qPCR detection of *Thelohania contejeani* and its application on clinical samples of *Austropotamobius pallipes* complex and other NICS

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

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The National Veterinery Insitute
Uppsala, Sweden

2021-06-02

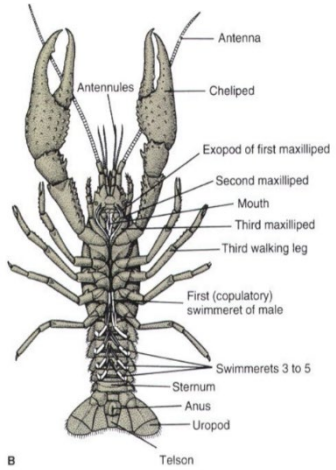
(photos Thorbjörn Hongslo).

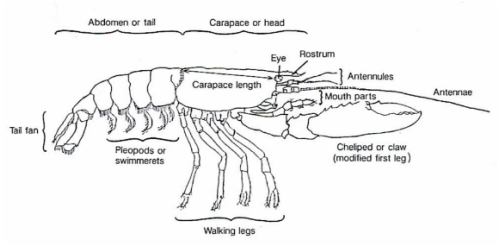
WSSV

Carapace soft tissue

pleopods

ventral abdomen skin

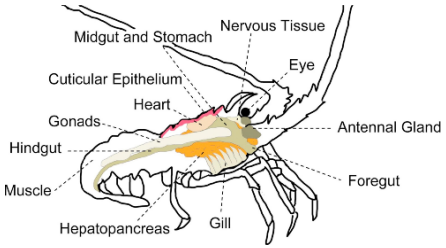




Psorospermium

Carapace soft tissue

Heart tissue

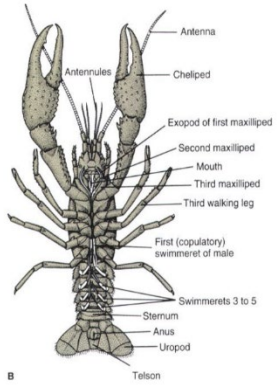


A. astaci

Carapace soft tissue

ventral abdomen skin &

& muscel tissue



Thelohania

Muscle tissue

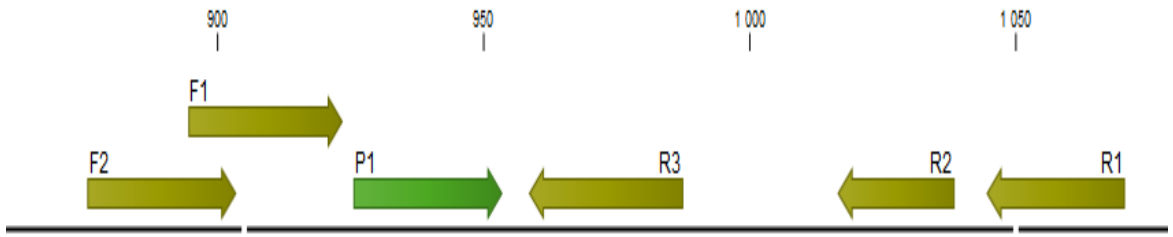
from abdomen,

