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Development of species-specific eDNA-based test systems for monitoring of non-indigenous Decapoda in Danish marine waters

NIVA Denmark Water Research

REPORT

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Summary

We report the development of seven eDNA-based species-specific test systems for monitoring of marine Decapoda in Danish marine waters. The seven species are 1) *Callinectes sapidus* (blå svømmekrabbe), 2) *Eriocheir sinensis* (kinesisk uldhåndskrabbe), 3) *Hemigrapsus sanguineus* (stribet klippekrabbe), 4) *Hemigrapsus takanoi* (pensel-klippekrabbe), 5) *Homarus americanus* (amerikansk hummer), 6) *Paralithodes camtschaticus* (Kamchatka-krabbe) and 7) *Rhithropanopeus harrisii* (østamerikansk brakvandskrabbe). The following three are new developments: *Callinectes sapidus*, *Hemigrapsus sanguineus* and *Hemigrapsus takanoi*, whilst the remaining four previously developed under earlier phases of the MONIS projects have been tested again. The additional tests on the four previously developed assays were performed with a broader diversity of Decapoda known from Danish seas, to re-evaluate the specificity of these assays. This additional testing revealed that two previously published assays were unspecific, and to accommodate this, two new assays are presented replacing those previously published. We recommend that the species-specific eDNA assays presented here will allow for continuous monitoring of these species as part of the NOVANA monitoring programme.

Four keywords	Fire emneord
1. Non-indigenous species	1. Ikke-hjemmehørende arter
2. eDNA	2. eDNA
3. Monitoring	3. Overvågning
4. Marine Strategy Framework Directive	4. Havstrategidirektivet

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MONIS 5

Development of species-specific eDNA-based test systems for monitoring of non-indigenous Decapoda in Danish marine waters

Client: Danish Environmental Protection Agency

Preface

We report the development of seven species-specific eDNA-based test systems for monitoring of non-indigenous marine Decapoda in Danish marine waters. The work has been funded by the Danish Environmental Protection Agency as an additional activity from the MONIS project ('Monitoring of Non-Indigenous Species in Danish Marine Waters') and been carried out collectively by NIVA Denmark (lead partner) and the Natural History Museum of Denmark (NHMD).

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'Fiskeri- og Søfartsmuseet' in Esbjerg for providing samples from Decapoda, and

Henrik Carl at the Natural History Museum of Denmark for loan of photographs, including the photo of *Eriocheir sinensis*.

More information about the MONIS project (phases 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' (Andersen *et al.* 2016),

'Development of species-specific eDNA-based test systems for monitoring of non-indigenous species in Danish marine waters' (Andersen *et al.* 2018),

'Tekniske anvisninger for eDNA-baseret overvågning af ikke-hjemmehørende marine arter' (Knudsen *et al*. 2018), and

'A baseline study of the occurrence of non-indigenous species in Danish harbours' (Andersen *et al.* 2019).

Copenhagen, 22 October 2020

Jesper H. Andersen
Project Manager

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Titel: Udvikling af artsspecifikke eDNA-baserede testsystemer til overvågning af ikke-hjemmehørende krabber i de danske farvande
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For at kunne spore ikke-hjemmehørende arter af marine invasive tibenede krebsdyr, ved hjælp af DNA-niveauer i filtrerede vandprøver, er der for denne rapport udviklet og testet tre nye artsspecifikke sporingssystemer, og for overblikkets skyld er fire tidligere udviklede og testede eDNA-sporingssystemer ligeledes efterkontrolleret, testet igen og enten inkluderet igen, eller erstattet af mere præcise eDNA-sporingssystemer.

Ved brug af kvantitativ PCR (quantitative polymerase chain reaction) (qPCR) er det med disse systemer nu muligt at spore eDNA i vandprøver fra seks ikke-hjemmehørende tibenede krebsdyr i danske farvande, og det er muligt at vurdere niveauerne af miljø-DNA (environmental DNA, eDNA) i vandprøverne fra de enkelte arter.

Alle syv sporingssystemer er blevet designet og testet både på DNA fra vævsprøver fra de eftersøgte arter, men også på DNA fra andre sameksisterende og hjemmehørende arter repræsenterende marine tibenede krebsdyr (Decapoda). De tre nye sporingssystemer er her eftervist som værende artsspecifikke og således i stand til at spore DNA fra 'pensel klippekrabbe', 'den blå svømmekrabbe' og 'stribet klippe-krabbe'.

