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Development of species-specific eDNA-based test systems for monitoring of freshwater crayfish

Norwegian Institute for Water Research

REPORT

Main Office
Gaustadalléen 21
NO-0349 Oslo, Norway
Phone (47) 22 18 51 00

NIVA Region South
Jon Lilletuns vei 3
NO-4879 Grimstad, Norway
Phone (47) 22 18 51 00

NIVA Region East
Sandvikaveien 59
NO-2312 Ottestad, Norway
Phone (47) 22 18 51 00

NIVA Region West
Thormøhlensgate 53 D
NO-5006 Bergen Norway
Phone (47) 22 18 51 00

NIVA Denmark
Njalsgade 76, 4th floor
DK 2300 Copenhagen S, Denmark
Phone (45) 39 17 97 33

Internet: www.niva.no

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Steen W. Knudsen, NIVA Denmark Sune Agersnap, Department of Bioscience, Aarhus University Peter Rask Møller, Natural History Museum, University of Copenhagen Jesper H Andersen, NIVA Denmark	Fresh water biology	Public
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Summary

This MONIS 5 report focuses on freshwater crayfish and was funded by the Danish Environmental Protection Agency. The aim of the present study was to develop species-specific systems for tracking environmental DNA (eDNA) from nine species of freshwater crayfish in water samples. Among these nine species, only one (*Astacus astacus*) can be considered indigenous in Scandinavia, and among the other eight non-indigenous species; two (*Astacus leptodactylus* and *Pacifastacus leniusculus*) are frequently encountered in Scandinavian freshwater systems. The remaining seven non-indigenous species are rarely encountered in Scandinavia but have been recorded in the past from other places in Europe. It is our aim that the nine species-specific eDNA assays presented here will allow for continuous monitoring of both the one indigenous species, the two non-indigenous species more frequently encountered and help produce early warnings of the seven non-indigenous species that might disperse to Denmark.

Four keywords	Fire emneord
1. Non-indigenous species	1. Ikke-hjemmehørende arter
2. eDNA	2. eDNA
3. Monitoring	3. Overvågning
4. Freshwater crayfish	4. Ferskvandskrebs

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Jesper Harbo Andersen
Project Manager

Nikolai Friberg
Research Manager

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Development of species-specific eDNA-based test systems for monitoring of freshwater crayfish

Preface

We report the development of 9 species-specific eDNA-based test systems for monitoring of freshwater crayfish. The work has been funded by the Danish Environmental Protection Agency as a spin-out activity from the MONIS project ('Monitoring of Non-Indigenous Species in Danish Marine Waters') and been carried out collectively by NIVA Denmark (lead partner), the Natural History Museum of Denmark and University of Aarhus.

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More information about the MONIS project cluster (phase 1-5) can be found in:

- 'Steps toward nation-wide monitoring of non-indigenous species in Danish marine waters under the Marine Strategy Framework Directive' by Andersen *et al.* (2016),
- 'Development of species-specific eDNA-based test systems for monitoring of non-indigenous species in Danish marine waters' by Andersen *et al.* (2018),
- 'Tekniske anvisninger for eDNA-baseret overvågning af ikke-hjemmehørende marine arter' (in Danish) by Knudsen et al. (2018), and
- 'A baseline study of the occurrence of non-indigenous species in Danish harbours' by Andersen *et al.* (2019).

Copenhagen, 11 December 2019

Jesper H. Andersen

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For at kunne spore både hjemmehørende og ikke-hjemmehørende arter af ferskvandskrebs, ved hjælp af DNA niveauer i filtrerede vandprøver, er der for denne rapport udviklet og testet ni arts-specifikke sporingssystemer.

Ved brug af kvantitativ PCR (polymerase chain reaction) (qPCR) er det med disse ni systemer muligt at spore DNA i vandprøver fra ni arter af ferskvandskrebs, og det er muligt at vurdere niveauerne af DNA i vandprøverne fra de enkelte arter af ferskvandskrebs. Alle ni sporingssystemer er blevet designet og testet både på DNA fra vævsprøver fra den eftersøgte art, men også på DNA fra andre sameksisterende ferskvandskrebs.

Alle ni sporingssystemer er her eftervist som værende artsspecifikke og i stand til at spore DNA fra hver af de ni arter af ferskvandskrebs. Specificiteten for hvert sporingssystem er eftervist med resultater fra sammenligning af nukleotid sekvenser hvor de enkelte primere og prober binder og med qPCR tests af forskelige kombinationer af primere og prober.

Sammenligningen med nukleotid sekvenser fra andre arter af krebs blev udført ved at identificere variable gen regioner i det mitokondrielle gen: cytochrome oxidase 1 (mtDNA-co1). Nukleotid sekvenser af mtDNA-co1 blev enten indhentet fra en genetisk database eller indhentet ved *de novo*-sekventering af DNA ekstraheret fra vævsprøver indsamlet fra de ni arter af ferskvandskrebs.

Blandt de ni sporingssystemer, som der er testet, er der for hvert art udvalgt et sporingssystem der er artsspecifikt, men samtidig også er sensitivt for lave niveauer af miljø-DNA.

1 Introduction

Native crayfish in Europe and thus Scandinavia are threatened by the introduction of non-indigenous crayfish species (Agersnap *et al*., 2017; Strand *et al*., 2019; Manfrin *et al*., 2019; Wittwer *et al*., 2019), and its companion the lethal crayfish plague (Vrålstad *et al*., 2009). Because of this invasion the Indigenous crayfish populations in Europe has been reduced considerably. The number of non-indigenous crayfish species in Europe now exceeds the number of indigenous crayfish species twofold. Early detection is crucial when it comes to stopping or reducing a new invasion. Monitoring the distribution and prevalence of both native and non-indigenous species of crayfish in Danish freshwater systems filtering water and evaluating levels of environmental DNA (eDNA) offers an alternative and potentially also cheaper approach for mapping the distribution and occurrence of crayfish in Denmark. In the wake of recent studies (Agersnap *et al*., 2017; Strand *et al*., 2019; Atkinson *et al*., 2019) this MONIS 5 study aims at developing and testing species-specific primer- and probe assays for nine species of crayfish that can occur in Northern European freshwaters. The non-indigenous species are listed on the union list of alien invasive species under the Regulation (EU) 1143/2014 on invasive alien species or on the Danish national list of invasive alien species.

Before any of the species-specific primer- probe assays can be used to detect environmental DNA in filtered water samples in a quantitative PCR (qPCR) setup, the assays must be tested with specific positive outcome in two stages: The *in silico* test and the *in vitro* test. In the *in silico* test stage each of the specific primer- probe assay must be tested in the initial computer setup with comparison of known DNA sequences from similar gene regions from closely related co-occurring species and the same gene region for the target species. These gene regions can be obtained from gene bank databases, such as the National Center for Biotechnology Information (NCBI). If comparative gene regions are scarcely represented in gene bank databases, *de novo* sequencing on extracted DNA from closely related co-occurring species should be done prior to comparison of sequence data. Samples from natural history museum collections provide the possibility of re-validating species identification, and samples from such collections should be prioritized for any eventual *de novo* sequencing and for any match in an *in vitro* setup that is supposed to check if closely related sister species can give rise to false positive amplification. The subsequent *in vitro* test must include the *in silico* designed primers and probes and ensure their specificity in a qPCR setup performed on DNA extracted from the target species and DNA from potential co-occurring non-target species. The most closely related sister species co-occurring in the geographical area where the filtered water samples are to be collected must be included in such an *in vitro* test.

A qPCR *in vitro* test will help to show whether the developed primers and probe can unintentionally return false positive amplification on DNA from co-occurring sister species. In case an *in vitro* test results in positive amplification on DNA from co-occurring sister species, this can be a result of the gene region targeted and the primer binding sites have less than 5 base pair differences in difference. The species-specific primers and probes presented in this report do not cover species that were unknown or are without sequence data deposited on gene databases at the onset of this project. Once an *in vitro* test has been completed with a validated and positive result for the species-specific assay that only returns positive amplification in a qPCR setup for the sought species, the assay is regarded as being species-specific at operational level. It is, however, recommended that species-specific assays that are tested positive at operational level are further validated through *in vivo* or *in situ* tests. *In vivo* or *in situ* test will require that the species-specific assay can return positive amplification in a qPCR setup performed on filtered and extracted water samples collected from locations where the target species is known to be present.

2 Methods

All testing of species-specific assays has been performed in the same way, using the same setup for both PCR (Polymerase Chain Reactions) and qPCR (quantitative PCR). The protocols for *in silico* design of primers and *in vitro* testing of designed primers and probes follow the setup and protocols described by Agersnap *et al.* (2017) and Knudsen *et al.* (2019).

Tissue samples were obtained from museum specimens of crayfish, and DNA was extracted from these tissue samples using the DNeasy Blood and Tissue kit (Qiagene provider).

The resulting primer and probes are presented in the following tables and nine sections. The first tables (Table 2.1 and 2.2) provide a quick overview of the nine species targeted in this study and presents the *in silico* designed and *in vitro* tested primer- probe assays. The nine sections following these two tables present each species-specific primer- probe assay for each of the nine species of freshwater crayfish. For each assay developed and tested the mitochondrial gene sequences used for *in silico* design are listed with accession numbers for the GenBank National Center for Biotechnology Information (NCBI) records. For sample abbreviations without GenBank accession numbers museum tissue sample numbers are listed and refer to samples held the Natural History Museum of Denmark.

2.1 Conditions of the specificity test – *in silico* testing

All species-specific primer and probe assays obtained from literature search were compared in a DNA sequence alignment viewer. Sequence alignment was performed using the MAFFT v6.822 (Katoh & Toh, 2010) alignment algorithm accessible as a plugin in Geneious v. R7 (Kearse *et al.,* 2012). The *in silico* design was based on initial primer suggestions inferred from using Primer3 v.0.4.0 (Koressaar and Remm, 2007), and by matching primers against the NCBI GenBank database using Primer-BLAST (Ye *et al*., 2012). The *in silico* design protocol follows the tests described by Knudsen et al. (2019) and by Agersnap *et al*. (2017).

All species- specific assays developed and tested in this study were performed by aligning mtDNA sequences from de novo sequenced material obtained from NHMD and by downloading mtDNA sequences from National Center for Biotechnology Information (NCBI) GenBank.

For the species of crayfish where limited sequence data for the mitochondrial cytochrome oxidase 1 (mtDNA-co1) region was available on NCBI GenBank, *de novo* sequencing of the mtDNA-co1 region was performed with the forward primer: LCO1490: 5'-GGTCAACAAATCATAAAGATATTGG-3' and reverse primer: HC02198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' and (Folmer *et al.,* 1994) in a PCR setup with 25 µL reaction volume comprising forward and reverse primers (ordered through TAG Copenhagen A/S), using 1 µL forward and 1 µL reverse primer (with 10 µM initial concentrations per primer), and 0.1 µL 5U/µL AmpliTaq Gold Polymerase (Thermofisher, Applied Biosystems), 11.6 µL ddH_2O, 2 µL 25 mM $MgCl_2$ and 2 µL template DNA extracted from tissue samples and diluted 1:10 prior to usage. The amplified products were visualized with gel electrophoresis in 2% agarose gel stained with GelRed. This PCR setup is similar to the PCR setup 01 described by Knudsen *et al.* (2019) and is referred to as 'setup 01' in this study. Amplified products were purified with a Qiagen PCR purification kit (Qiagen, cat. No. 28106) and *de novo* Sanger sequenced using the sequencing service provided by Macrogen Europe (Amsterdam). Sanger sequencing was performed in both forward and reverse directions and resulting sequence reads were assembled and manually inspected in the software Geneious vR7.

Table 2.1. *Table of primer and probe qPCR detection systems developed by MONIS 5 invasive cray-fish with a summary of the final product. 'PM' indicates a probe modification. All oligos are written in a 5' -> 3' direction. The primers are named with a combination of an abbreviated genus name an abbreviated species name and the mitochondrial gene region that is targeted by the assay and a letter indicating whether it is a (F)orward, (R)everse or (P)robe, and an arbitrary number. The abbre-viated genus name follows previous taxonomy for the genera:* Pontastacus *(now synonymized with* Astacus*) and* Orconectes *(now synonymized with* Faxonius*). To be able to match primers and probes with already developed and tested reagents in this project, these primer and probe names have been retained for this report. These primer and probe names can be considered altered if these re-sults are to be published in peer reviewed scientific literature.*

No (1)	Species	Primer (forward and revers) and probe name	Sequence in 5'->3' direction with FAM and BHQ1 modifications indicated
Krebs_01	*Astacus astacus*	Astast_COI_F01	5'-CGATTTTAGGGGCGGTAAAT-3'
		Astast_COI_R01	5'-CACCTGCCAACACAGGTAGA-3'
		Astast_COI_P01	5'-FAM-TCGAATACCTCTTTTTGTTTGATCTGT-BHQ-1-3'
Krebs_02	*Pacifastacus leniusculus*	Paclen_CO1_F02	5'-TGTAGTCACGGCACATGCTT-3'
		Paclen_CO1_R01	5'-CCGCTGCTAGAGGAGGATAA-3'
		Paclen_CO1_P01	5'-FAM-AAAGAGGAGTGGGTACTGGATGAAC-BHQ1-3'
Krebs_03	*Astacus leptodactylus*	Ponlep_CO1_F03	5'-TTTGGGACTTGAGCAGGAAT-3'
		Ponlep_CO1_R03	5'-CTGGTTGTCCGAGTTCAACA-3'
		Ponlep_CO1_P03	5'-FAM-TGGGAACCTCTTTAAGAATAATTATTCG-BHQ-1-3'
Krebs_04	*Procambarus clarkii*	Procla_co1_F04	5'-GCGGGAGCATCTGTAGATTT-3'
		Procla_co1_R04	5'-ATAGCTCCTGCCAACACAGG-3'
		Procla_co1_P04	5'-FAM-ACGAACAGTAGGGATAACCATGGAT-BHQ1-3'
Krebs_05	*Procambarus fallax*	Profal_co1_F01	5'-AGTTGAGAGGGGAGTAGGAAC-3'
		Profal_co1_R01	5'-AGTTATACCAGCTGCCCGTA-3'
		Profal_co1_P01	5'-FAM-AACTGTTTATCCTCCTTTAGCTTCTGC-BHQ1-3'
Krebs_06	*Faxonius juvenilis*	Orcjuv_co1_F06	5'-CGGGAAGGTTAATTGGAGATGA-3'
		Orcjuv_co1_R09	5'-CCTGTTCCAACTCCTCTTTCTAC-3'
		Orcjuv_co1_P06	5'-FAM-TGGGGGATTTGGTAACTGGTTAATTCCT-BHQ1-3'
Krebs_07	*Faxonius limosus*	Orclim_co1_F03	5'-GTTGGGTCAGCTGGGAAGTT-3'
		Orclim_co1_R01	5'-GTCATTCCTGTGGCCCGTAT-3'
		Orclim_co1_P03	5'-FAM-TGGAGGATTTGGTAATTGGTTAATTCCT-BHQ1-3'
Krebs_08	*Faxonius rusticus (2)*	Orcrus_co1_F03	5'-CGGGAAGGTTAATTGGAGATGAC-3'
		Orcrus_co1_R02	5'-AAATCTACTGACGCCCCTGC-3'
		Orcrus_co1_P02	5'-FAM-ACAGTGTATCCTCCTCTCGCTTCTGCA-BHQ1-3'
Krebs_09	*Faxonius virilis*	Faxvir_co1_F05	5'-CAGGAAGATTGATTGGGGACGA-3'
		Faxvir_co1_R01	5'-GTTATCCCTGCAGCCCGTAT-3'
		Faxvir_co1_P01	5'-FAM-TTGGAGGTTTCGGGAACTGGCTGATTC-BHQ1-3'

1) The assay name and number are arbitrarily assigned for this report.

2) The primers and probes developed for detection of eDNA from Faxonius rusticus *are unable to distinguish between DNA from* Faxonius rusticus *and* Faxonius limosus *when the qPCR is run for more than 35 cycles. A cycle of quantification cut-off (Cq-cut-off) of 35 is required when this* Faxrus *assay is used.*

Table 2.2. *TS = Tissue sample collected and available for DNA-specificity test, NTS = Tissue sample tested in PCR and qPCR setup, level of specificity = the results from the Table 1.2 List of crayfish species in Danish freshwater habitats targeted for eDNA monitoring in the MONIS 5 project. Species-specific eDNA assays (primers and probes) have been developed and tested in a laboratory setup (in silico and in vitro testing) during the MONIS 5 project. 'Assay ready' indicates whether the assay can be considered ready for test at operational level - i.e. subsequent testing in ensuing project. TS = Target Species; NTS = Non-Target Species. 'At gl' indicates the assay is ready for use with specificity at genus level – i.e. the assay cannot discriminate between eDNA from different species within the listed genus. in vitro qPCR test on DNA extracted from tissue sample, Assay ready = the evaluation of the in vitro test, whether or not the assay can be applied for tests on water samples. NT= not tested.*

No(1)	Genus	Species	Danish commmon name	TS collected	NTS collected and tested(2)	Level of specificity	Assay ready
01	*Astacus*	*astacus*	Flodkrebs	Yes	Yes	species	Yes
02	*Pacifastacus*	*leniusculus*	Signalkrebs	Yes	Yes	species	Yes
03	*Astacus*	*leptodactylus*	Galizisk sumpkrebs	Yes	Yes	species	Yes
04	*Procambarus*	*clarkii*	Lousianna krebs	Yes	Yes	species	Yes
05	*Procambarus*	*fallax*	Marmorkrebs	Yes	Yes	species	Yes
06	*Faxonius*	*juvenilis*	Kentucky flodkrebs	Yes	Yes	species	Yes
07	*Faxonius*	*limosus*	Amerikans flodkrebs	Yes	Yes	Species	Yes
08	*Faxonius*	*rusticus*	Rustfarvet flodkrebs	Yes	Yes	species	Yes (3)
09	*Faxonius*	*virilis*	Viril flodkrebs	Yes	Yes	Species	Yes

1) The species number is an arbitrary number assigned through this report.
2) Whether non-target species have been collected refers to whether species from potentially co-occurring and evolutionary closely related species in Danish Seas have been collected, and if the assay has been tested on the Non-Target-Species. The 'NA' indicates that the species was unavailable for testing.
3) Tested in qPCR setup with the primer combination being species-specific below Cq=35. Amplification results from Cq>35 needs to be regarded as false positive signals, as they potentially can stem from a different invasive species of Faxonius.