back and tail

Composite sample

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

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



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

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

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	

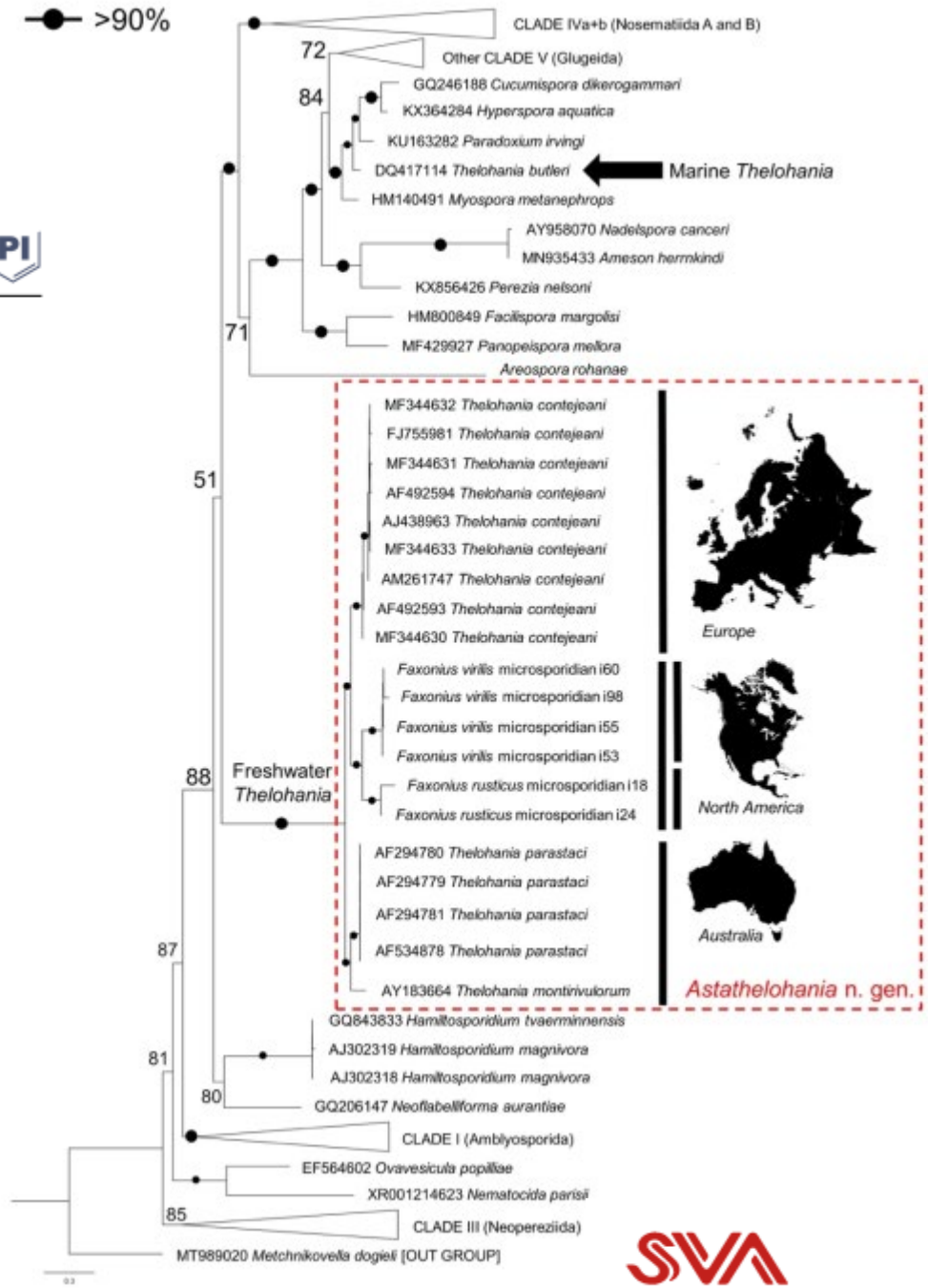
microorganisms

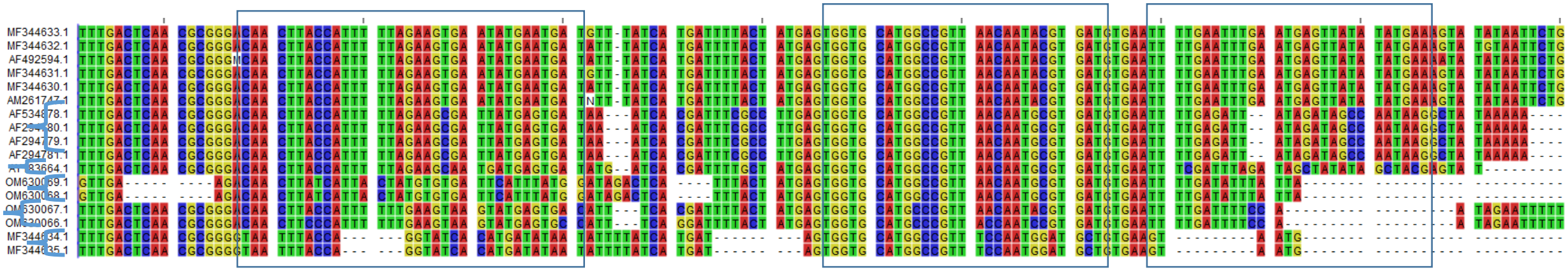
MDPI

Article

Revising the Freshwater *Thelohania* to *Astathelohania* gen. et comb. nov., and Description of Two New Species

Cheyenne E. Stratton [1], Lindsey S. Reisinger [1], Donald C. Behringer [1,2] and Jamie Bojko [3,4,*]





Astathelohania

Thelohania contejeani

Thelohania parastaci

Astathelohania montirivulorum

Astathelohania virili n. sp.