Specificiteten for hvert sporingssystem er eftervist først ud fra sammenligning af nukleotidsekvenser hvor de enkelte primere og en passende 'hydrolysis probe' kan binde til et specifikt område i det mitokondrielle genom for den eftersøgte organisme. Primer og probe kombinationen er derpå testet under standardiserede temperaturomstændigheder i en qPCR opsætning, hvor den unikke sammensætning af nukleinsyrer i DNA sekvensen er med til at sikre at kun DNA fra den eftersøgte art registreres. Med qPCR-tests af forskelige kombinationer af primere og prober kan effektiviteten og specificiteten af kombinationerne så vurderes for at finde frem til den optimale kombination der senerehen bør bruges på indsamlede vandprøver. Sammenligningen med nukleotid-sekvenser fra andre arter af tibenede krebsdyr blev udført ved at identificere variable gen-regioner i de mitokondrielle genregioner: cytochrome oxidase 1 (mtDNA-co1) og cytochrome b (mtDNA-cytb). Nukleotid sekvenser af mtDNA-co1 og mtDNA-cytb blev enten indhentet fra en genetisk database eller indhentet ved *de novo*-sekventering af DNA ekstraheret fra vævsprøver indsamlet fra de syv arter.

Blandt de sporingssystemer der er testet, er der for hver art udvalgt et sporingssystem, da det i studiet her er eftervist som værende artsspecifikt, men samtidig også er udvalgt efter i hvor høj grad det er sensitivt for lave niveauer af miljø-DNA. De tidligere udviklede eDNA-sporingssystemer, der er blevet udviklet imod 'amerikansk hummer', 'østamerikansk brakvandskrabbe', 'kinesisk uldhåndskrabbe' og 'Kamchatka krabbe' er testet igen i denne rapport, og nye specifikke sporingssystemer præsenteres for to af arterne, for at sikre bedre præcision. Med inklusion og reevaluaring af alle artsspecifikke qPCR-systemer udviklet i MONIS-regi for sporing af marine krabber, er alle systemer for artsspecifik sporing af marine krabber samlet i samme rapport. I denne rapport er disse qPCR systemers specificitet efterprøvet igen mod en større diversitet af ikke-eftersøgte arter. To tidligere publicerede assays er her erstattet af nye og mere specifikke assays.

1 Introduction

Currently seven non-indigenous species of marine Decapoda is known from Danish marine waters (Tendal & Jensen 2017). These are listed here with the author name included for the first description of the species and the Danish common name in square brackets:

1. *Callinectes sapidus* Rathbun, 1896 [blå svømmekrabbe]*
2. *Eriocheir sinensis* H. Milne Edwards, 1853 [kinesisk uldhåndskrabbe]
3. *Hemigrapsus sanguineus* (De Haan, 1835; in De Haan, 1833-1850) [stribet klippekrabbe]*
4. *Hemigrapsus takanoi* Asakura & Watanabe, 2005 [pensel-klippekrabbe]*
5. *Homarus americanus* H. Milne-Edwards, 1837 [amerikansk hummer]**
6. *Paralithodes camtschaticus* (Tilesius, 1815) [Kamchatka-krabbe]**
7. *Rhithropanopeus harrisii* (Gould, 1841) [østamerikansk brakvandskrabbe]

Primers and probes for quantitative polymerase chain reaction (qPCR) have previously been developed and tested for *E. sinensis*, *H. americanus*, *P. camtschaticus* and *R. harrisii* to be specific against eDNA from these four species in a previous report from the MONIS project (Andersen et al., 2016, 2017). We have tested these four assays again against a broader representation of the diversity of Decapoda in Danish seas than previously and present these new updated results in this report too. Two previously presented species-specific assays have been discarded, and instead two new assays are included in replacement. The previously published assays (Andersen et al., 2018) that were found unspecific for *Homarus americanus* and *Paralithodes camschaticus* are not included in this report. Instead only the new and specific assays are included.

The aim of the present study is to develop and validate additional specific primer and probes for qPCR detection of eDNA that has a mitochondrial genomic origin from the three species: *Hemigrapsus sanguineus*, *Hemigrapsus takanoi* and *Callinectes sapidus*, since they are known to occur in Danish waters Tissue samples and DNA extractions have been performed on samples from *Hemigrapsus sanguineus* and *Hemigrapsus takanoi* collected in November 2019. Tissue samples from *Callinectes sapidus* were obtained from old preserved museum specimens stored at the Natural History Museum of Denmark (NHMD).

**) The three species with new assays.*
***) The species where the previous published assay was found to be unspecific. New assays were therefore developed and tested for the present report.*

2 Methods

All testing of species-specific assays has been performed in the same way, using the same set up 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 set up and protocols described by Agersnap *et al.* (2017) and Knudsen *et al.* (2019).

Tissue samples of marine Decapoda were obtained from museum specimens, or from aquarium specimens and DNA was extracted from these tissue samples using the DNeasy Blood and Tissue kit (Qiagene provider) following the manufacturers protocol.