The primers were designed by aligning sequences obtained from samples collected by Sune Agersnap and William B. Larsen:

- *Astacus astacus*: 151_76_4810
- *Faxonius immunis*: 151_70_Oroimm, 151_72_Oroimm142
- *Faxonius juvenilis*: 151_66_Faxjuv146, 151_67_Faxjuv137, 151_75_Faxjuv145
- *Faxonius limosus*: 151_68_Faxlim138, 151_71_Faxlim141
- *Pacifastacus leniusculus*: 151_80_5691
- *Pontastacus leptodactylus*: 151_77_4773, 151_78_4847, 151_79_4777
- *Procambarus clarkii*: 151_69_Procla139, 151_74_Procla144
- *Procambarus fallax*: 151_73_Profal143

With additional sequences obtained from NCBI GenBank:

- *Astacus astacus*: GU727619, JN254659-JN254681
- *Astacus leptodactylus*: MF288079-MF288086,
- *Austropotamobius italicus*: AY121127, HM622614

- *Austropotamobius pallipes*: AY667114-AY667115
- *Austropotamobius torrentium*: AY667128, AM180946
- *Cherax destructor*: KJ950555, KM039112
- *Faxonius limosus*: JF911554
- *Faxonius rusticus*: AY701249, KX238168
- *Orconectes immunis*: JF438005-JF438006
- *Orconectes juvenilis*: AF474352, AY701233, JF437985, KT282396-KT282407, KT282419-KT282428
- *Orconectes limosus*: JF437992-JF437993
- *Orconectes rusticus*: AY701248-AY701249
- *Orconectes virilis*: FJ608577, EU442743
- *Pacifastacus gambelii*: KF827994-KF827995
- *Pacifastacus leniusculus*: AF525226-AF525227, MF288087, JF437999, JF437995-JF437998, JF438000
- *Procambarus fallax*: HM358011, KF033123

All assays were tested under the same PCR and qPCR setups. The DNA template added varied from assay to assay to make the target species different. All primers tested and inferred with Primer3 v.0.4.0 (Koressaar & Remm, 2007) are listed for each species of crayfish.

2.2 Conditions of the specificity test – *in vitro* testing

DNA from reference tissue samples were either supplied from external sources (Table 2.3) or extracted from tissue samples using the DNeasy Blood and tissue kit (Qiagen) according to manufacturer's specifications.

Initial primer specificity, test of annealing temperature and primer concentration was performed in a 25 µL reaction volume comprising forward and reverse primers (ordered through TAG Copenhagen A/S), using 1 µL forward and 1 µL reverse primer (with 10 µM initial concentrations per primer), and 0.1 µL 5U/µL AmpliTaq Gold Polymerase (Thermofisher, Applied Biosystems), 11.6 µL ddH$_2$O, 2 µL 25 mM MgCl$_2$ and 2 µL template DNA extracted from tissue samples and diluted 1:10 prior to usage. This initial PCR was performed on various combinations of the primers designed for the mitochondrial gene region targeted. This is similar to the PCR setup 01 described by Knudsen et al. (2019) and is referred to as 'PCR setup 01' in the present study as well. For each species, different primer combinations were tested to ensure they could amplify the targeted mitochondrial gene region in DNA extracted from tissue from the targeted species. These PCR setups were performed on both DNA extracted from tissue from the target species, as well as on DNA extracted from other non-target species. For the primer combinations that returned species-specific amplification species-specific FAM-BHQ1 modified probes were ordered, to allow for subsequent testing of specificity against the gene region in the targeted species in a qPCR setup. This qPCR setup is similar to setup 02 described by Knudsen *et al.* (2019). The qPCR was setup to test the different primers and the probe on DNA extracted from tissue from both target species and from non-target species. The primer and probe combinations that returned only species-specific amplification were selected as the species-specific assays to use in future assessments of eDNA levels from freshwater crayfish.

qPCR reactions were run on a Stratagene Mx3005P qPCR Machine (Agilent, Santa Clara, California, United States). Primer probe specificity test was run using 1 µL forward and 1 µL reverse primer (with 10 µM initial concentrations per primer) and 1 µL probe (with 2.5 µM initial concentration) in a 25 µL reaction volume, including 10 µL Applied Biosystems TaqMan Environmental Mastermix 2.0 (Thermo Fisher Scientific, Waltham, Massachusetts, United States), 10 µL ddH$_2$O and 2 µL 1:10 diluted tem-

plate DNA from tissue extractions, ranging in concentrations of DNA between 50 ng/mL and 20000 ng/mL. Target- and non-target species were run in duplicate reactions and two negative controls. All data obtained from the qPCR setups were exported as Excel files from the Mx3005 P software, and analysed in R v3.3 (R Core Team, 2016) using the packages: "ggplot2" (Wickham, 2016), "pdp" (Greenwell, 2017) and "readxl" (Wickham and Bryan, 2017).

Table 2.3. *Species used for in silico design of primers and probes and any eventual tissue samples obtained from the Natural History Museum of Denmark and any eventual mitochondrial DNA sequences obtained from databases.*

Species	Museum catalog number (1)	Accession number(2)	Abbr code
Astacus astacus	4810	GU727619	Astast
Astacus leptodactylus	4773	AF525228	Astlep
Cherax destructor	NA	HG799087	Chedes
Cherax quadricarinatus	NA	NA	Chequa
Cherax quinquecarinatus	NA	NA	Chequi
Faxonius immunis	Oroimm142	NA	Oroimm
Faxonius juvenilis	Faxjuv146	AF474352	Orojuv
Faxonius limosus	Faxlim138	JF437992	Orolim
Faxonius rusticus	Faxrus189	AY701248	Faxrus
Faxonius virilis	Faxvir187	NA	Faxvir
Pacifastacus fortis	NA	NA	Pacfor
Pacifastacus leniusculus	5691	AF525226	Paclen
Procambarus clarkii	Procla139	JN000900	Procla
Procambarus fallax	Profal143	HM358011	Profal

1) The museum catalog number refers to the invertebrate collection at the Zoological Museum at the University of Copenhagen and samples collected by S.W. Knudsen, W.B. Hansen and S. Agersnap.
2) The accession numbers refer to sequences obtained from the National Center for Biotechnology Information database.

3 Results

The species-specific assays are listed for each species with alignments and amplification curves obtained from each of the qPCR tests performed.

3.1 Species no. krebs_01: *Astacus astacus*

Binomial nomenclature and author: *Astacus astacus* (Linnaeus, 1758) – see figure 3.1.
English common name: Noble crayfish
Danish common name: Europæisk flodkrebs

Figure 3.1. Astacus astacus*. Photo provided by the Danish Environmental Protection Agency.*

In the genus *Astacus* there are two species found in Denmark. *Astacus astacus* (Fig. 3.1) and *Astacus leptodactylus*. A species-specific assay was developed and tested by Agersnap et al. (2017) (Figure 3.2 and Table 3.1). For this study the same assay was tested again.

Astast_COI_F0336 5'-GATTAGAGGAATAGTAGAGAG-3'
Astast_COI_R0397 5'-CTGATGCTAAAGGGGGATAA-3'
Astast_COI_P0357 5'-FAM-AGGAGTAGGGACAGGATGAACT-BHQ1-3'

An additional assay was developed in the present study (Figure 3.3 and Table 3.2):

Astast_COI_F01 5'-CGATTTTAGGGGCGGTAAAT-3'
Astast_COI_R01 5'-CACCTGCCAACACAGGTAGA-3'
Astast_COI_P01 5'-FAM-TCGAATACCTCTTTTTGTTTGATCTGT-BHQ-1-3'

Table 3.1. *Previous developed primers and probes specific for* A. astacus *(Agersnap et al., 2017).*

Species	Gene	Size base pair (bp)	Temp (°C)	Length (bp)	GC (%)
Astacus astacus	*mtDNA-co1*	65			
Astast_COI_F0336	5'-GATTAGAGGAATAGTAGAGAG-3'		44.4	21	38.1
Astast_COI_R0397	5'-CTGATGCTAAAGGGGGATAA-3'		56.8	20	45.0
Astast_COI_P0357	5'-FAM-AGGAGTAGGGACAGGATGAACT-BHQ1-3'		58.2	22	50.0

Table 3.2. *Primers and probes specific for* A. astacus *designed and tested in the present study.*

Species	Gene	Size base pair (bp)	Temp (°C)	Length (bp)	GC (%)
Astacus astacus	*mtDNA-co1*	144 base pair (bp)			
Astast_COI_F01	5'-CGATTTTAGGGGCGGTAAAT-3'		60.2	20	45.0
Astast_COI_R01	5'-CACCTGCCAACACAGGTAGA-3'		59.7	20	55.0
Astast_COI_P01	5'-FAM-TCGAATACCTCTTTTTGTTTGATCTGT-BHQ-1-3'		62.5	27	33.3

Table 3.3. In vitro *testing performed on DNA extracted from tissue samples and nucleotide sequences used for design and alignment of primers.*

Related species	Tested	Amplifi-cation	Acc. number or sequence
Astacus astacus	Yes	Yes	GU727619, JN254659-JN254681, 151_76_4810
Astacus leptodactylus	Yes	No	MF288079-MF288086
Cherax destructor	No	NA	KJ950555, KM039112
Cherax quadricarinatus	No	NA	NA
Cherax quinquecarinatus	No	NA	NA
Faxonius immunis	Yes	No	151_70_Oroimm, 151_72_Oroimm142, JF438005-JF438006
Faxonius juvenilis	Yes	No	151_66_Faxjuv146, 151_67_Faxjuv137, 151_75_Faxjuv145, AF474352, AY701233, JF437985, KT282396-KT282407, KT282419-KT282428
Faxonius limosus	Yes	No	JF911554, 151_68_Faxlim138, 151_71_Faxlim141
Faxonius rusticus	No	NA	AY701249, KX238168, AY701248-AY701249
Faxonius virilis	Yes	No	FJ608577, EU442743
Pacifastacus fortis	No	NA	NA
Pacifastacus leniusculus	Yes	No	AF525226-AF525227, MF288087, JF437999, JF437995-JF437998, JF438000, 151_80_5691
Procambarus clarkii	Yes	No	151_69_Procla139, 151_74_Procla144
Procambarus fallax	Yes	No	151_73_Profal143

** Additional sequences from each species and additional not listed species were used in the alignment for increased accuracy and diversity coverage within and among other crayfish species.*

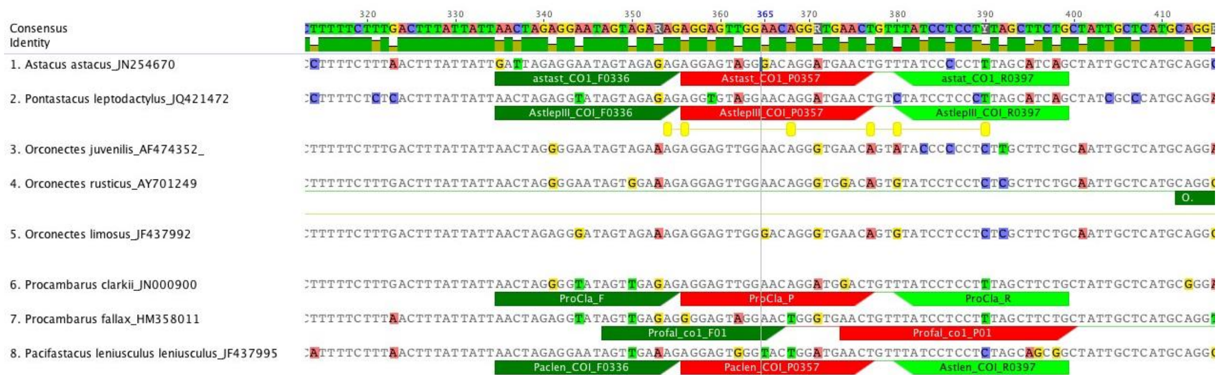

Figure 3.2. Alignment of crayfish species for the mtDNA-co1 gene for the assay presented by Ager- *snap et al. (2017). Primers are marked with green annotations. The probe is indicated with a red annotation. Sequences were obtained acquired from NCBI GenBank and aligned in Geneious.*

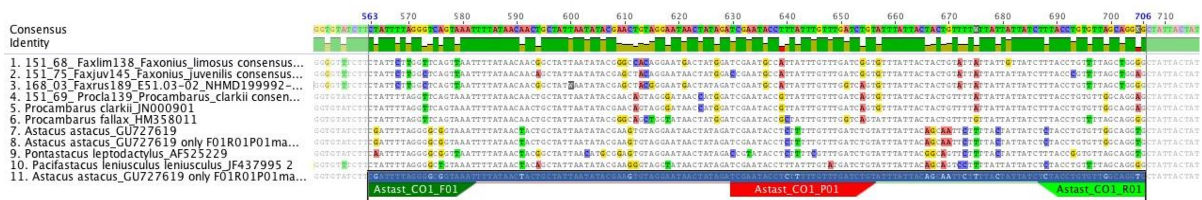

Figure 3.3. *Alignment of crayfish species for the mtDNA-co1 gene for the F01-R01-P01 assay developed and tested in the present study. Primers are marked with green annotations. The probe is indicated with a red annotation. Sequences were obtained acquired from NCBI GenBank and aligned in Geneious v R7.*

Primers and probes tested

The following primers and probes were *in silico* designed and tested *in vitro* in a PCR reaction setup (PCR setup 01) to find a species-specific combination of primers and probes: Astast_CO1_F01: 3'-CGATTTTAGGGGCGGTAAAT-5', Astast_CO1_F02: 3'-GCAGGCGCATCTGTAGACTT-5', Astast_CO1_F04: 3'-TATCCCCCTTTAGCATCAGC-5', Astast_CO1_F05: 3'-TTTTGATTGCTCCCCTTTTC-5', Astast_CO1_P01: 3-FAM-'TCGAATACCTCTTTTTGTTTGATCTGT-BHQ1-5', Astast_CO1_P02: 3-FAM-'TTTCATTACACTTGGCAGGTGTATCTT-BHQ1-5', Astast_CO1_R01: 3'-CACCTGCCAACACAGGTAGA-5', Astast_CO1_R02: 3'-ATTTACCGCCCCTAAAATCG-5', Astast_COI_F0336: 3'-GATTAGAGGAATAGTAGAGAG-5', Astast_COI_P0357: 3-FAM-'AGGAGTAGGGACAGGATGAACT-BHQ1-5', Astast_COI_R0397: 3'-CTGATGCTAAAGGGGGATAA-5'. The initial PCR results from the test performed using these primers are not included in this report

Assay specificity results

The species-specific assay (Astast_COI_F0336, Astast_COI_R0397, Astast_COI_P0357) (Agersnap et al., 2017) was found to be unspecific (Figure 3.4). The assay designed and tested in this study (Astast_COI_F01, Astast_COI_R01, Astast_COI_P01) amplified for the two replicates of *Astacus astacus* at a Cq of 28 and 28 (Figure 3.5). The new F01-R01-P01-assay tested in this study was found to be species-specific only against the targeted species (Figure 3.6).

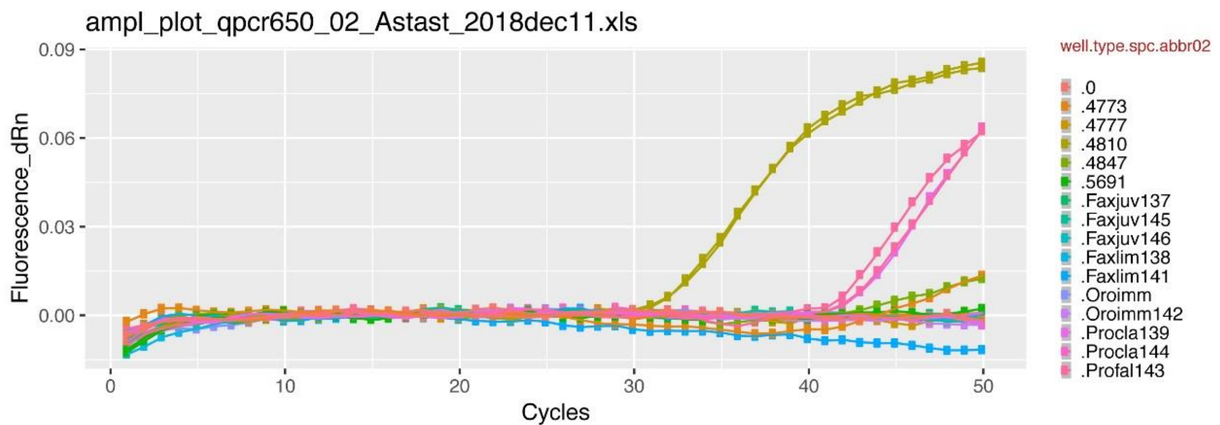

Figure 3.4. *Amplification of* Astacus astacus *species using the Agersnap et al. (2017) assay. Target species* A. astacus *is shown in yellow-green (Aa_4810) and non-target sister species in other colours. This assay also amplifies DNA from* Procambarus fallax *(Profal143) magenta lines. The other colors represent '.0' the negative control, 'Faxjuv146'* Faxonius juvenilis *[Kentucky_River_crayfish], 'Fax-juv137'* Faxonius juvenilis *[Kentucky_River_crayfish], 'Faxlim138'* Faxonius limosus *[spinycheek_crayfish], 'Procla139'* Procambarus clarkii *[Lousianna_flodkrebs], 'Oroimm'* Faxonius immunis *[calico_crayfish], 'Faxlim141'* Faxonius limosus *[spinycheek_crayfish], 'Oroimm142'* Faxonius immunis *[calico_crayfish], 'Profal143'* Procambarus fallax *[marmorkrebs], 'Procla144'* Procambarus clarkii *[Lousianna_flodkrebs], 'Faxjuv145'* Faxonius juvenilis *[Kentucky_River_crayfish], '4810'* Astacus astacus *[Flodkrebs3], '4773'* Pontastacus leptodactylus *[Galizisk sumpkrebs1], '4847'* Pontastacus leptodactylus *[Galizisk sumpkrebs2], '4777'* Pontastacus leptodactylus *[Galizisk sumpkrebs3], '5691'* Pacifastacus leniusculus *[Signalkrebs1].*

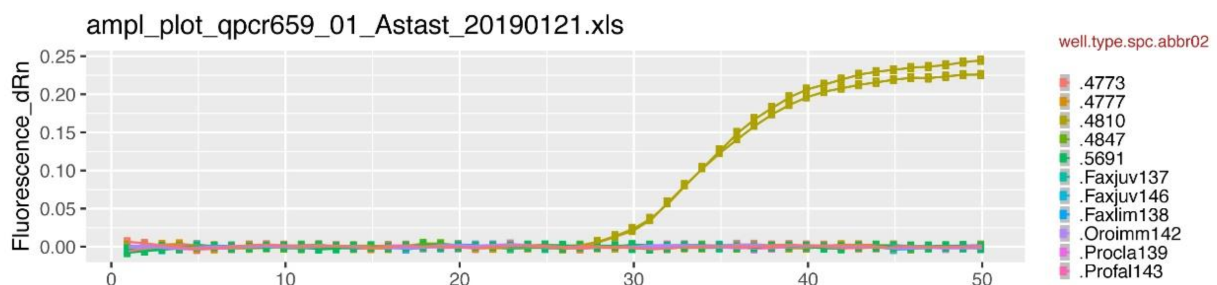

Figure 3.5. *Amplification of* Astacus astacus *species using a new assay developed in the present study. Target species* A. astacus *is shown in green-yellow (4810) and non-target sister species in other col-ours. This assay is able to distinguish between* Astacus astacus *and other species of crayfish, but re-turns a relative low level of fluorescence. The other colors represent '.0' the negative control, 'Faxjuv146'* Faxonius juvenilis *[Kentucky_River_crayfish], 'Faxjuv137'* Faxonius juvenilis *[Ken-tucky_River_crayfish], 'Faxlim138'* Faxonius limosus *[spinycheek_crayfish], 'Procla139'* Procambarus clarkii *[Lousianna_flodkrebs], 'Oroimm'* Faxonius immunis *[calico_crayfish], 'Faxlim141'* Faxonius limosus *[spinycheek_crayfish], 'Oroimm142'* Faxonius immunis *[calico_crayfish], 'Profal143'* Procam-barus fallax *[marmorkrebs], 'Procla144'* Procambarus clarkii *[Lousianna_flodkrebs], 'Faxjuv145'* Faxo-nius juvenilis *[Kentucky_River_crayfish], '4810'* Astacus astacus *[Flodkrebs3], '4773'* Pontastacus lep-todactylus *[Galizisk sumpkrebs1], '4847'* Pontastacus leptodactylus *[Galizisk sumpkrebs2], '4777'* Pontastacus leptodactylus *[Galizisk sumpkrebs3], '5691'* Pacifastacus leniusculus *[Signalkrebs1].*