Astathelohania rusti n. sp.

Vairimorpha austropotamobii

Future collaborations??

it would be very beneficial if someone would share

well characterized samples

or

test the method and share results with us

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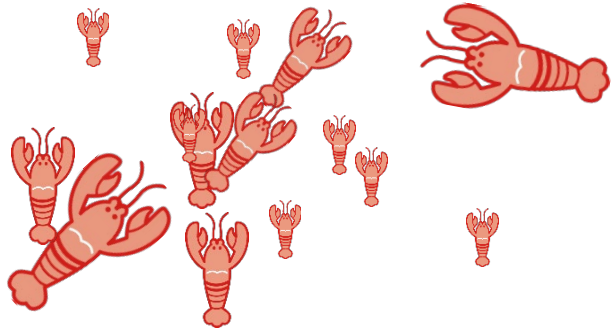


anna.aspan@sva.se

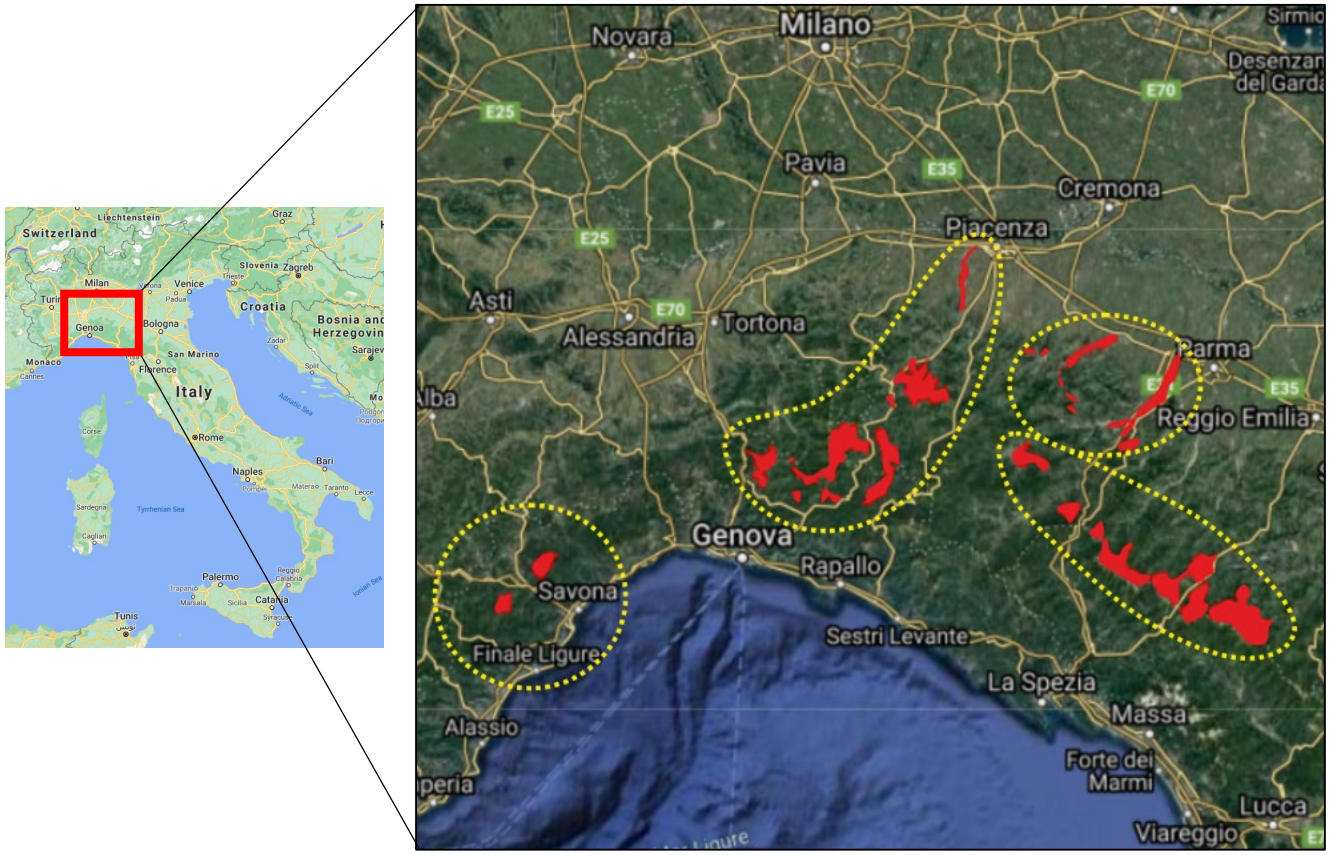
Monitoring of crayfish populations in Northern Italy

