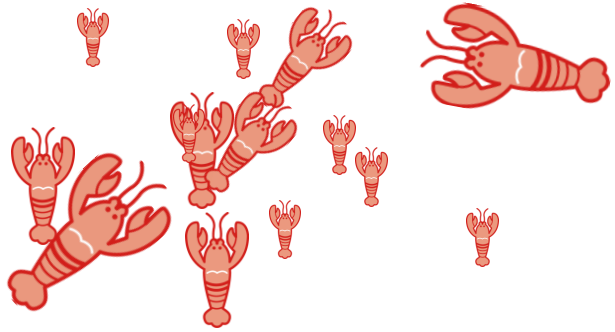
it would be very beneficial if someone would share

well characterized samples

or

test the method and share results with us

tomas.jinnerot@sva.se



anna.aspan@sva.se