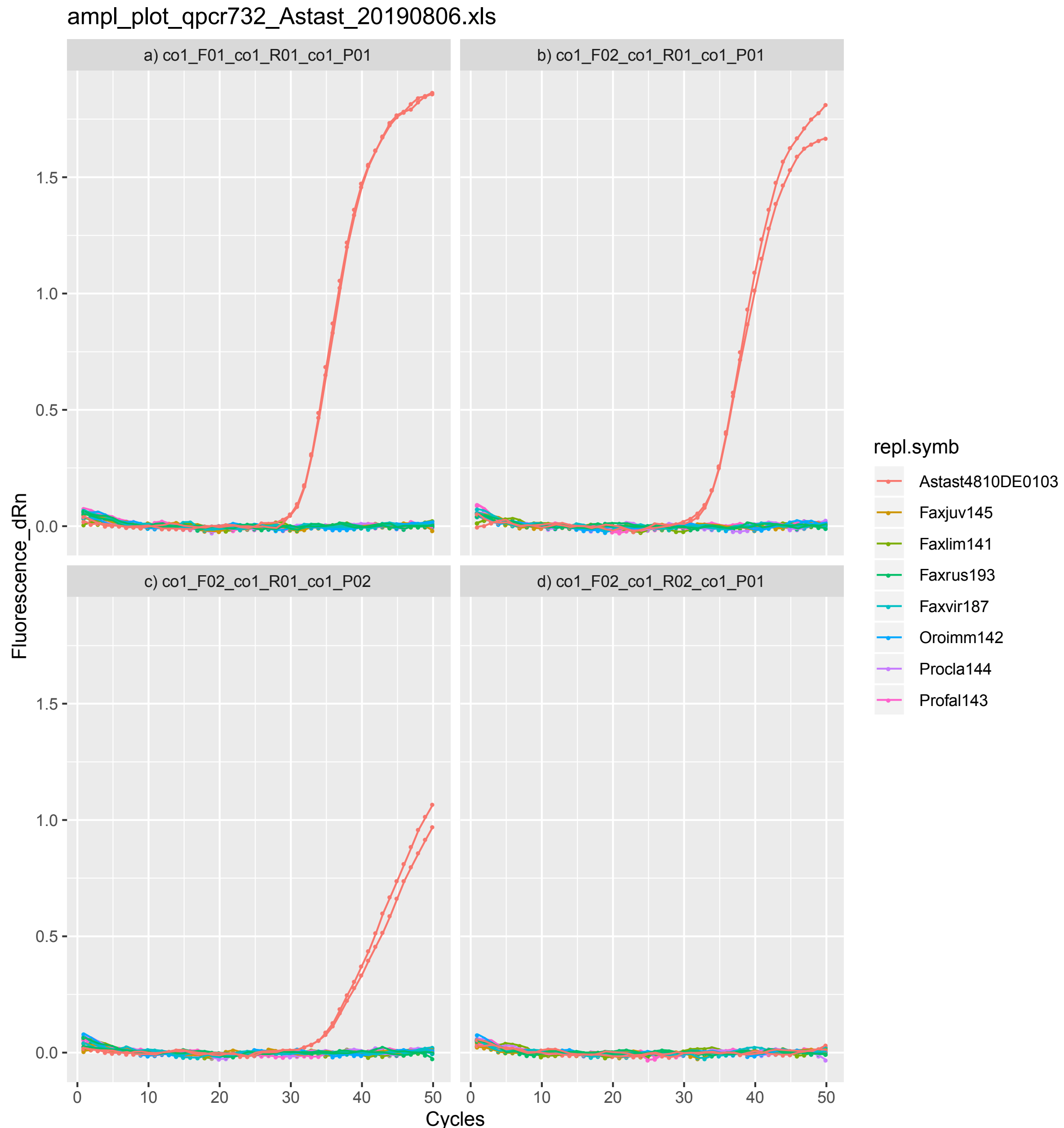

Figure 3.6. *Amplification of* Astacus astacus *species using four new assays developed in the present study. Target species* A. astacus *is shown in red (4810) and non-target sister species in other colours. The four assays (a-d) show the different combinations of primer and probe tested. The assay using Astast_co1_F01+ Astast_co1_R01+ Astast_co1_P01 (a) returns species-specific detection with the highest relative fluorescence level and lowest Cq. This assay was preferred among the four tested.*

3.2 Species no. krebs_02: Pacifastacus leniusculus

Binomial nomenclature and author: *Pacifastacus leniusculus* (Dana, 1852) – see figure 3.7.
English common name: Signal crayfish
Danish common name: Signalkrebs

Figure 3.7. Pacifastacus leniusculus. *Photo provided by the Danish Environmental Protection Agency.*

A species-specific assay against *Pacifastacus leniusculus* (Fig. 3.7) was developed and tested by Agersnap *et al.* (2017) (Figure 3.8 and Table 3.4). For this study the same assay was tested again.

Paclen_COI_F0336 5'-AACTAGAGGAATAGTTGAAAG-3'
Astlen_COI_R0397 5'-CCGCTGCTAGAGGAGGATAA-3'
Paclen_COI_P0357 5'-FAM-AGGAGTGGGTACTGGATGAACT-BHQ1-3'

A new species-specific assay was also developed (Figure 3.9 and Table 3.5) and tested in this study (Table 3.6) and compared with the assay from Agersnap *et al.* (2017).

Paclen_CO1_F02 5'-TGTAGTCACGGCACATGCTT-3'
Paclen_CO1_R01 5'-CCGCTGCTAGAGGAGGATAA-3'
Paclen_CO1_P01 5'-FAM-AAAGAGGAGTGGGTACTGGATGAAC-BHQ1-3'

Table 3.4. *Previous developed primers and probes specific for* P. leniusculus *(Agersnap et al., 2017).*

Species	**Gene**	**Size** base pair (bp)	**Temp (°C)**	**Length (bp)**	**GC (%)**
Pacifastacus leniusculus	*mtDNA-co1*	65 base pair (bp)			
Paclen_COI_F0336	5'-AACTAGAGGAATAGTTGAAAG-3'		47.5	21	33.3
Astlen_COI_R0397	5'-CCGCTGCTAGAGGAGGATAA-3'		59.6	20	55.0
Paclen_COI_P0357	5'-FAM-AGGAGTGGGTACTGGATGAACT-BHQ1-3'		59.0	22	50.0

Table 3.5. *Primers and probes specific for* P. leniusculus *designed and tested in the present study.*

Species	**Gene**	**Size** base pair (bp)	**Temp (°C)**	**Length (bp)**	**GC (%)**
Pacifastacus leniusculus	*mtDNA-co1*	236 base pair (bp)			
Paclen_CO1_F02	5'-TGTAGTCACGGCACATGCTT-3'		60.3	20	50.0
Paclen_CO1_R01	5'-CCGCTGCTAGAGGAGGATAA-3'		59.6	20	55.0
Paclen_CO1_P01	5'-FAM-AAAGAGGAGTGGGTACTGGATGAAC-BHQ1-3'		63.0	25	48.0

Table 3.6. In vitro *testing performed on DNA extracted from tissue samples and nucleotide sequences used for design and alignment of primers.*

Related species	Tested	Amplification	Acc. number or sequence
Astacus astacus	Yes	No	GU727619, JN254659-JN254681, 151_76_4810
Astacus leptodactylus	Yes	No	MF288079-MF288086
Cherax destructor	No	NA	KJ950555, KM039112
Cherax quadricarinatus	No	NA	NA
Cherax quinquecarinatus	No	NA	NA
Faxonius immunis	Yes	No	151_70_Oroimm, 151_72_Oroimm142, JF438005-JF438006
Faxonius juvenilis	Yes	No	151_66_Faxjuv146, 151_67_Faxjuv137, 151_75_Faxjuv145, AF474352, AY701233, JF437985, KT282396-KT282407, KT282419-KT282428
Faxonius limosus	Yes	No	JF911554, 151_68_Faxlim138, 151_71_Faxlim141
Faxonius rusticus	No	No	AY701249, KX238168, AY701248-AY701249
Faxonius virilis	Yes	No	FJ608577, EU442743
Pacifastacus fortis	No	NA	NA
Pacifastacus leniusculus	Yes	Yes	AF525226-AF525227, MF288087, JF437999, JF437995-JF437998, JF438000, 151_80_5691
Procambarus clarkii	Yes	No	151_69_Procla139, 151_74_Procla144
Procambarus fallax	Yes	No	151_73_Profal143

** Additional sequences from each species and additional not listed species were used in the alignment for increased accuracy and diversity coverage within and among other crayfish species.*

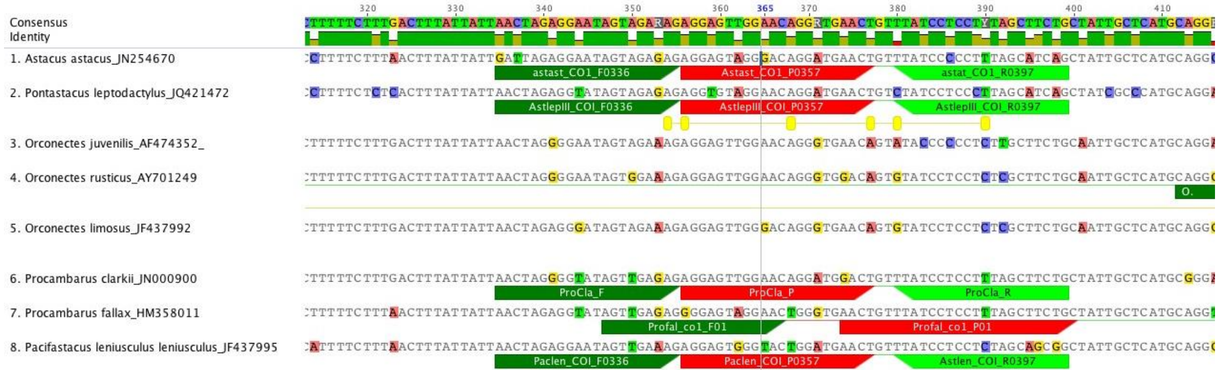

Figure 3.8. *Alignment of crayfish species for the mtDNA-co1 gene for the primer binding region for the assay presented by Agersnap et al. (2017). Primers are marked with green annotations. The probe is indicated with a red annotation. Sequences were obtained acquired from NCBI GenBank and aligned in Geneious v. R7.*

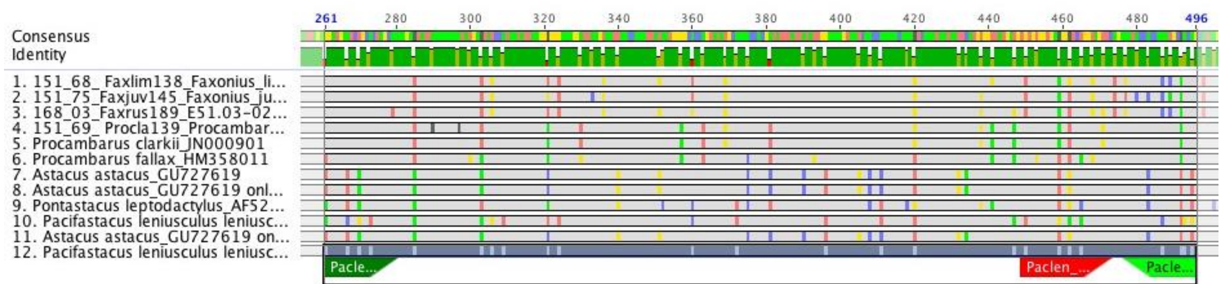

Figure 3.9. *Alignment of crayfish species for the mtDNA-co1 gene for the Paclen_CO1_R01+Paclen_CO1_P01+Paclen_CO1_F02 specific primer-probe assay developed in the present study. Primers are marked with green annotations. The probe is indicated with a red annotation. Sequences were obtained acquired from NCBI GenBank and aligned in Geneious v R7.*

Primers and probes tested

The following primers and probes were *in silico* designed and tested in a PCR reaction setup as 'PCR setup 01' to find a species-specific combination of primers and probes: Astlen_COI_R0397: 3'-CCGCTGCTAGAGGAGGATAA-5', Paclen_CO1_F01: 3'-TTGTAGTCACGGCACATGCT-5', Paclen_CO1_F02: 3'-TGTAGTCACGGCACATGCTT-5', Paclen_CO1_P01: 3-FAM-'AAAGAGGAGTGGGTACTGGATGAAC-BHQ1-5', Paclen_CO1_P03: 3-FAM-'TTCCTTTAATATTAGGGGCTCCTGA-BHQ1-5', Paclen_CO1_R01: 3'-CCGCTGCTAGAGGAGGATAA-5', Paclen_CO1_R03: 3'-TATTTATCCGGGGGAATGCT-5', Paclen_CO1_R05: 3'-ATTTATCCGGGGGAATGCTA-5', Paclen_COI_F0336: 3'-AACTAGAGGAATAGTTGAAAG-5', Paclen_COI_P0357: 3-FAM-'AGGAGTGGGTACTGGATGAACT-BHQ1-5'.

The initial PCR results from the test performed using these primers are not included in this report

Assay specificity results

The specific assay developed by Agersnap *et al*. (2017) was not able to discriminate between *Pacifastacus leniusculus* and *Faxonius juvenilis*. Using the assay developed by Agersnap *et al*. (2017) two replicates of *Pacifastacus leniusculus* amplified at a Cq of 31 and 32, and also resulted in amplification of *Faxonius juvenilis* at a Cq of 39 and 40 (Figure 3.10). Using the newly developed assays tested in this study F02+P01+R01 (Figure 3.11) returned species-specific amplification at a Cq of 30 and 30 for both replicates. This F02+P01+R01 assay (Figure 3.11) only amplified DNA from the intended target species.

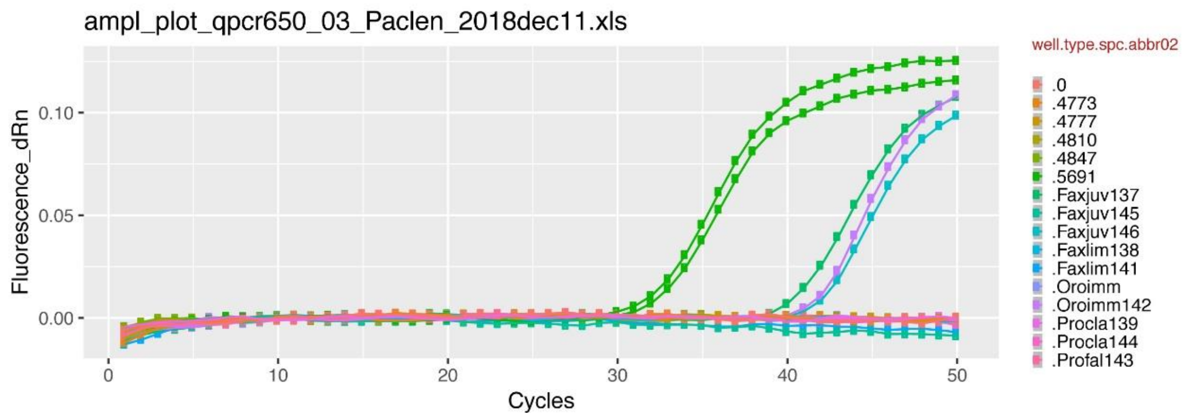

Figure 3.10. *Amplification of* Pacifastacus leniusculus *species using the Agersnap et al. (2017) assay. Target species* Pacifastacus leniusculus *is shown in green. This assay also amplifies DNA from* Faxonius juvenilis *(Faxjuv146 and Faxjuv145). The other colors represent '.0' the negative control, 'Faxjuv146'* Faxonius juvenilis *[Kentucky_River_crayfish], 'Faxjuv137'* Faxonius juvenilis *[Kentucky_River_crayfish], 'Faxlim138'* Faxonius limosus *[spinycheek_crayfish], 'Procla139'* Procambarus clarkii *[Lousianna_flodkrebs], 'Oroimm'* Faxonius immunis *[calico_crayfish], 'Faxlim141'* Faxonius limosus *[spinycheek_crayfish], 'Oroimm142'* Faxonius immunis *[calico_crayfish], 'Profal143'* Procambarus fallax *[marmorkrebs], 'Procla144'* Procambarus clarkii *[Lousianna_flodkrebs], 'Faxjuv145'* Faxonius juvenilis *[Kentucky_River_crayfish], '4810'* Astacus astacus *[Flodkrebs3], '4773'* Pontastacus leptodactylus *[Galizisk sumpkrebs1], '4847'* Pontastacus leptodactylus *[Galizisk sumpkrebs2], '4777'* Pontastacus leptodactylus *[Galizisk sumpkrebs3], '5691'* Pacifastacus leniusculus *[Signalkrebs1].*

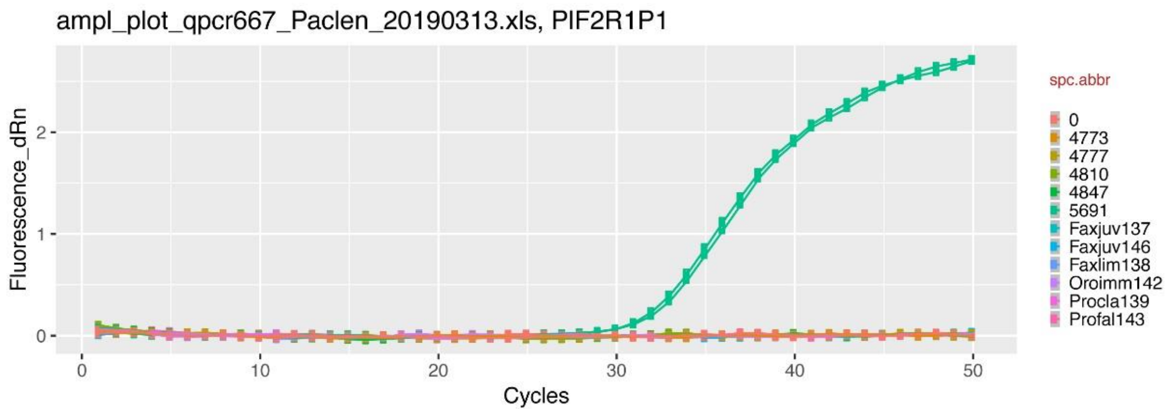

Figure 3.11. *Amplification of* Pacifastacus leniusculus *species using the Paclen_CO1_F02+R01+P01 assay developed in the present study. Target species* Pacifastacus leniusculus (5691) (Danish common name: Signal krebs) *is shown in blue-green color. The other colors represent '.0' the negative control, 'Faxjuv146'* Faxonius juvenilis *[Kentucky_River_crayfish], 'Faxjuv137'* Faxonius juvenilis *[Kentucky_River_crayfish], 'Faxlim138'* Faxonius limosus *[spinycheek_crayfish], 'Procla139'* Procambarus clarkii *[Lousianna_flodkrebs], 'Oroimm'* Faxonius immunis *[calico_crayfish], 'Faxlim141'* Faxonius limosus *[spinycheek_crayfish], 'Oroimm142'* Faxonius immunis *[calico_crayfish], 'Profal143'* Procambarus fallax *[marmorkrebs], 'Procla144'* Procambarus clarkii *[Lousianna_flodkrebs], 'Faxjuv145'* Faxonius juvenilis *[Kentucky_River_crayfish], '4810'* Astacus astacus *[Flodkrebs3], '4773'* Pontastacus leptodactylus *[Galizisk sumpkrebs1], '4847'* Pontastacus leptodactylus *[Galizisk sumpkrebs2], '4777'* Pontastacus leptodactylus *[Galizisk sumpkrebs3], '5691'* Pacifastacus leniusculus *[Signalkrebs1].*

3.3 Species no. krebs_03: *Astacus leptodactylus*

Binomial nomenclature and author: *Astacus leptodactylus* Eschscholtz, 1823 – see figure 3.12.
English common name: Narrow-clawed crayfish
Danish common name: Galizisk sumpkrebs

Figure 3.12. Astacus leptodactylus*. Photo provided by the Danish Environmental Protection Agency.*

Astacus leptodactylus (also known as *Pontastacus leptodactylus*) (Fig. 3.12) is considered a cryptogenic species, that comprises three subspecies (subclade I, II and III). *Astacus leptodactylus* have previously been assigned to the genus *Pontastacus*. It is currently only possible to distinguish between these three subclades by using genetic sequencing. Two specific assays were developed and tested by Agersnap *et al*. (2017) (Figure 3.13 and Table 3.7). For this study the same two assays were tested again.