IZSVe is monitoring crayfish populations in Italy since 2012 to detect *T. contejeani* by macroscopic observation, followed by characterization through:

- histology
- citology
- molecular biology

LIFE18 NAT/IT/000806 - LIFE+ CLAW

- European LIFE project
- Start 10.2019 and end 09.2024



Evaluation of *T. contejeani* qPCR with IZSVe samples

Samples choice: *Austropotamobius pallipes* complex and NICS (44 crayfish specimens)

↓

DNA extraction from abdominal muscle tissue (QIAamp DNA Mini Kit - QIAGEN)

↓

Amplification on Bio-Rad CFX96

↓

Comparison with our previous results (molecular and histological analyses)

Crayfish species	Samples tested
Austropotamobius pallipes complex	34
Pacifastacus leniusculus	5
Cambaroides japonicus	2
Procambarus clarkii	2
Orconectes limosus	1

Previous analyses performed on IZSVe samples

- 35 of the samples tested by qPCR were previously characterized using two end-point PCR methods specific for *T. contejeani*, targeting the SSU rRNA gene:

El-Matbouli and Soliman, 2006

T. contejeani- specific primers	
F3	5'-AGCTAGTATGTAGGGTAAGGGC-3'
B3	5'-ACTCTTGGAGCTGGAATTACCG-3'

PCR product:

261 bp

Imhoff et al., 2010

Generic microsporidian outer primers	
V1f	5'-CACCAGGTTGATTCTGCCTGAC-3'
1492r	5'-GGTTACCTTGTTACGACTT-3'

Weiss et al., 1994

T. contejeani- specific inner primers	
MIC5-1	5'-ATAACAGGTCAGTGATGCCCT-3'
MIC3-4	5'-ACCCTAATATCCATCTGAGA-3'

PCR product:

215 bp

Sanger sequencing

32 positive and 3 negative samples

Previous analyses performed on IZSVe samples

- We also tested by qPCR 9 samples not previously characterized by *T. contejeani* - specific PCR

Sample ID	Host	Histological examination	PCR (Weiss et al., 1994)
39/ITT/22 27	*C. japonicus*	*Undescribed microsporidia*	Undescribed microsporidia
39/ITT/22 28	*C. japonicus*	*Undescribed microsporidia*	Undescribed microsporidia
39/ITT/22 31	*A. pallipes* complex	Microsporidiosis referable to *Nosema austropotamobii*	*N. austropotamobii* (100% Identity)
39/ITT/22 32	*A. pallipes* complex	Microsporidiosis referable to *Nosema austropotamobii*	not analysed
39/ITT/22 46	*P. leniusculus*	not analysed (macroscopically negative for microsporidia)	not analysed
39/ITT/22 47	*P. leniusculus*	not analysed (macroscopically negative for microsporidia)	not analysed
39/ITT/22 48	*P. clarkii*	not analysed (macroscopically negative for microsporidia)	not analysed
39/ITT/22 49	*P. clarkii*	not analysed (macroscopically negative for microsporidia)	not analysed
39/ITT/22 50	*O. limosus*	not analysed (macroscopically negative for microsporidia)	not analysed

qPCR results

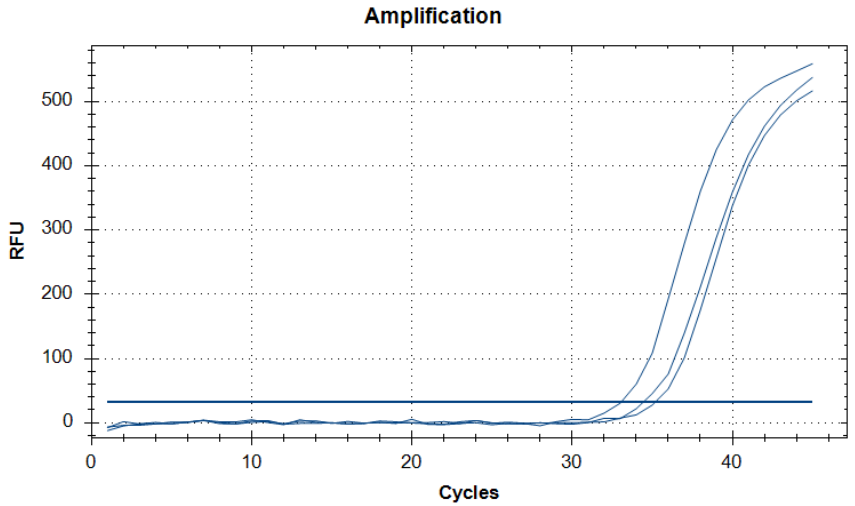
qPCR results: 36 positive (Ct range 8 - 37) and 8 negative samples.