For subclade I:
AstlepI_COI_F0336 5'-AACTAGGGGTATAGTAGAGAG-3'
AstlepI_COI_R0397 5'-CTGATGCTAAAGGGGGATAA-3'
AstlepI_COI_P0357 5'-FAM-AGGAGTAGGGACCGGATGAACT-BHQ1-3'

For subclade III:
AstlepIII_COI_F0336 5'-AACTAGAGGTATAGTAGAGGG-3'
AstlepIII_COI_R0397 5'-CTGATGCTAGGGGAGGATAA-3'
AstlepIII_COI_P0357 5'-FAM-GGGTGTAGGAACTGGATGAACC-BHQ1-3'

In addition to the assays developed by Agersnap *et al*. (2017) a new combination of primers and a probe was developed for subclade I, II and III (Figure 3.14 and Table 3.8) to be able to test on other species of crayfish (Table 3.9):

Ponlep_CO1_F03 5'-TTTGGGACTTGAGCAGGAAT-3'
Ponlep_CO1_R03 5'-CTGGTTGTCCGAGTTCAACA-3'
Ponlep_CO1_P03 5'-FAM-TGGGAACCTCTTTAAGAATAATTATTCG-BHQ-1-3'

Table 3.7. *Previous developed primers and probes specific for* A. leptodactylus *(Agersnap et al., 2017).*

Species	Gene	Size base pair (bp)	Temp (°C)	Length (bp)	GC (%)
Astacus leptodactylus	*mtDNA-co1*	65 base pair (bp)			
AstlepI_COI_F0336	5'-AACTAGGGGTATAGTAGAGAG-3'		46.5	21	42.9
AstlepI_COI_R0397	5'-CTGATGCTAAAGGGGGATAA-3'		56.8	20	45.0
AstlepI_COI_P0357	5'-FAM-AGGAGTAGGGACCGGATGAACT-BHQ1-3'		62.4	22	54.6
Astacus leptodactylus	*mtDNA-co1*		65 base pair (bp)		
AstlepIII_COI_F0336	5'-AACTAGAGGTATAGTAGAGGG-3'		46.5	21	42.9
AstlepIII_COI_R0397	5'-CTGATGCTAGGGGAGGATAA-3'		56.9	20	50.0
AstlepIII_COI_P0357	5'-FAM-GGGTGTAGGAACTGGATGAACC-BHQ1-3'		61.9	22	54.6

Table 3.8. *Primers and probes specific for* A. leptodactylus *designed and tested in the present study.*

Species	Gene	Size base pair (bp)	Temp (°C)	Length (bp)	GC (%)
Astacus leptodactylus	*mtDNA-co1*	70 base pair (bp)			
Ponlep_CO1_F03	5'-TTTGGGACTTGAGCAGGAAT-3'		59.7	20	45.0
Ponlep_CO1_R03	5'-CTGGTTGTCCGAGTTCAACA-3'		59.7	20	50.0
Ponlep_CO1_P03	5'-FAM-TGGGAACCTCTTTAAGAATAATTATTCG-BHQ-1-3'		62.0	28	32.1

Table 3.9. In vitro *testing performed on DNA extracted from tissue samples and nucleotide sequences used for design and alignment of primers.*

Related species	Tested	Amplification	Acc. number or sequence
Astacus astacus	Yes	No	GU727619, JN254659-JN254681, 151_76_4810
Astacus leptodactylus	Yes	Yes	MF288079-MF288086
Faxonius immunis	Yes	No	151_70_Oroimm, 151_72_Oroimm142, JF438005-JF438006
Faxonius juvenilis	Yes	No	151_66_Faxjuv146, 151_67_Faxjuv137, 151_75_Faxjuv145, AF474352, AY701233, JF437985, KT282396-KT282407, KT282419-KT282428
Faxonius limosus	Yes	No	JF911554, 151_68_Faxlim138, 151_71_Faxlim141
Faxonius rusticus	No	NA	AY701249, KX238168, AY701248-AY701249
Faxonius virilis	Yes	No	FJ608577, EU442743
Pacifastacus fortis	No	NA	NA
Pacifastacus leniusculus	Yes	No	AF525226-AF525227, MF288087, JF437999, JF437995-JF437998, JF438000, 151_80_5691
Procambarus clarkii	Yes	No	151_69_Procla139, 151_74_Procla144
Procambarus fallax	Yes	No	151_73_Profal143

** Additional sequences from each species and additional not listed species were used in the alignment for increased accuracy and diversity coverage within and among other crayfish species.*

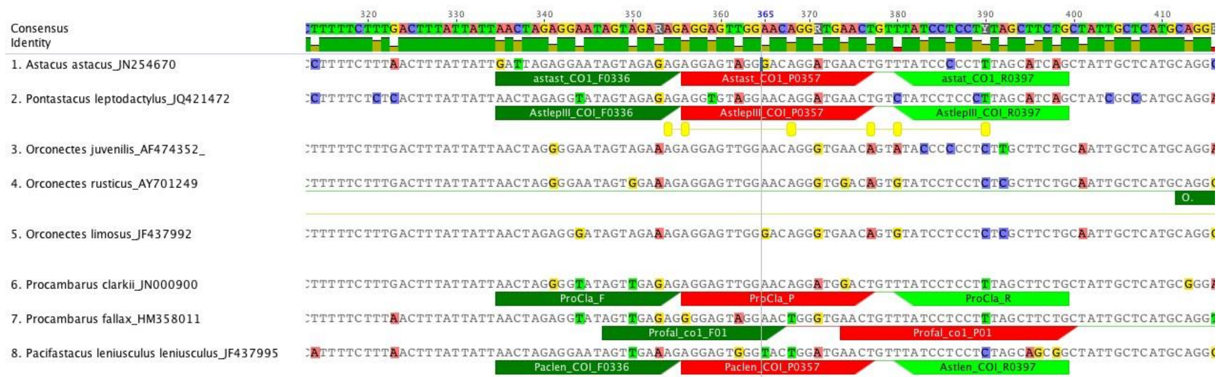

Figure 3.13. *Alignment of crayfish species for the mtDNA-co1 gene for the assay developed by Agersnap et al. (2017). Primers are marked with green annotations. The probe is indicated with a red annotation. Sequences were obtained acquired from NCBI GenBank and aligned in Geneious v. R7.*

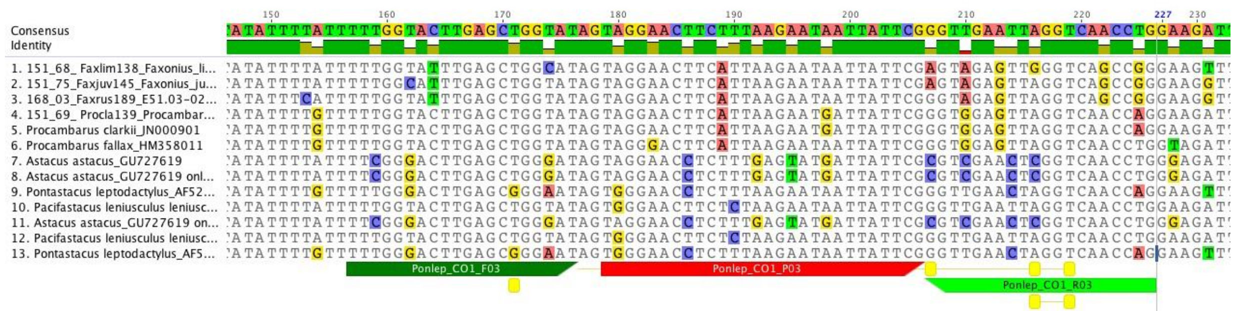

Figure 3.14. *Alignment of crayfish species for the mtDNA-co1 gene for the Ponlep_co1_F03+Ponlep_co1_R03+Ponlep_co1_P03-assay developed in the present study. Primers are marked with green annotations. The probe is indicated with a red annotation. Sequences were obtained acquired from NCBI GenBank and aligned in Geneious v. R7.*

Primers and probes tested

The following primers and probes were *in silico* designed and tested *in vitro* in PCR reactions set up as 'PCR setup 01' to find a species-specific combination of primers and probes: Ponlep_CO1_F01: 3'-TGCAGGGGCTTCTGTAGATT-5', Ponlep_CO1_F02: 3'-GGGGTGTAGGAACTGGATGA-5', Ponlep_CO1_F03: 3'-TTTGGGACTTGAGCAGGAAT-5', Ponlep_CO1_P02: 3-FAM-'GTTTATCCTCCCC-TAGCATCAGCTA-BHQ1-5', Ponlep_CO1_P03: 3-FAM-'TGGGAACCTCTTTAAGAATAATTATTCG-BHQ1-5', Ponlep_CO1_R01: 3'-AAATTGACCGCCCCTAAAAT-5', Ponlep_CO1_R03: 3'-CTGGTTGTCCGAGTTCAACA-5', Ponlep_CO1_R04: 3'-TCTTCCTGGTTGTCCGAGTT-5', Ponlep_CO1_R05: 3'-CTTCCTGGTTG-TCCGAGTTC-5', AstlepI_COI_F0336: 3'-AACTAGGGGTATAGTAGAGAG-5', AstlepI_COI_P0357: 3-FAM-'AGGAGTAGGGACCGGATGAACT-BHQ1-5', AstlepI_COI_R0397: 3'-CTGATGCTAAAGGGGGATAA-5', AstlepIII_COI_F0336: 3'-AACTAGAGGTATAGTAGAGGG-5', AstlepIII_COI_P0357: 3-FAM-'GGGTGTAGGAACTGGATGAACC-BHQ1-5', AstlepIII_COI_R0397: 3'-CTGATGCTAGGGGAGGATAA-5'.

The initial PCR results from the test performed using these primers are not included in this report

Assay specificity results

Using the Agersnap *et al*. (2017) assay did not return any specific amplification (Figure 3.15 and 3.16). The newly designed assay developed in the present study resulted in amplification of two replicates of *Astacus leptodactylus subclade I and subclade III* at a Cq of 24 and 26, the replicates of *Astacus*

leptodactylus subclade II amplified at Cq 34 and 35 (Figure 3.17). None of the native species amplified with this primer-probe assay.

The designed eDNA target assay for *Astacus leptodactylus* is expected to only amplify DNA from the target species when tested on laboratory or environmental water samples.

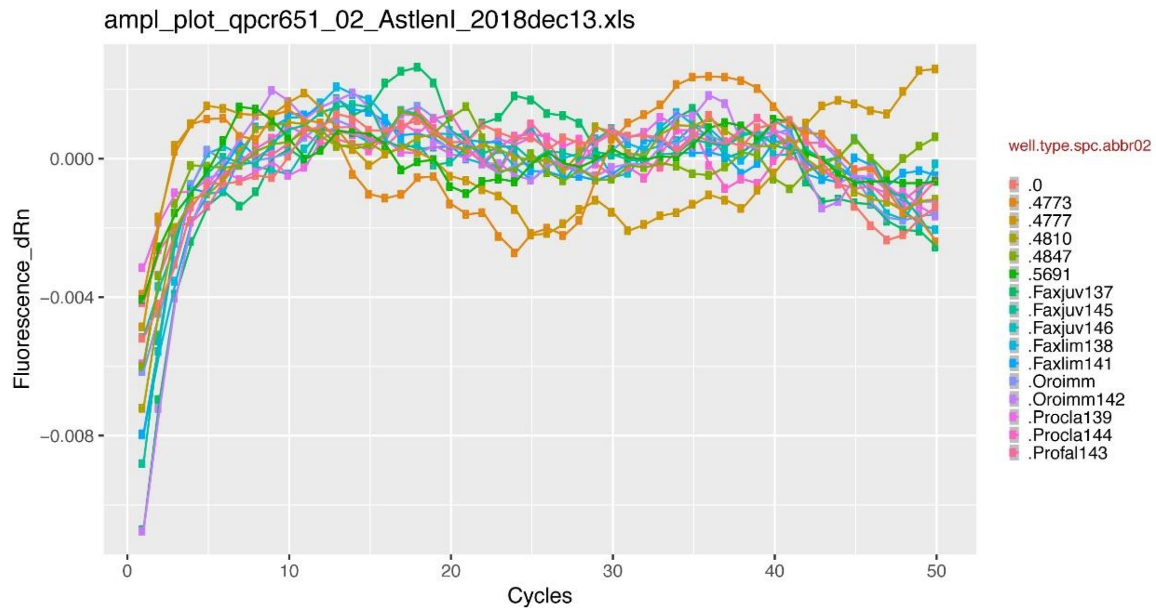

Figure 3.15. *Amplification of* Astacus leptodactylus *subclade I species using the Agersnap* et al*. (2017) assay. This assay test failed in amplifying any DNA from any species of crayfish. The other colors represent '.0' the negative control, 'Faxjuv146'* Faxonius juvenilis *[Kentucky_River_crayfish], 'Faxjuv137'* Faxonius juvenilis *[Kentucky_River_crayfish], 'Faxlim138'* Faxonius limosus *[spinycheek_crayfish], 'Procla139'* Procambarus clarkii *[Lousianna_flodkrebs], 'Oroimm'* Faxonius immunis *[calico_crayfish], 'Faxlim141'* Faxonius limosus *[spinycheek_crayfish], 'Oroimm142'* Faxonius immunis *[calico_crayfish], 'Profal143'* Procambarus fallax *[marmorkrebs], 'Procla144'* Procambarus clarkii *[Lousianna_flodkrebs], 'Faxjuv145'* Faxonius juvenilis *[Kentucky_River_crayfish], '4810'* Astacus astacus *[Flodkrebs3], '4773'* Pontastacus leptodactylus *[Galizisk sumpkrebs1], '4847'* Pontastacus leptodactylus *[Galizisk sumpkrebs2], '4777'* Pontastacus leptodactylus *[Galizisk sumpkrebs3], '5691'* Pacifastacus leniusculus *[Signalkrebs1].*

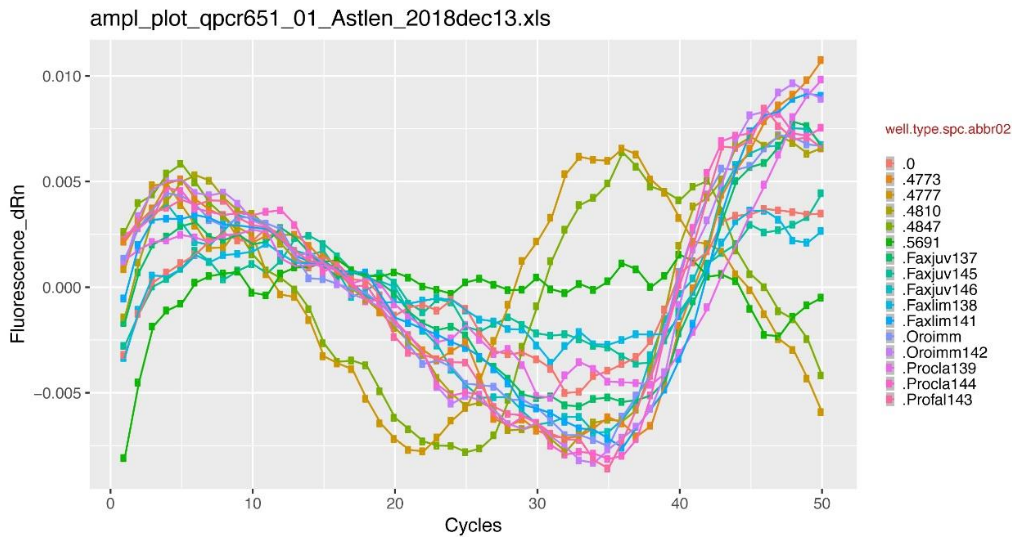

Figure 3.16. *Amplification of* Astacus leptodactylus *subclade III species using the Agersnap* et al. *(2017) assay. This assay failed in amplifying any DNA from any species of crayfish. The colors represent '.0' the negative control, 'Faxjuv146'* Faxonius juvenilis *[Kentucky_River_crayfish], 'Faxjuv137'* Faxonius juvenilis *[Kentucky_River_crayfish], 'Faxlim138'* Faxonius limosus *[spinycheek_crayfish], 'Procla139'* Procambarus clarkii *[Lousianna_flodkrebs], 'Oroimm'* Faxonius immunis *[calico_crayfish], 'Faxlim141'* Faxonius limosus *[spinycheek_crayfish], 'Oroimm142'* Faxonius immunis *[calico_crayfish], 'Profal143'* Procambarus fallax *[marmorkrebs], 'Procla144'* Procambarus clarkii *[Lousianna_flodkrebs], 'Faxjuv145'* Faxonius juvenilis *[Kentucky_River_crayfish], '4810'* Astacus astacus *[Flodkrebs3], '4773'* Pontastacus leptodactylus *[Galizisk sumpkrebs1], '4847'* Pontastacus leptodactylus *[Galizisk sumpkrebs2], '4777'* Pontastacus leptodactylus *[Galizisk sumpkrebs3], '5691'* Pacifastacus leniusculus *[Signalkrebs1].*

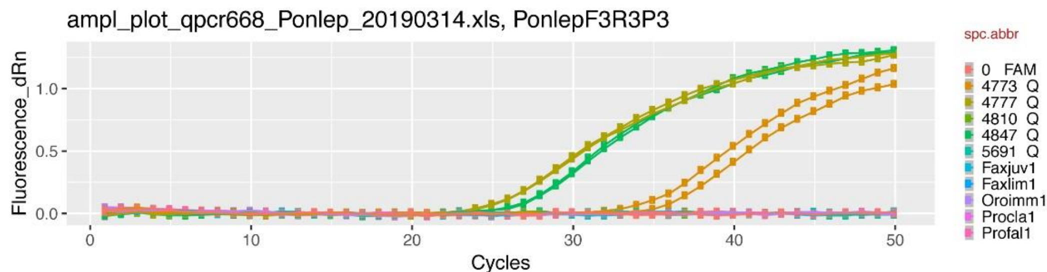

Figure 3.17. *Amplification of* Astacus leptodactylus *subclade I, II and III (Astacus leptodactylus) species using the F03+R03+P03 assay developed in this study that targets the mtDNA-CO1 region. The orange (4773), the yellow-green (4777) and the green (4847) amplification plot lines represent amplification on samples from* Astacus leptodactylus *(Danish common name: Galizisk sumpkrebs1),* Astacus leptodactylus *(Danish common name: Galizisk sumpkrebs2),* Astacus leptodactylus *(Danish common name: Galizisk sumpkrebs3), respectively. The other colors represent '.0' the negative control, 'Faxjuv146'* Faxonius juvenilis *[Kentucky_River_crayfish], 'Faxjuv137'* Faxonius juvenilis *[Kentucky_River_crayfish], 'Faxlim138'* Faxonius limosus *[spinycheek_crayfish], 'Procla139'* Procambarus clarkii *[Lousianna_flodkrebs], 'Oroimm'* Faxonius immunis *[calico_crayfish], 'Faxlim141'* Faxonius limosus *[spinycheek_crayfish], 'Oroimm142'* Faxonius immunis *[calico_crayfish], 'Profal143'* Procambarus fallax *[marmorkrebs], 'Procla144'* Procambarus clarkii *[Lousianna_flodkrebs], 'Faxjuv145'* Faxonius juvenilis *[Kentucky_River_crayfish], '4810'* Astacus astacus *[Flodkrebs3], '4773'* Astacus leptodactylus *[Galizisk sumpkrebs1], '4847'* Astacus leptodactylus *[Galizisk sumpkrebs2], '4777'* Astacus leptodactylus *[Galizisk sumpkrebs3], '5691'* Pacifastacus leniusculus *[Signalkrebs1].*

3.4 Species no. krebs_04: *Procambarus clarkii*

Binomial nomenclature and author: *Procambarus clarkii* (Girard, 1852) – see figure 3.18.
English common name: Red swamp crayfish/ Louisiana crayfish
Danish common name: Louisiana flodkrebs

Figure 3.18. Procambarus clarkii. *Photo provided by the Danish Environmental Protection Agency.*

Procambarus clarkii is non-indigenous in Europe (Figure 3.18). A specific assay was developed and tested by Treguier *et al.* (2014) (Figure 3.19 and Table 3.10). For this study the same assay was tested again.

ProCla_F 5'-AACTAGGGGTATAGTTGAGAG-3'
ProCla_R 5'-CAGAAGCTAAAGGAGGATAA-3'
ProCla_P 5'-FAM-AGGAGTTGGAACAGGATGGACT-BHQ1-'3

In addition, a four-primer probe assay was designed and tested on DNA extracted from other species of crayfish (Table 3.12), and one assay was found to be more specific and returning a relatively higher fluorescence (Figure 3.20 and Table 3.11).

Table 3.10. *Previous developed primers and probes specific for* P. clarkii *(Treguier et al., 2014).*

Species	Gene	**Size** base pair (bp)	Temp (°C)	Length (bp)	GC (%)
Procambarus clarkii	*mtDNA-co1*	65 bp			
ProCla_F	5'-AACTAGGGGTATAGTTGAGAG-3'		49.6	21	42.9
ProCla_R	5'-CAGAAGCTAAAGGAGGATAA-3'		51.6	20	40.0
ProCla_P	5'-FAM-AGGAGTTGGAACAGGATGGACT-BHQ1-'3		61.3	22	50.0

Table 3.11. *Primers and probes specific for* P. clarkii *designed and tested in the present study.*

Species	**Gene**	**Size** base pair (bp)	**Temp (°C)**	**Length (bp)**	**GC (%)**
Procambarus clarkii	*mtDNA-co1*		203 bp		
Procla_co1_F04	5'-GCGGGAGCATCTGTAGATTT-3'		59.3	20	50.0
Procla_co1_R04	5'-ATAGCTCCTGCCAACACAGG-3'		60.3	20	55.0
Procla_co1_P04	5'-FAM-ACGAACAGTAGGGATAACCATGGAT-BHQ1-3'		63.0	25	44.0

Table 3.12. In vitro *testing performed on DNA extracted from tissue samples and nucleotide sequences used for design and alignment of primers.*

Related species	Tested	Amplification	Acc. number or sequence
Astacus astacus	Yes	No	GU727619, JN254659-JN254681, 151_76_4810
Astacus leptodactylus	Yes	No	MF288079-MF288086
Cherax destructor	No	NA	KJ950555, KM039112
Cherax quadricarinatus	No	NA	NA
Cherax quinquecarinatus	No	NA	NA
Faxonius immunis	Yes	No	151_70_Oroimm, 151_72_Oroimm142, JF438005-JF438006
Faxonius juvenilis	Yes	No	151_66_Faxjuv146, 151_67_Faxjuv137, 151_75_Faxjuv145, AF474352, AY701233, JF437985, KT282396-KT282407, KT282419-KT282428
Faxonius limosus	Yes	No	JF911554, 151_68_Faxlim138, 151_71_Faxlim141
Faxonius rusticus	No	No	AY701249, KX238168, AY701248-AY701249
Faxonius virilis	Yes	No	FJ608577, EU442743
Pacifastacus fortis	No	NA	NA
Pacifastacus leniusculus	Yes	No	AF525226-AF525227, MF288087, JF437999, JF437995-JF437998, JF438000, 151_80_5691
Procambarus clarkii	Yes	Yes	151_69_Procla139, 151_74_Procla144
Procambarus fallax	Yes	No	151_73_Profal143

** Additional sequences from each species and additional not listed species were used in the alignment for increased accuracy and diversity coverage within and among other crayfish species.*

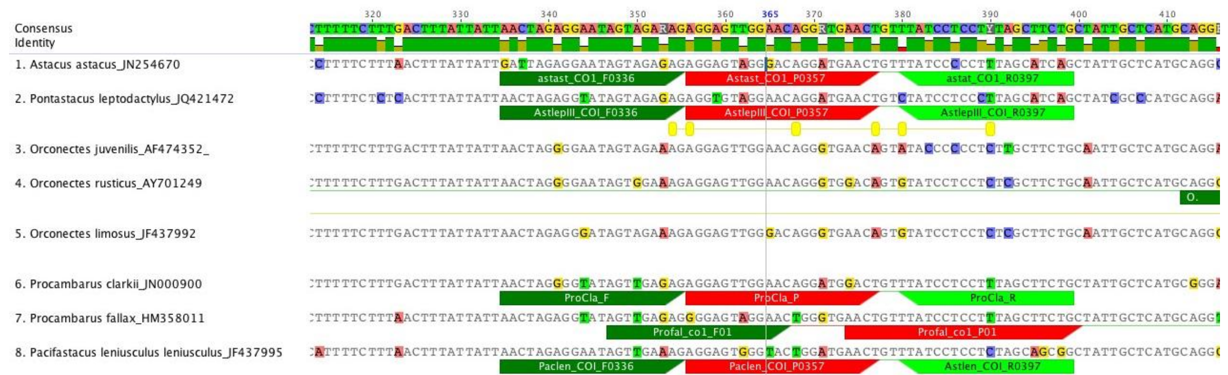

Figure 3.19. *Alignment of crayfish species for the mtDNA-co1 gene for the species-specific assay developed and tested by Tregiuer* et al. *(2014). Primers are marked with green annotations. The probe is indicated with a red annotation. Sequences were obtained acquired from NCBI GenBank and aligned in Geneious v. R7.*

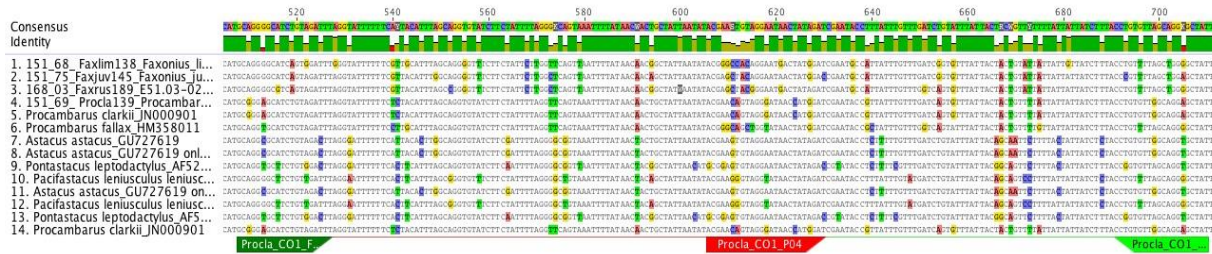

Figure 3.20. *Alignment of crayfish species for the mtDNA-co1 gene for the fragment where the species-specific Procla_co1_F04+Procla_co1_R04+Procla_co1_P04-assay developed in the present study binds. Primers are marked with green annotations. The probe is indicated with a red annotation. Sequences were obtained acquired from NCBI GenBank and aligned in Geneious v. R7.*

Primers and probes tested

The following primers and probes were *in silico* designed and tested *in vitro* in PCR reactions set up as 'PCR setup 01' to find a species-specific combination of primers and probes: Procla_CO1_F02: 3'-ATTGGTGGGTTTGGAAATTG-5', Procla_CO1_F04: 3'-GCGGGAGCATCTGTAGATTT-5', Procla_CO1_F05: 3'-AGGGATAACCATGGATCGAA-5', Procla_CO1_P01: 3-FAM-'CAGGATGGACTGTTTATCCTCCTTT-BHQ1-5', Procla_CO1_P04: 3-FAM-'ACGAACAGTAGGGATAACCATGGAT-BHQ1-5', Procla_CO1_R01: 3'-CGCATGAGCAATAGCAGAAG-5', Procla_CO1_R03: 3'-TCCATCCTGTTCCAACTCCT-5', Procla_CO1_R04: 3'-ATAGCTCCTGCCAACACAGG-5', ProCla_F: 3'-AACTAGGGGTATAGTTGAGAG-5', ProCla_P: 3-FAM-'AGGAGTTGGAACAGGATGGACT-BHQ1-5', ProCla_R: 3'-CAGAAGCTAAAGGAGGATAA-5'.

The initial PCR results from the test performed using these primers are not included in this report.

Assay specificity results

The two replicates of *Procambarus clarkii* amplified at a Cq of 21 and 21 (Figure 3.21) when the previously published assay was tested (Treguier *et al*., 2014), but also returned amplification for DNA extracted from Faxonius juvenilis. The additional primer probe assays developed and tested in the present study (Figure 3.22) all returned species-specific amplification. Among the four primer and probe combinations tested (Figure 3.22a-d) the assay combination:

Procla_co1_F04+Procla_co1_R04+Procla_co1_P04 (Figure 3.22d) returned the lowest Ct value and highest relative fluorescence.

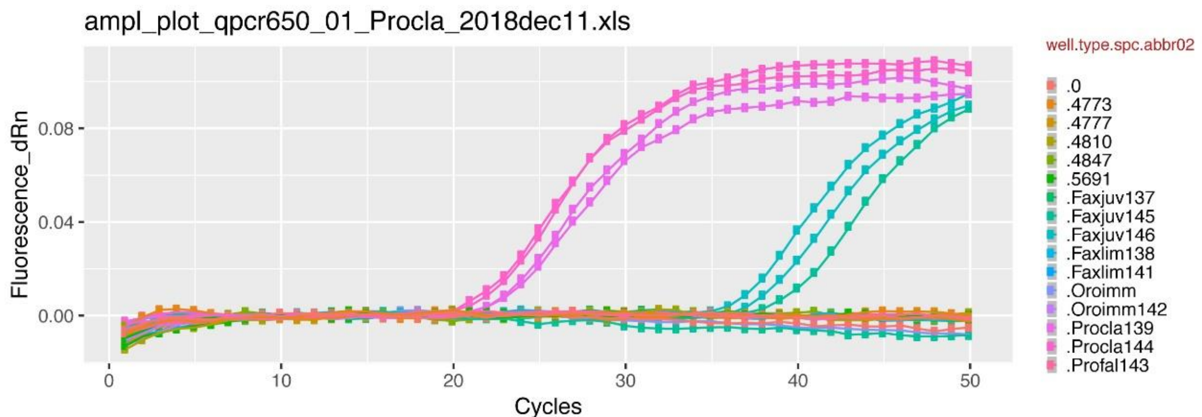

Figure 3.21. *Amplification of* Procambarus clarkii *species using the Treguier* et al. *(2014) assay. Target species* Procambarus clarkii *is shown in magenta (Procla144) and purple (Procla139). This assay also amplifies DNA from* Faxonius juvenilis *(Faxjuv146 and Faxjuv137) cyan curves. The other colors represent '.0' the negative control, 'Faxjuv146'* Faxonius juvenilis *[Kentucky_River_crayfish], 'Faxjuv137'* Faxonius juvenilis *[Kentucky_River_crayfish], 'Faxlim138'* Faxonius limosus *[spinycheek_crayfish], 'Procla139'* Procambarus clarkii *[Lousianna_flodkrebs], 'Oroimm'* Faxonius immunis *[calico_crayfish], 'Faxlim141'* Faxonius limosus *[spinycheek_crayfish], 'Oroimm142'* Faxonius immunis *[calico_crayfish], 'Profal143'* Procambarus fallax *[marmorkrebs], 'Procla144'* Procambarus clarkii *[Lousianna_flodkrebs], 'Faxjuv145'* Faxonius juvenilis *[Kentucky_River_crayfish], '4810'* Astacus astacus *[Flodkrebs3], '4773'* Pontastacus leptodactylus *[Galizisk sumpkrebs1], '4847'* Pontastacus leptodactylus *[Galizisk sumpkrebs2], '4777'* Pontastacus leptodactylus *[Galizisk sumpkrebs3], '5691'* Pacifastacus leniusculus *[Signalkrebs1].*

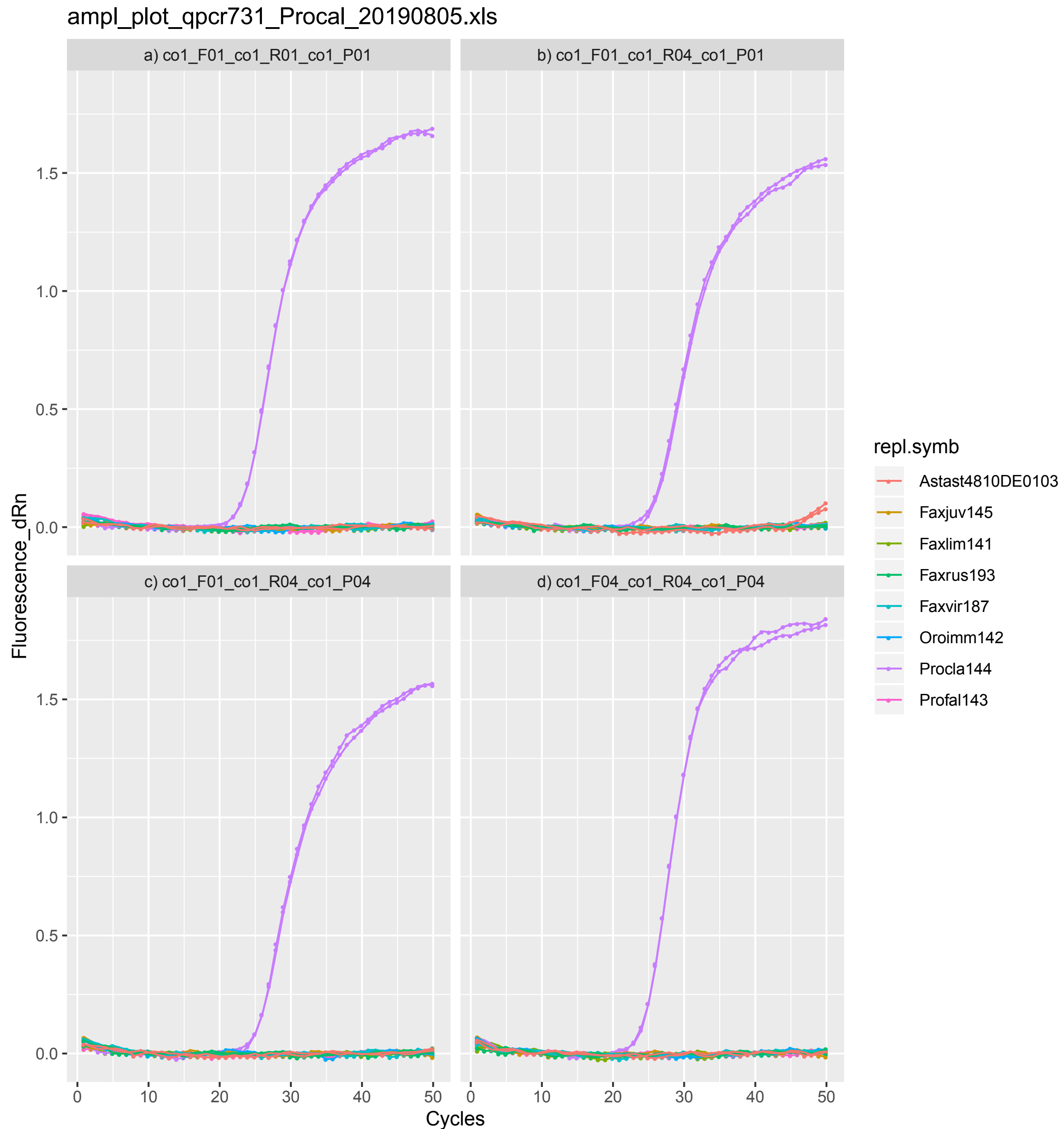

Figure 3.22. Amplification of Procambarus clarkii *species using the four species-specific primer and probe combinations designed in this study (a-d). Among the four primer combinations tested the combination of Procla_co1_F04+Procla_co1_R04+Procla_co1_P04 (d) returned the lowest Ct value and highest relative fluorescence. This assay was thus selected as the most specific for this study. The other colors represent: NTC' the negative control, 'Faxjuv145'* Faxonius juvenilis *[Kentucky_River_crayfish], 'Faxlim141'* Faxonius limosus *[spinycheek_crayfish], 'Faxrus193'* Faxonius rusticus *[rusty_crayfish], 'Faxvir187'* Faxonius virilis *[virile_crayfish], 'Oroimm142'* Faxonius immunis *[cali-co_crayfish], 'Procla144'* Procambarus clarkii *[Lousianna_flodkrebs], 'Profal143'* Procambarus fallax *[marmorkrebs].*

3.5 Species no. krebs_05: *Procambarus fallax*

Binomial nomenclature and author: *Procambarus fallax* (Hagen, 1870) – see figure 3.23.
English common name: Marbled crayfish
Danish common name: Marmorkrebs

Figure 3.23. Procambarus fallax. *Photo provided by the Danish Environmental Protection Agency.*

Procambarus fallax is non-indigenous in Europe (Figure 3.23). A specific assay was developed and tested for this project (Figure 3.24 and Table 3.13) and tested on DNA extracted from other species of crayfish (table 3.14).