Out of the 35 samples previously characterized by end-point PCR:

- All our 32 positive samples were detected correctly by the qPCR
- Our 3 negative samples were positive by qPCR

➡ Higher sensitivity

Sample ID	Crayfish species	Histological examination	qPCR results (Ct)	End-point PCR (El-Matbouli and Soliman, 2006; Imhoff et al., 2010)
39/ITT/22 11	*A. pallipes* complex	not analysed	34.48	-
39/ITT/22 18	*P. leniusculus*	no visible microsporidia	33.1	-
39/ITT/22 19	*P. leniusculus*	no visible microsporidia	35.23	-



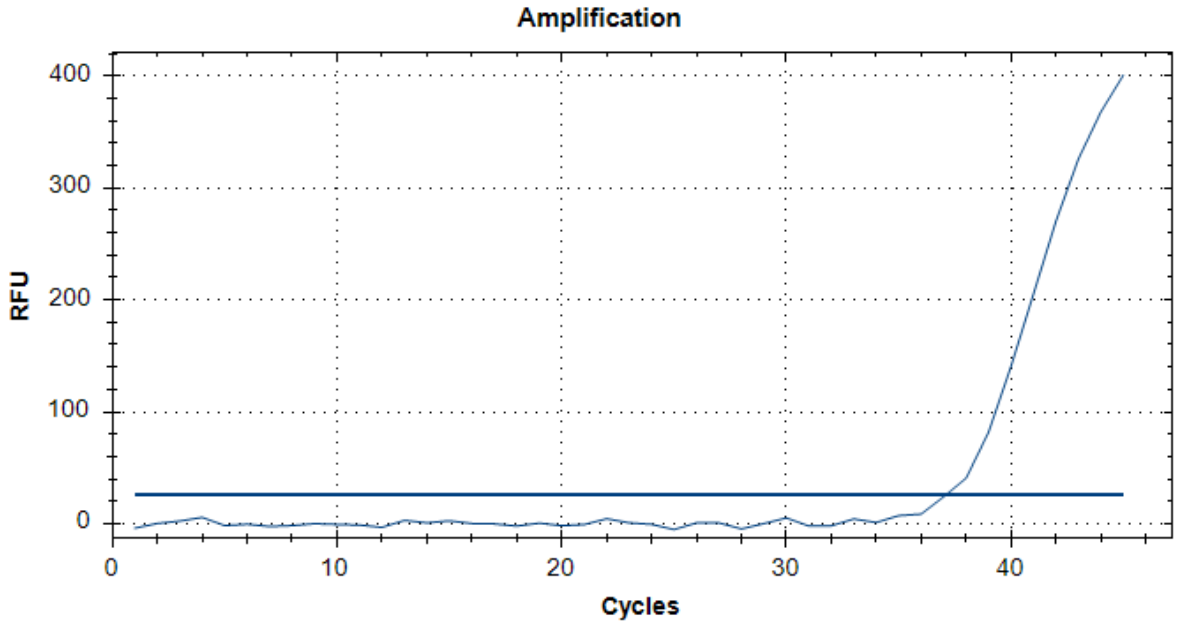
⬇

Samples sent to Sweden for further analyses

qPCR results

- About the 9 samples not previously characterized by *T. contejeani* - specific PCR:
  - 8 were completely negative for *T. contejeani* by qPCR
  - 1 was positive for *T. contejeani* by qPCR (Ct value: 37)

39/ITT/22.32: *A. pallipes* positive for *N. austropotamobii* ➡ co-infection



Co-infection of *T. contejeani* and *N. austropotamobii*

- In Italy similar macroscopic clinical signs can be produced by *Nosema* (*Vairimorpha) austropotamobii*.