Profal_co1_F01 5'-AGTTGAGAGGGGAGTAGGAAC-3'
Profal_co1_R01 5'-AGTTATACCAGCTGCCCGTA-3'
Profal_co1_P01 5'-FAM-AACTGTTTATCCTCCTTTAGCTTCTGC-BHQ1-3'

Table 3.13. *Assay developed during this study.*

Species	**Gene**	**Size** base pair (bp)	**Temp (°C)**	**Length (bp)**	**GC (%)**
Procambarus fallax	*mtDNA-co1*	181 bp			
Profal_co1_F01	5'-AGTTGAGAGGGGAGTAGGAAC-3'		56.5	21	52.4
Profal_co1_R01	5'-AGTTATACCAGCTGCCCGTA-3'		57.4	20	50.0
Profal_co1_P01	5'-FAM-AACTGTTTATCCTCCTTTAGCTTCTGC-BHQ1-3'		62.6	27	40.7

Table 3.14. In vitro *testing performed on DNA extracted from tissue samples and nucleotide sequences used for design and alignment of primers.*

Related species	Tested	Amplification	Acc. number or sequence
Astacus astacus	Yes	No	GU727619, JN254659-JN254681, 151_76_4810
Astacus leptodactylus	Yes	No	MF288079-MF288086
Cherax destructor	No	NA	KJ950555, KM039112
Cherax quadricarinatus	No	NA	NA
Cherax quinquecarinatus	No	NA	NA
Faxonius immunis	Yes	No	151_70_Oroimm, 151_72_Oroimm142, JF438005-JF438006
Faxonius juvenilis	Yes	No	151_66_Faxjuv146, 151_67_Faxjuv137, 151_75_Faxjuv145, AF474352, AY701233, JF437985, KT282396-KT282407, KT282419-KT282428
Faxonius limosus	Yes	No	JF911554, 151_68_Faxlim138, 151_71_Faxlim141
Faxonius rusticus	Yes	No	AY701249, KX238168, AY701248-AY701249
Faxonius virilis	Yes	No	FJ608577, EU442743
Pacifastacus fortis	No	NA	NA
Pacifastacus leniusculus	Yes	No	AF525226-AF525227, MF288087, JF437999, JF437995-JF437998, JF438000, 151_80_5691
Procambarus clarkii	Yes	No	151_69_Procla139, 151_74_Procla144
Procambarus fallax	Yes	Yes	151_73_Profal143

** Additional sequences from each species and additional not listed species were used in the alignment for increased accuracy and diversity coverage within and among other crayfish species.*

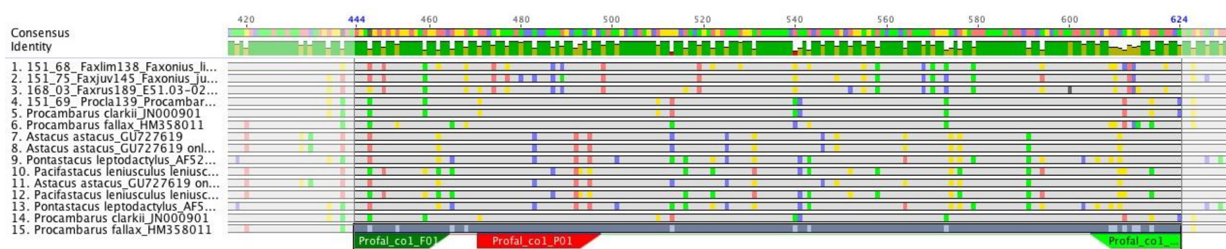

Figure 3.24. *Alignment of crayfish species for the mtDNA-co1 gene for the Profal_co1_F01+Profal_co1_R01+Profal_co1_P01 assay developed in the present study. Primers are marked with green annotations. The probe is indicated with a red annotation. Sequences were obtained acquired from NCBI GenBank and aligned in Geneious v. R7.*

Primers and probes tested

The following primers and probes were *in silico* designed and tested *in vitro* in PCR reactions set up as 'PCR setup 01' to find a species-specific combination of primers and probes:

Profal_co1_F01: 3'-AGTTGAGAGGGGAGTAGGAAC-5', Profal_co1_F02: 3'-TTGGTGGGTTTGGGAATTGA-5', Profal_co1_F03: 3'-ACTGGGTGAACTGTTTATCCTCC-5', Profal_co1_F04: 3'-GCTCCAGATATAGCTTTCCCTCG-5', Profal_co1_F05: 3'-TTCGGGTGGAGTTAGGTCAA-5', Profal_co1_P01: 3-FAM-'AACTGTTTATCCTCCTTTAGCTTCTGC-BHQ1-5', Profal_co1_P02: 3-FAM-'GCTCCAGATATAGCTTTCCCTCGAATA-BHQ1-5', Profal_co1_P04: 3-FAM-'ATATACGGGCAGCTGGTATAACTATG-BHQ1-5', Profal_co1_R01: 3'-AGTTATACCAGCTGCCCGTA-5', Profal_co1_R02: 3'-GGAGGATAAACAGTTCACCCAGT-5', Profal_co1_R03: 3'-TCCATAGTTATACCAGCTGCCC-5', Profal_co1_R04: 3'-ACTGACCAAACAAATAGCGGT-5', Profal_co1_R05: 3'-AGTTCCTACTCCCCTCTCAACT-5'.

The initial PCR results from the test performed using these primers are not included in this report

Assay specificity results

The two replicates of *Procambarus fallax* amplified at a Cq of 24 and 25, the replicates of (Figure 3.25a). None of the native species amplified with this Profal_co1_F01+Profal_co1_R01+Profal_co1_P01 (Figure 3.25a) primer-probe assay. One additional assay also returned species-specific amplification (Figure 3.25f). The designed eDNA target assay for *Procambarus fallax* is expected to only amplify DNA from the target species when tested on laboratory or environmental water samples.

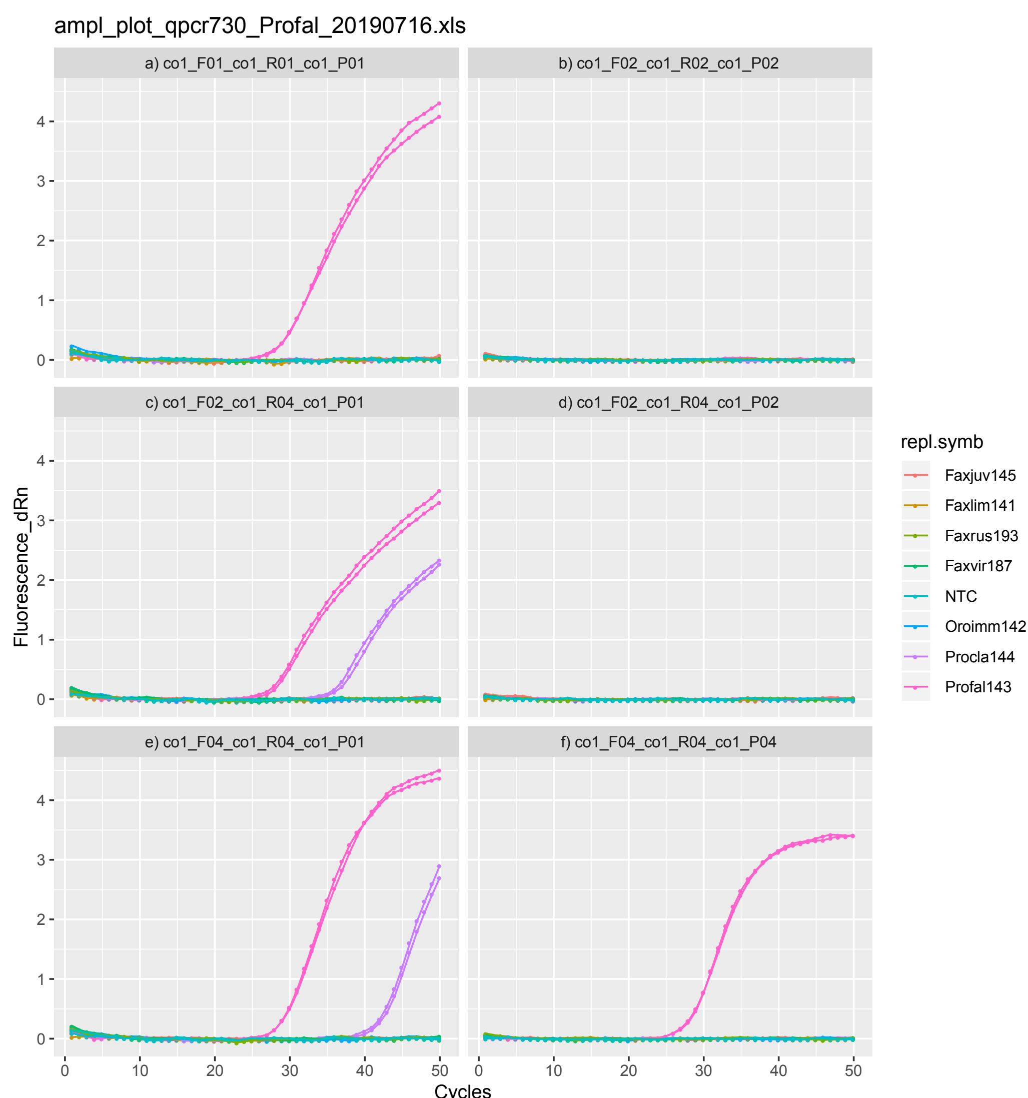

Figure 3.25. *Amplification of* Procambarus fallax (magenta red line) *species using the six species-specific primer and probe combinations designed and tested in this study (a-f). Among the six primer combinations tested the combination of Profal_co1_F01+Profal_co1_R01+Profal_co1_P01 (a) returned specific amplification at the lowest Ct value and highest relative fluorescence. This assay was thus selected as the most specific for this study. The other colors represent: NTC' the negative control, 'Faxjuv145'* Faxonius juvenilis *[Kentucky_River_crayfish], 'Faxlim141'* Faxonius limosus *[spinycheek_crayfish], 'Faxrus193'* Faxonius rusticus *[rusty_crayfish], 'Faxvir187'* Faxonius virilis *[virile_crayfish], 'Oroimm142'* Faxonius immunis *[calico_crayfish], 'Procla144'* Procambarus clarkii *[Lousianna_flodkrebs], 'Profal143'* Procambarus fallax *[marmorkrebs].*

3.6 Species no. krebs_06: *Faxonius juvenilis*

Binomial nomenclature and author: *Faxonius juvenilis* (Hagen, 1870) – see figure 3.26.
English common name: Kentucky River crayfish
Danish common name: Kentucky River crayfish

Figure 3.26. Faxonius juvenilis*. Photo provided by the Danish Environmental Protection Agency.*

Faxonius juvenilis is non-indigenous in Europe (Figure 3.26). A specific assay was developed and tested for this project (Figure 3.27 and Table 3.15) and tested on DNA extracted from other species of freshwater crayfish (Table 3.16).

Orcjuv_co1_F06 5'-CGGGAAGGTTAATTGGAGATGA-3'
Orcjuv_co1_R09 5'-CCTGTTCCAACTCCTCTTTCTAC-3'
Orcjuv_co1_P06 5'-FAM-TGGGGGATTTGGTAACTGGTTAATTCCT-BHQ1-3'

Table 3.15. *Assay developed during this study.*

Species	Gene	**Size** base pair (bp)	Temp (°C)	Length (bp)	GC (%)
Faxonius juvenilis	*mtDNA-co1*	244 bp			
Orcjuv_co1_F06	5'-CGGGAAGGTTAATTGGAGATGA-3'		62.3	22	45.5
Orcjuv_co1_R09	5'-CCTGTTCCAACTCCTCTTTCTAC-3'		58.5	23	47.8
Orcjuv_co1_P06	5'-FAM-TGGGGGATTTGGTAACTGGTTAATTCCT-BHQ1-3'		68.6	28	42.9

Table 3.16. In vitro *testing performed on DNA extracted from tissue samples and nucleotide sequences used for design and alignment of primers.*

Related species	Tested	Amplification	Acc. number or sequence
Astacus astacus	Yes	No	GU727619, JN254659-JN254681, 151_76_4810
Astacus leptodactylus	Yes	No	MF288079-MF288086
Cherax destructor	No	NA	KJ950555, KM039112
Cherax quadricarinatus	No	NA	NA
Cherax quinquecarinatus	No	NA	NA
Faxonius immunis	Yes	No	151_70_Oroimm, 151_72_Oroimm142, JF438005-JF438006
Faxonius juvenilis	Yes	Yes	151_66_Faxjuv146, 151_67_Faxjuv137, 151_75_Faxjuv145, AF474352, AY701233, JF437985, KT282396-KT282407, KT282419-KT282428
Faxonius limosus	Yes	No	JF911554, 151_68_Faxlim138, 151_71_Faxlim141
Faxonius rusticus	Yes	No	AY701249, KX238168, AY701248-AY701249
Faxonius virilis	Yes	No	FJ608577, EU442743
Pacifastacus fortis	No	NA	NA
Pacifastacus leniusculus	Yes	No	AF525226-AF525227, MF288087, JF437999, JF437995-JF437998, JF438000, 151_80_5691
Procambarus clarkii	Yes	No	151_69_Procla139, 151_74_Procla144
Procambarus fallax	Yes	No	151_73_Profal143

** Additional sequences from each species and additional not listed species were used in the alignment for increased accuracy and diversity coverage within and among other crayfish species.*

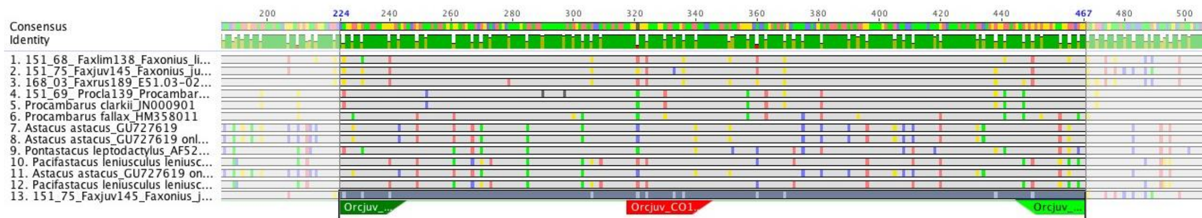

Figure 3.27. *Alignment of crayfish species for the mtDNA-co1 gene. Primers are marked with green annotations. The probe is indicated with a red annotation. Sequences were obtained acquired from NCBI GenBank and aligned in Geneious.*

Primers and probes tested

The following primers and probes were *in silico* designed and tested *in vitro* in PCR reactions set up as 'PCR setup 01' to find a species-specific combination of primers and probes:

Orcjuv_co1_F01: 3'-CGGGGAGGTTAATTGGAGATGA-5', Orcjuv_co1_F02: 3'-GGGGCTTAACAGGGG-TAGTA-5', Orcjuv_co1_F03: 3'-GGATACCTCGGCGTTATTCAGA-5', Orcjuv_co1_F04: 3'-TCGGCGT-TATTCAGATTACCCA-5', Orcjuv_co1_F05: 3'-TCGAGTAGAGTTAGGTCAGCC-5', Orcjuv_CO1_F06: 3'-CGGGAAGGTTAATTGGAGATGA-5', Orcjuv_CO1_F08: 3'-AGGGGAATAGTAGAAAGAGGAGT-5', Orcjuv_CO1_F09: 3'-GCATTTGAGCTGGTATAGTAGGA-5', Orcjuv_co1_P04: 3-FAM-'TACCTACTTCAA-TAGAGTGGCAGCATT-BHQ1-5', Orcjuv_CO1_P06: 3-FAM-'TGGGGGATTTGGTAACTGGTTAATTCCT-BHQ1-5', Orcjuv_CO1_P09: 3-FAM-'GAGTAGAGTTAGGTCAGCCGGGAAGGT-BHQ1-5', Orcjuv_co1_R01: 3'-AAAGCCATATCAGGTGCCCC-5', Orcjuv_co1_R02: 3'-ACGCCGAGGTATCCCATTAAG-5', Orcjuv_co1_R03: 3'-GGTGGAAAAGAATGCTGCCA-5', Orcjuv_co1_R04: 3'-AGTGTGATCAGCAGG-TGGAA-5', Orcjuv_co1_R05: 3'-TCACCCTGTTCCAACTCCTC-5', Orcjuv_CO1_R07: 3'-ACCCTGTTCCAACTCCTCTTTC-5', Orcjuv_CO1_R08: 3'-ACCCCTGCCAAATGTAACGA-5', Orcjuv_CO1_R09: 3'-CCTGTTCCAACTCCTCTTTCTAC-5'.

The initial PCR results from the test performed using these primers are not included in this report

Assay specificity results

The two replicates of *Faxonius juvenilis* amplified at a Cq of 25 (Figure 3.28b). Two additional assays also returned species-specific amplification (Figure 3.28a and c).

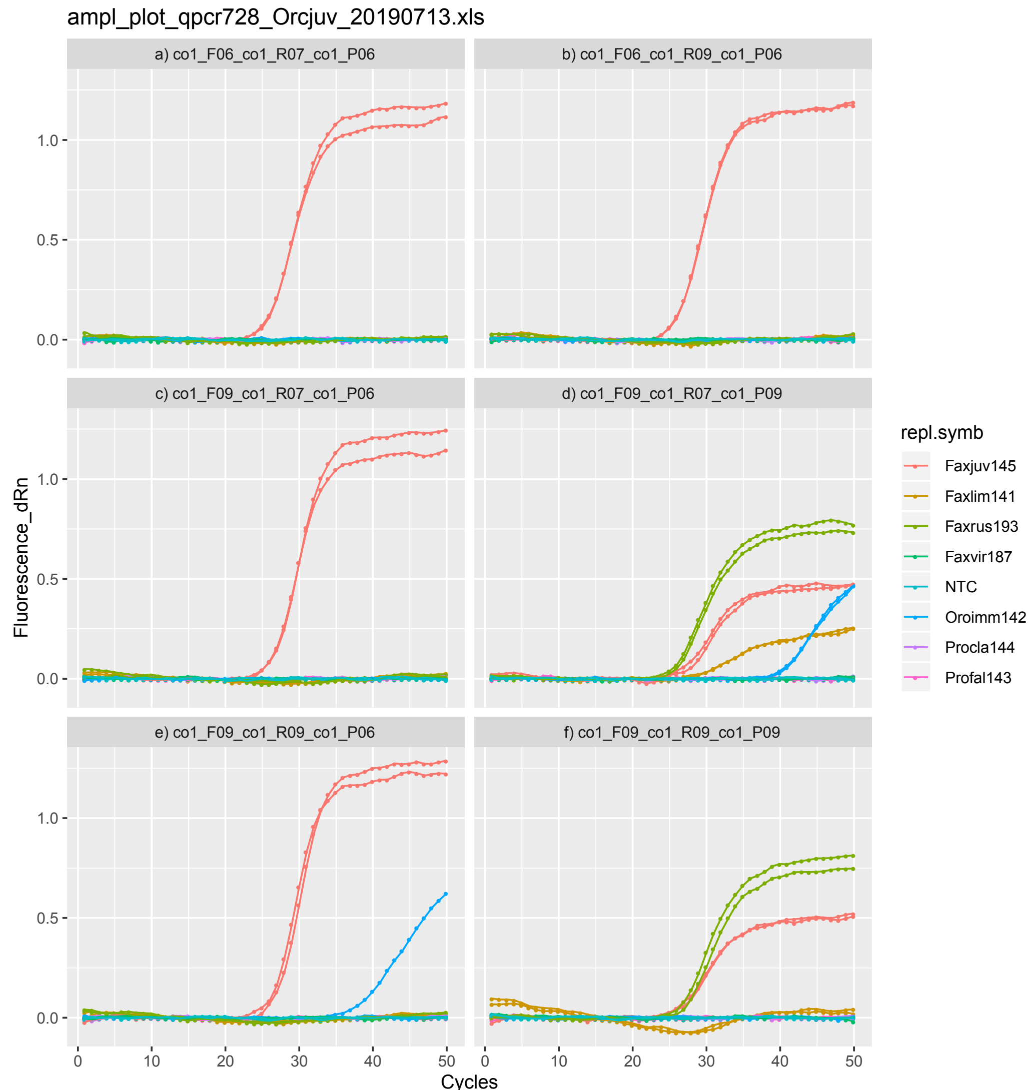

Figure 3.28. *Amplification of* Faxonius juvenilis *species using different primers and probes Target species* Faxonius juvenilis *is shown in red and non-target sister species in other colors. The co1_F06-co1_R09-co1_P06 assay (b) is specific against only* Faxonius juvenilis *and returns amplification at the earliest Cq-treshold and gives the highest relative flourescense. The other colors represent: NTC' the negative control, 'Faxjuv145'* Faxonius juvenilis *[Kentucky_River_crayfish], 'Faxlim141'* Faxonius limosus *[spinycheek_crayfish], 'Faxrus193'* Faxonius rusticus *[rusty_crayfish], 'Faxvir187'* Faxonius virilis *[virile_crayfish], 'Oroimm142'* Faxonius immunis *[calico_crayfish], 'Procla144'* Procambarus clarkii *[Lousianna_flodkrebs], 'Profal143'* Procambarus fallax *[marmorkrebs]. Among the other primer probe assays tested two additional combinations also returns specificity (a and c), but with a slightly later onset of Ct.*

3.7 Species no. krebs_07: *Faxonius limosus*

Binomial nomenclature and author: *Faxonius limosus* (Rafinesque, 1817) – see figure 3.29.
English common name: Spiny-cheek crayfish
Danish common name: Amerikansk flodkrebs

Figure 3.29. Faxonius limosus*. Photo provided by the Danish Environmental Protection Agency.*

Faxonius limosus is non-indigenous in Europe (Figure 3.29). A specific assay was developed and tested for this project (Figure 3.30 and Table 3.17) and tested on DNA extracted from other species of freshwater crayfish (Table 3.18).

Orclim_co1_F03 5'-GTTGGGTCAGCTGGGAAGTT-3'
Orclim_co1_R01 5'-GTCATTCCTGTGGCCCGTAT-3'
Orclim_co1_P03 5'-FAM-TGGAGGATTTGGTAATTGGTTAATTCCT-BHQ1-3'

Table 3.17. *Assay developed during this study.*

Species	Gene	Size base pair (bp)	Temp (°C)	Length (bp)	GC (%)
Faxonius limosus	*mtDNA-co1*	411 bp			
Orclim_co1_F03	5'-GTTGGGTCAGCTGGGAAGTT-3'		61.5	20	55.0
Orclim_co1_R01	5'-GTCATTCCTGTGGCCCGTAT-3'		62.1	20	55.0
Orclim_co1_P03	5'-FAM-TGGAGGATTTGGTAATTGGTTAATTCCT-BHQ1-3'		65.4	28	35.7

Table 3.18. In vitro *testing performed on DNA extracted from tissue samples and nucleotide sequences used for design and alignment of primers.*

Related species	Tested	Amplification	Acc. number or sequence
Astacus astacus	Yes	No	GU727619, JN254659-JN254681, 151_76_4810
Astacus leptodactylus	Yes	No	MF288079-MF288086
Cherax destructor	No	NA	KJ950555, KM039112
Cherax quadricarinatus	No	NA	NA
Cherax quinquecarinatus	No	NA	NA
Faxonius immunis	Yes	No	151_70_Oroimm, 151_72_Oroimm142, JF438005-JF438006
Faxonius juvenilis	Yes	No	151_66_Faxjuv146, 151_67_Faxjuv137, 151_75_Faxjuv145, AF474352, AY701233, JF437985, KT282396-KT282407, KT282419-KT282428
Faxonius limosus	Yes	Yes	JF911554, 151_68_Faxlim138, 151_71_Faxlim141
Faxonius rusticus	No	NA	AY701249, KX238168, AY701248-AY701249
Faxonius virilis	Yes	No	FJ608577, EU442743
Pacifastacus fortis	No	NA	NA
Pacifastacus leniusculus	Yes	No	AF525226-AF525227, MF288087, JF437999, JF437995-JF437998, JF438000, 151_80_5691
Procambarus clarkii	Yes	No	151_69_Procla139, 151_74_Procla144
Procambarus fallax	Yes	Yes	151_73_Profal143

** Additional sequences from each species and additional not listed species were used in the alignment for increased accuracy and diversity coverage within and among other crayfish species.*

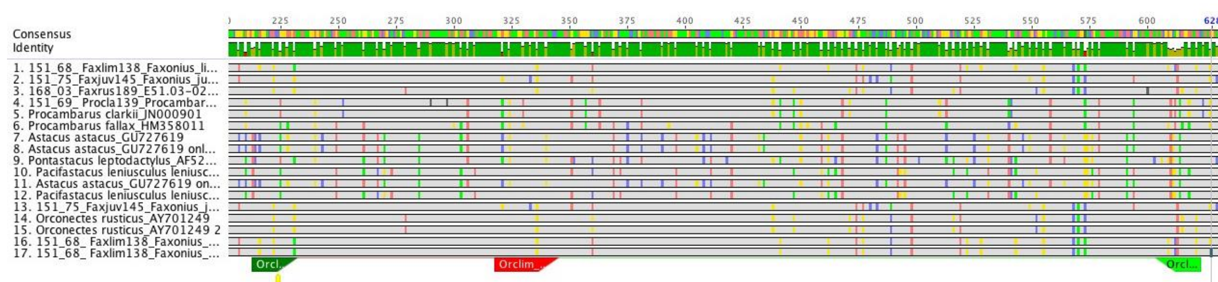

Figure 3.30. *Alignment of crayfish species for the mtDNA-co1 gene. For the Orclim_co1_F03, Orclim_co1_R01, and Orclim_co1_P03 primers and probe. Primers are marked with green annotations. The probe is indicated with a red annotation. Sequences were obtained acquired from NCBI GenBank and aligned in Geneious.*

Primers and probes tested

The following primers and probes were *in silico* designed and tested *in vitro* in PCR reactions set up as 'PCR setup 01' to find a species-specific combination of primers and probes:

Orclim_co1_F01: 3'-AGGGGCATCAGTGGATTTGG-5', Orclim_co1_F02: 3'-TAGAGTTGGGTCAGCTGGGA-5', Orclim_co1_F03: 3'-GTTGGGTCAGCTGGGAAGTT-5', Orclim_co1_F04: 3'-TGGGACAGGGTGAACAGTGT-5', Orclim_co1_F05: 3'-CGAGTAGAGTTGGGTCAGCTG-5', Orclim_co1_P03: 3-FAM-'TGGAGGATTTGGTAATTGGTTAATTCCT-BHQ1-5', Orclim_co1_P04: 3-FAM-'CTCTCGCTTCTGCAATTGCTCATG-BHQ1-5', Orclim_co1_R01: 3'-GTCATTCCTGTGGCCCGTAT-5', Orclim_co1_R02: 3'-ACCCTGTCCCAACTCCTCTT-5', Orclim_co1_R03: 3'-AAAGCCATATCAGGTGCCCC-5', Orclim_co1_R04: 3'-CCAAATCCACTGATGCCCCT-5', Orclim_co1_R05: 3'-CACTGTTCACCCTGTCCCAA-5'.

The initial PCR results from the test performed using these primers are not included in this report

Assay specificity results

The two replicates of *Faxonius limosus* amplified at a Cq of 24 and 25, the replicates of (Figure 3.31). None of the native species amplified with this primer-probe assay.

The designed eDNA target assay for *Faxonius limosus* is expected to only amplify DNA from the target species when tested on laboratory or environmental water samples.

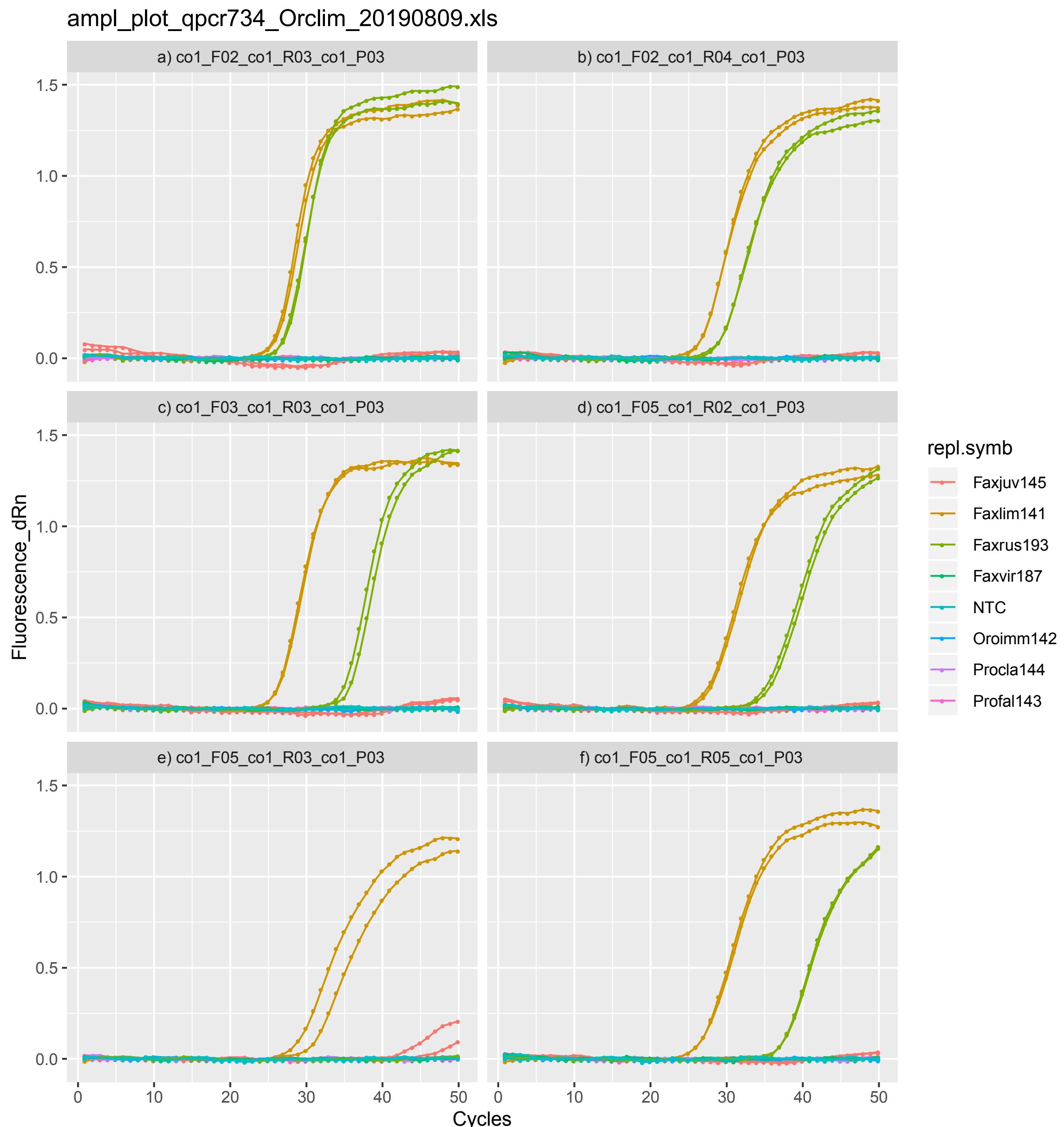

Figure 3.31. *Amplification of* Faxonius *limosus species (Faxlim141) using the assay developed in this study. Target species* Faxonius limosus *is shown in orange and non-target sister species in other colors. The co1_F03-co1_R05-co1_P02 assay (e) is specific against* Faxonius rusticus *and* Faxonius limosus *and returns amplification at the earliest Cq-treshold and gives the highest relative flourescense. 'NTC' the negative control, 'Faxjuv145'* Faxonius juvenilis *[Kentucky_River_crayfish], 'Faxlim141'* Faxonius limosus *[spinycheek_crayfish], 'Faxrus193'* Faxonius rusticus *[rusty_crayfish], 'Faxvir187'* Faxonius virilis *[virile_crayfish], 'Oroimm142'* Faxonius immunis *[calico_crayfish], 'Procla144'* Procambarus clarkii *[Lousianna_flodkrebs], 'Profal143'* Procambarus fallax *[marmorkrebs].*

3.8 Species no. krebs_08: *Faxonius rusticus*

Binomial nomenclature and author: *Faxonius rusticus* (Girard, 1852) – see figure 3.32.
English common name: Rusty crayfish
Danish common name: Rustfarvet flodkrebs

Figure 3.32. Faxonius rusticus*. Photo provided by the Danish Environmental Protection Agency.*

Faxonius rusticus is non-indigenous in Europe (Figure 3.32). A specific assay was developed and tested for this project (Figure 3.33 and Table 3.19) and tested on DNA extracted from other species of freshwater crayfish (Table 3.20).

Orcrus_co1_F03 5'-CGGGAAGGTTAATTGGAGATGAC-3'
Orcrus_co1_R02 5'-AAATCTACTGACGCCCCTGC-3'
Orcrus_co1_P02 5'-FAM-ACAGTGTATCCTCCTCTCGCTTCTGCA-BHQ1-3'

Table 3.19. *Assay developed during this study*

Species	Gene	**Size** base pair (bp)	Temp (°C)	Length (bp)	GC (%)
Faxonius rusticus	*mtDNA-co1*	304 bp			
Orcrus_co1_F03	5'-CGGGAAGGTTAATTGGAGATGAC-3'		62.5	23	43.5
Orcrus_co1_R02	5'-AAATCTACTGACGCCCCTGC-3'		61.5	20	55.0
Orcrus_co1_P02	5'-FAM-ACAGTGTATCCTCCTCTCGCTTCTGCA-BHQ1-3'		69.3	27	51.9

Table 3.20. In vitro *testing performed on DNA extracted from tissue samples and nucleotide sequences used for design and alignment of primers.*

Related species	Tested	Amplification	Acc. number or sequence
Astacus astacus	Yes	No	GU727619, JN254659-JN254681, 151_76_4810
Astacus leptodactylus	Yes	No	MF288079-MF288086
Cherax destructor	No	NA	KJ950555, KM039112
Cherax quadricarinatus	No	NA	NA
Cherax quinquecarinatus	No	NA	NA
Faxonius immunis	Yes	No	151_70_Oroimm, 151_72_Oroimm142, JF438005-JF438006
Faxonius juvenilis	Yes	No	151_66_Faxjuv146, 151_67_Faxjuv137, 151_75_Faxjuv145, AF474352, AY701233, JF437985, KT282396-KT282407, KT282419-KT282428
Faxonius limosus	Yes	Yes	JF911554, 151_68_Faxlim138, 151_71_Faxlim141
Faxonius rusticus	Yes	Yes	AY701249, KX238168, AY701248-AY701249
Faxonius virilis	Yes	No	FJ608577, EU442743
Pacifastacus fortis	No	NA	NA
Pacifastacus leniusculus	Yes	No	AF525226-AF525227, MF288087, JF437999, JF437995-JF437998, JF438000, 151_80_5691
Procambarus clarkii	Yes	No	151_69_Procla139, 151_74_Procla144
Procambarus fallax	Yes	No	151_73_Profal143

** Additional sequences from each species and additional not listed species were used in the alignment for increased accuracy and diversity coverage within and among other crayfish species.*

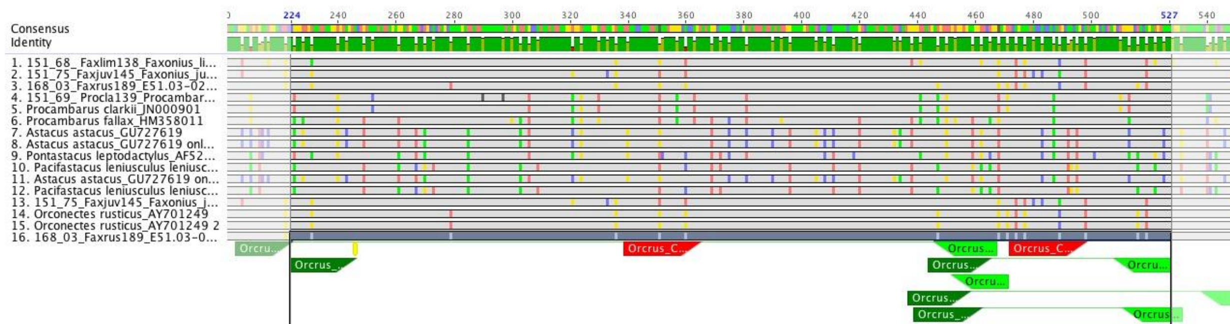

Figure 3.33. *Alignment of crayfish species for the mtDNA-co1 gene, with the species-specific primers and probe (Orcrus_co1_F03, Orcrus_co1_R02, Orcrus_co1_P02) mapped Primers are marked with green annotations. The probe is indicated with a red annotation. Sequences were obtained acquired from NCBI GenBank and aligned in Geneious.*

Primers and probes tested

The following primers and probes were *in silico* designed and tested *in vitro* in PCR reactions set up as 'PCR setup 01' to find a species-specific combination of primers and probes:

Orcrus_CO1_F01: 3'-CGGGTAGAGTTAGGTCAGCC-5', Orcrus_CO1_F02: 3'-AGTGGAAAGAGGAGT-TGGAACA-5', Orcrus_CO1_F03: 3'-CGGGAAGGTTAATTGGAGATGAC-5', Orcrus_CO1_F04: 3'-GGGGAATAGTGGAAAGAGGAGT-5', Orcrus_CO1_F05: 3'-GGAATAGTGGAAAGAGGAGTTGGA-5', Orcrus_CO1_P01: 3-FAM-'AATTCCTTTAATGTTAGGGGCGCCTGA-BHQ1-5', Orcrus_CO1_P02: 3-FAM-'ACAGTGTATCCTCCTCTCGCTTCTGCA-BHQ1-5', Orcrus_CO1_R01: 3'-CCTGTTCCAACTCCTCTTTCCA-5', Orcrus_CO1_R02: 3'-AAATCTACTGACGCCCCTGC-5', Orcrus_CO1_R03: 3'-CCACCCTGTTCCAACTCCTC-5', Orcrus_CO1_R04: 3'-ACCCCGGCTAAATGTAACGA-5', Orcrus_CO1_R05: 3'-ACCTAAATCTACTGA-

CGCCCC-5', Orusticus_COI_5F: 3'-CAGGGGCGTCAGTAGATTTAGGTAT-5', Orusticus_COI_5R: 3'-CAT-TCGATCTATAGTCATTCCCGTAG-5'.

The initial PCR results from the test performed using these primers are not included in this report.

Assay specificity results

The two replicates of *Faxonius rusticus* amplified at a Cq of 22 and 22 (Figure 3.34). However, the primer combination is not specific for *Faxonius rusticus* alone, but also amplifies on DNA from *Faxonius* limosus at Cq>38.