Pretto et al. 2018. *Austropotamobius pallipes* complex severely affected by: A. *T. contejeani;* B. *N. austropotamobii*

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A formal redefinition of the genera *Nosema* and *Vairimorpha* (Microsporidia: Nosematidae) and reassignment of species based on molecular phylogenetics

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[e] Department of Environmental Science, The Connecticut Agricultural Experiment Station, 123 Huntington Street, New Haven, CT 06511, USA

- Recent evidence of co-infection in the same host
- To detect *N. austropotamobii* we used generic microsporidian primers from Weiss et al., 1994

Generic microsporidian outer primers	
V1f	5'-CACCAGGTTGATTCTGCCTGAC-3'
1492r	5'-GGTTACCTTGTTACGACTT-3'

SSU rRNA gene

T. contejeani	***N. austropotamobii***
1267 - 1326 bp	1102 - 1165 bp

Co-infection of *T. contejeani* and *N. austropotamobii*

- Out of the samples considered, we had 9 samples with confirmed co-infection. The qPCR detected *T. contejeani* correctly in all of them.

Sample ID	Host	Histological examination	*T. contejeani* specific PCR (El-Matbouli and Soliman, 2006)		Generic microsporidian primers (Weiss et al., 1994)		*T. contejeani* qPCR
			Gel	Sequencing (% Identity)	Gel	Sequencing (% Identity)	Ct
39/ITT/22 16	*A. pallipes* complex	Microsporidiosis referable to *N. austropotamobii* & *T. contejeani*	+	*T. contejeani* (100%)	+	*N. austropotamobii* (100%)	28.78
39/ITT/22 23	*A. pallipes* complex	Microsporidiosis referable to *N. austropotamobii*	+	*T. contejeani* (100%)	+	*N. austropotamobii* (100%)	31.16
39/ITT/22 24	*A. pallipes* complex	Microsporidiosis referable to *N. austropotamobii*	+	*T. contejeani* (100%)	+	*N. austropotamobii* (99.91%)	29.65
39/ITT/22 25	*A. pallipes* complex	Microsporidiosis referable to *N. austropotamobii*	+	*T. contejeani* (100%)	+	*N. austropotamobii* (100%)	28.16
39/ITT/22 26	*A. pallipes* complex	Microsporidiosis referable to *N. austropotamobii*	+	*T. contejeani* (100%)	+	not sequenced	28.69
39/ITT/22 30	*A. pallipes* complex	Microsporidiosis referable to *N. austropotamobii* & *T. contejeani*	+	*T. contejeani* (100%)	+	*N. austropotamobii* (99.91%)	25.97
39/ITT/22 37	*A. pallipes* complex	Microsporidiosis referable to *N. austropotamobii* & *T. contejeani*	+	*T. contejeani* (100%)	+	*N. austropotamobii* (100%)	26.81
39/ITT/22 39	*A. pallipes* complex	Microsporidiosis referable to *N. austropotamobii* & *T. contejeani*	+	not sequenced	+	*N. austropotamobii* (99%)	29.5
39/ITT/22 41	*A. pallipes* complex	not analysed (macroscopically positive for microsporidia)	+	*T. contejeani* (100%)	+	*N. austropotamobii* (100%)	32.15

- Specificity:
  - DNA from 1 *A. pallipes* (39/ITT/22.31) highly infected with *N. austropotamobii* (confirmed by sequencing)
  - Bioinformatic analysis

→ completely negative by qPCR

Conclusions: sensitivity and specificity

Based on both bioinformatic analysis and experimental testing performed on DNA extracted from crayfish **abdominal muscle tissue**:

- This qPCR is more sensitive than the two end-point methods considered (El-Matbouli and Soliman, 2006 and Imhoff et al., 2010)
- Detection of *T. contejeani* was successful also in specimens with confirmed coinfection of *T. contejeani* and *N. austropotamobii*
- Specificity of primers and probe was confirmed obtaining negative results on at least one specimen positive for *N. austropotamobii* and two specimens positive for other microsporidia

Conclusions: future developments

- Further analyses are needed to verify the sensitivity and specificity of this method on DNA extracted from:
  - environmental samples
  - complex matrices

- Development of a species-specific PCR/qPCR for the detection of *Nosema austropotamobii* is ongoing

- Future collaborations: are you interested in sharing samples?

Thank you for your attention

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LIFE18 NAT/IT/000806 – LIFE+ CLAW

- European LIFE project
- Start 10.2019 and end 09.2024
- Co-financed by EU for the 60%
- Total value: 3,711,742 €
- 10 Partners