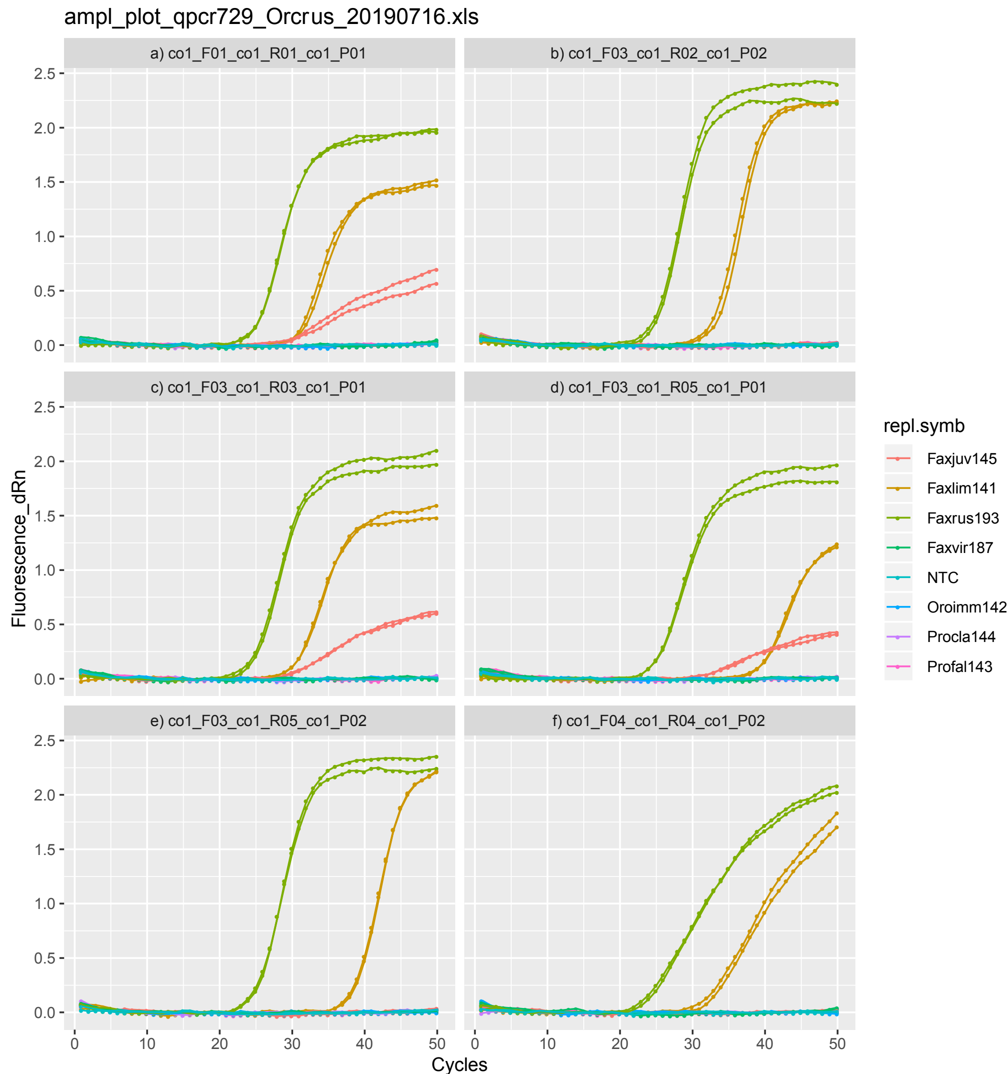

Figure 3.34. *Amplification of* Faxonius rusticus *species using different primers and probes. Target species* Faxonius rusticus *is shown in green and non-target sister species in other colors. The co1_F03-co1_R05-co1_P02 assay (e) is specific against* Faxonius rusticus *and* Faxonius limosus *and returns amplification at the earliest Cq-treshold and gives the highest relative flourescense. 'NTC' the negative control, 'Faxjuv145'* Faxonius juvenilis *[Kentucky_River_crayfish], 'Faxlim141'* Faxonius limosus *[spinycheek_crayfish], 'Faxrus193'* Faxonius rusticus *[rusty_crayfish], 'Faxvir187'* Faxonius virilis *[virile_crayfish], 'Oroimm142'* Faxonius immunis *[calico_crayfish], 'Procla144'* Procambarus clarkii *[Lousianna_flodkrebs], 'Profal143'* Procambarus fallax *[marmorkrebs].*

3.9 Species no. krebs_09: *Faxonius virilis*

Binomial nomenclature and author: *Faxonius virilis* (Hagen, 1870) – see figure 3.35.
English common name: Virile crayfish
Danish common name: Viril krebs

Figure 3.35. Faxonius virilis*. Photo provided by the Danish Environmental Protection Agency.*

Faxonius virilis is non-indigenous in Europe (Figure 3.35). A specific assay was developed and tested for this project (Figure 3.36 and Table 3.21)) and tested on DNA extracted from other species of freshwater crayfish (Table 3.22).

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Faxvir_co1_F05 5'-CAGGAAGATTGATTGGGGACGA-3'
Faxvir_co1_R01 5'-GTTATCCCTGCAGCCCGTAT-3'
Faxvir_co1_P01 5'-FAM-TTGGAGGTTTCGGGAACTGGCTGATTC-BHQ1-3'

Table 3.21. *Assay developed during this study.*

Species	Gene	**Size** base pair (bp)	Temp (°C)	Length (bp)	GC (%)
Faxonius virilis	*mtDNA-co1*	400 bp			
Faxvir_co1_F05	5'-CAGGAAGATTGATTGGGGACGA-3'		65.5	22	50.0
Faxvir_co1_R01	5'-GTTATCCCTGCAGCCCGTAT-3'		61.2	20	55.0
Faxvir_co1_P01	5'-FAM-TTGGAGGTTTCGGGAACTGGCTGATTC-BHQ1-3'		73.1	27	51.9

Table 3.22. In vitro *testing performed on DNA extracted from tissue samples and nucleotide sequences used for design and alignment of primers.*

Related species	Tested	Amplification	Acc. number or sequence
Astacus astacus	Yes	No	GU727619, JN254659-JN254681, 151_76_4810
Astacus leptodactylus	Yes	No	MF288079-MF288086
Cherax destructor	No	NA	KJ950555, KM039112
Cherax quadricarinatus	No	NA	NA
Cherax quinquecarinatus	No	NA	NA
Faxonius immunis	Yes	No	151_70_Oroimm, 151_72_Oroimm142, JF438005-JF438006
Faxonius juvenilis	Yes	No	151_66_Faxjuv146, 151_67_Faxjuv137, 151_75_Faxjuv145, AF474352, AY701233, JF437985, KT282396-KT282407, KT282419-KT282428
Faxonius limosus	Yes	No	JF911554, 151_68_Faxlim138, 151_71_Faxlim141
Faxonius rusticus	Yes	No	AY701249, KX238168, AY701248-AY701249
Faxonius virilis	Yes	Yes	FJ608577, EU442743
Pacifastacus fortis	No	NA	NA
Pacifastacus leniusculus	Yes	No	AF525226-AF525227, MF288087, JF437999, JF437995-JF437998, JF438000, 151_80_5691
Procambarus clarkii	Yes	No	151_69_Procla139, 151_74_Procla144
Procambarus fallax	Yes	No	151_73_Profal143

** Additional sequences from each species and additional not listed species were used in the alignment for increased accuracy and diversity coverage within and among other crayfish species.*

Figure 3.36. *Alignment of crayfish species for the mtDNA-co1 gene, with the species-specific primers and probe (Faxvir_co1_F05, Faxvir_co1_R01, Faxvir_co1_P01) mapped. Primers are marked with green annotations. The probe is indicated with a red annotation. Sequences were obtained acquired from NCBI GenBank and aligned in Geneious v. R7.*

Primers and probes tested

The following primers and probes were *in silico* designed and tested *in vitro* in PCR reactions set up as 'PCR setup 01' to find a species-specific combination of primers and probes:

Faxvir_CO1_F01: 3'-CGAGTAGAGTTAGGCCAGCC-5', Faxvir_CO1_F02: 3'-ATACGGGCTGCAGGGATAAC-5', Faxvir_CO1_F03: 3'-AGCCAGGAAGATTGATTGGGG-5', Faxvir_CO1_F04: 3'-CGGGCTGCA-GGGATAACTAT-5', Faxvir_CO1_F05: 3'-CAGGAAGATTGATTGGGGACGA-5', Faxvir_CO1_P01: 3-FAM-'TTGGAGGTTTCGGGAACTGGCTGATTC-BHQ1-5', Faxvir_CO1_P02: 3-FAM-'CGTATACCGTTATTT-GTTTGGTCAGTGT-BHQ1-5', Faxvir_CO1_R01: 3'-GTTATCCCTGCAGCCCGTAT-5', Faxvir_CO1_R02: 3'-CTCCAGCTAAAACGGGCAAAG-5', Faxvir_CO1_R03: 3'-ATAGTTATCCCTGCAGCCCG-5', Faxvir_CO1_R04: 3'-GATCCCCACCACCTGCAG-5', Faxvir_CO1_R05: 3'-ATCCCTGCAGCCCGTATATT-5'.

The initial PCR results from the test performed using these primers are not included in this report.

Assay specificity results

The two replicates of *Faxonius virilis* amplified at a Cq of 28 (Figure 3.37). The primer combination F05-R01-P01 is specific for *Faxonius virilis*.

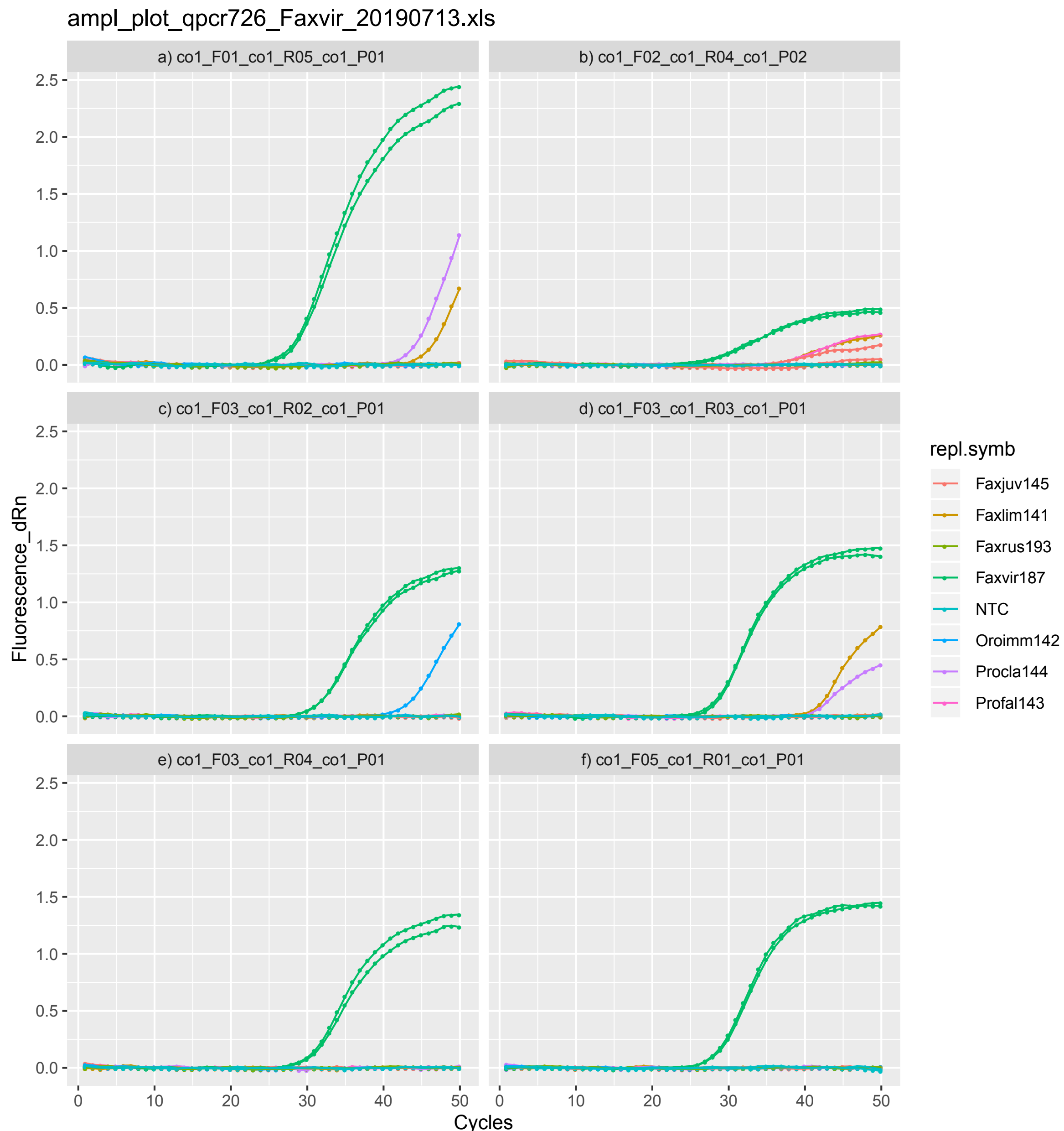

Figure 3.37. *Amplification of* Faxonius virilis *species using different primers and probes Target species* Faxonius virilis *is shown in green and non-target sister species in other colors. The co1_F05-co1_R01-co1_P01 assay (f) is specific against Faxonius virilis and returns amplification at the earliest Cq-treshold and gives the highest relative flourescense. The other colors represent: 'NTC' the negative control, 'Faxjuv145'* Faxonius juvenilis *[Kentucky_River_crayfish], 'Faxlim141'* Faxonius limosus *[spiny-cheek_crayfish], 'Faxrus193'* Faxonius rusticus *[rusty_crayfish], 'Faxvir187'* Faxonius virilis *[vir-ile_crayfish], 'Oroimm142'* Faxonius immunis *[calico_crayfish], 'Procla144'* Procambarus clarkii *[Lousianna_flodkrebs], 'Profal143'* Procambarus fallax *[marmorkrebs].*

4 Discussion and conclusions

Apart from the assay developed against *Faxonius rusticus,* all nine assays presented are specific for the intended target species alone. The assay developed *for Faxonius rusticus* can be considered species-specific when a Ct-cut off is set at 35 cycles.

The species-specific assays presented by Agersnap *et al.* (2017) and Tregiuer *et al.* (2014) were found to perform with an insufficient specificity (Fig. 3.4, 3.10, 3.15, 3.16 and 3.21) when compared to the assays developed in the present study (Fig. 3.5, 3.6, 3.11, 3.17 and 3.22).

Optimal concentrations for individual reactions in a qPCR setup should be determined for both primers and for the probe for each of the assays before a large-scale analysis of filtered water samples is attempted. Inferring optimal concentrations will ensure that primers and probes can detect very low levels of eDNA in water samples. How low can be determined with qPCR tests with inclusion of standard dilution series that will make it possible to determine the limit of detection (LOD) and the limit of quantification (LOQ), see the study by Agersnap *et al.* (2017) and Knudsen *et al.* (2019) for details on the methodology.

The present study aimed at being able to detect eDNA from the non-indigenous *Procambarus fallax* (Hagen, 1870). However, in 2017, this freshwater crayfish was found to be a species complex comprised of two species – i.e. *Procambarus fallax* (Hagen, 1870) and *Procambarus virginalis* (Lyko, 2017). The primers and probes designed to be specific against *Procambarus fallax* were designed from nucleotide sequences deposited on NCBI GenBank prior to 2017. This means that the species-specific primers designed and tested against *Procambarus fallax* in the present study also can be considered capable of detecting eDNA from *Procambarus virginalis*. The primers and probes tested in this study on tissue from *Procambarus fallax* must be considered unable to distinguish between *Procambarus fallax* and *Procambarus virginalis*. Because of this the primers and probe designed for *Procambarus fallax* in this study, will return positive qPCR amplifycation on eDNA from both *Procambarus fallax* and *Procambarus virginalis*, and will not be able to determine whether the eDNA detected stems from *Procambarus fallax* or *Procambarus virginalis*. Since both species are considered non-indigenous in Scandinavian freshwater streams this issue can be considered irrelevant.

In initial tests performed in other laboratories in Denmark (Eurofins and Amphi Consult) the new assays targeting *Astacus astacus* and *Pacifastacus leniusculus* was inable to amplify on the positive DNA controls prepared by the MONIS project. However, secondary tests using a standard dilution series of positive controls was not able to reproduce the failure in these two assays, as seen by Eurofins and Amphi Consult. The additional tests performed on these two assays included four replicates of positive controls comprised of DNA extracted from two different individuals of both *Astacus astacus* and *Pacifastacus leniusculus*. As these two assays appear to have failed in successfully amplifying the positive controls in two external laboratories it is recommended that the positive controls are monitored closely, when these two assays are used.

The pathogenic fungus *Aphanomyces astaci* (i.e. crayfish plague) is considered a considerable threat to the survival of the endemic populations of *Astacus astacus* (i.e. Danish common name: 'flodkrebs'), in relation to this treat from *Aphanomyces astaci* it is worth noting that a recent study detected eDNA from *Procambarus virginalis* in Northern Germany and also found *Aphanomyces astaci* (i.e. crayfish plague) to be absent whenever *Procambarus virginalis* was detected (Mauvisseau *et al.,* 2019).

We conclude as follows:

- Of the many primer- probe combinations tested for each of the nine species of freshwater crayfish, a species-specific combination was found for each species.
- The species-specific primers developed and tested for *Astacus astacus*, *Astacus leniusculus*, *Pontastacus leptodactylus* and *Procambarus clarkii* in this study, appear to be more sensitive to low levels of eDNA and to perform better than the species-specific primers and probes previously published (Agersnap *et al*., 2017 and Treguier *et al*., 2014).
- The nine species-specific primer probe systems presented in this report can be used for monitoring freshwater crayfish occurrences in Northern Europe but will require further testing if they are to be used on water samples collected outside Northern Europe. Such further testing will help to infer whether primers and probes reported in this study also are species-specific when used for freshwater samples collected other parts of Europe?, where species tested might have differences in the gene region targeted by the primers and probes designed and tested in this study.
- The two new assays for *Astacus astacus* and *Pacifastacus leniusculus* must be monitored carefully for the positive controls when used, in case the inability to make these two assays work in external laboratories is a continuous problem. As the laboratory in Copenhagen have been unable to reproduce this failure in these two assays, we still recommend applying these two assays for detection of *Astacus astacus* and *Pacifastacus leniusculus*.

Further, we suggest carrying out a number of follow-up activities:

- The sensitivity of each of the developed specific assays is influenced by the concentrations of each of the reagents in the qPCR set up. The performance of each assays the and ability to detect the eDNA targeted can be increased by making sure the primers and probes are added to each reaction in optimal concentrations. This will ensure that even low levels of targeted eDNA is possible to detect in the qPCR setup. The optimal concentrations for the primers can be inferred from a relatively simple qPCR setup that checks how a gradient in concentrations of added primer and probe influences the performance of detection. Such a test of optimal concentrations is performed on known concentrations of positive controls to be able to compare the efficiency of the amplifications on the gradient of the primers. A test that infers the optimal concentrations of the primers can be performed as described in previous studies on species specific eDNA detection (Agersnap *et al*., 2017; Knudsen *et al*., 2019). To increase sensitivity and performance of the primers and probes presented in this report, it is strongly recommended that the optimal concentration in final qPCR reaction volumes is determined for each primer and probe, before any tests are performed on filtered water samples.
- Furthermore, it is strongly recommended that all tests performed on filtered water samples using these primer- and probe combinations are performed with the inclusion of a standard dilution series of a dsPCR amplicon that can serve as both positive control, but also will help evaluate the limit of detection (LOD) and limit of quantification (LOQ). For further details on setting up such standard dilution series we here refer to previous methods (Agersnap *et al*., 2017; Knudsen *et al.*, 2019) and for inferring LOD and LOQ we refer to previous published studies on analysis of eDNA levels inferred with qPCR (Ellison *et al.*, 2016 and Ficotela *et al*., 2014).

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Njalsgade 76, 4th floor
2300 Copenhagen S
Denmark
Telephone: +45 39 17 97 33
E-mail: mail@niva-dk.dk
CVR/VAT no.: 35431063
www.niva-denmark.dk