The resulting primer and probes are presented in the following tables and sections. The first tables (Tables 1-4) provide a quick overview of the non-indigenous species targeted in this study and present the *in silico* designed and *in vitro* tested primer-probe assays. The sections following these four tables present each species-specific primer- probe assay for each of the non-indigenous species of marine Decapoda encountered in Danish marine waters. 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 instead, referring to samples held at the Natural History Museum of Denmark (NHMD).

Table 1: *Table of primer and probe qPCR detection systems developed during the MONIS5 project focusing on eDNA from three species of non-indigenous marine Decapoda. 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 og (P)robe, and an arbitrary number. 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.*

No (1)	Species	Primer (forward and reverse) and probe name	Sequence in 5'->3' direction with FAM and BHQ1 modifications indicated
AID01	*Callinectes sapidus*	Calsap_co1_F01	5'-GGGCCTCAGTTGATCTTGGT-3'
		Calsap_co1_R01	5'-GTAGAGAACAGGGTCGCCTC-3'
		Calsap_co1_P01	5'-FAM-ATACCTCATTCTTCGACCCAGCTGGAG-BHQ1-3'
AID02	*Hemigrapsus sanguineus*	Hemsan_COI_F01	5'-CCTGGGCCGGTATAGTAGGT-3'
		Hemsan_COI_R01	5'-GGGGCTCCGAGTATAAGTGG-3'
		Hemsan_COI_P01	5'-FAMCGAGCAGAATTAAGACAACCAGGAAGC-BHQ1-3'
AID03	*Hemigrapsus takanoi*	Hemtak_co1_F05	5'-AGGTTTTGACTTCTTCCTCCTTCT-3'
		Hemtak_co1_R05	5'-CTGCGAGTGGAGGGTAAACG-3'
		Hemtak_co1_P05	5'-FAM-TAGAAAGAGGTGTAGGTACAGGATGGA-BHQ1-3'

1) *The assay name and number are abbreviated from the assay identification (AID) numbers used in the MONIS4 (M4) report (Andersen et al., 2018).*

Table 2: *Table of updated primer and probe qPCR detection systems previously developed during the MONIS 3-4 projects focusing on eDNA from non-indigenous marine Decapoda. With new assays added for* Homarus americanus *and* Paralithodes camschaticus*. '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 og (P)robe, and an arbitrary number. 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 results are to be published in peer reviewed scientific literature.*

No (1)	Species	Primer (forward and reverse) and probe name	Sequence in 5'->3' direction with FAM and BHQ1 modifications indicated
M4_AID 18	*Eriocheir sinensis*	Erisin_cytb_F02	ACCCCTCCTCATATCCAACCA
		Erisin_cytb_R02	AAGAATGGCCACTGAAGCGG
		Erisin_cytb_P02	FAM-TTTGCTTACGCTATTTTACGATCAATTCCT-BHQ1
AID05	*Homarus americanus (2)*	Homame_cytb_F02	TTTTAGTAGCAGCAGCGACTCTT
		Homame_cytb_R14	CCAAGAAGGTAGGGATTTAGAAGA
		Homame_cytb_P12	FAM- TGCAAGACATATTGATAAAGTTCCATTCCA-BHQ1
AID06	*Paralithodes camtschaticus (2)*	Parcam_co1_F12	CGTCCACAAGGAATAACCTTAGAC
		Parcam_co1_R12	AACTGGGTCTCCTCCTCCTG
		Parcam_co1_P12	FAM-TTTGTGTGATCCGTATTTATTACTGCAA-BHQ1
M4_AID 14	*Rhithropanopeus harrisii*	Rhihar_co1_F03	GTCAACCTGGTACTCTCATTGGT
		Rhihar_co1_R03	ACGAGGAAATGCTATATCAGGGG
		Rhihar_co1_P03	FAM-TGTTGTAGTAACAGCTCACGCCTTTGT-BHQ1

1) *The assay name and number are abbreviated from the assay identification (AID) numbers used in the MONIS4 (M4) report (Andersen et al. 2018).*
2) *The assay presented here in this report is different from the previously published (Andersen et al., 2018) assay, as the previous published assay was found to be unspecific. Instead only the new specific assay is included in the present report.*

Table 3: *List of species targeted in the present MONIS5 project. 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 in vitro tests performed in this study. Species-specific eDNA assays (primers and probes) have been developed and tested in laboratorial 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 an ensuing project. TS = Target Species; NTS = Non-Target Species. 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.*

Genus	Species	Danish common name	TS collected	NTS collected and tested(2)	Level of specificity	Assay ready
Callinectes	*sapidus*	Blå svømmekrabbe	Yes	Yes	Species	Yes
Hemigrapsus	*sanguineus*	Stribet klippekrabbe	Yes	Yes	Species	Yes
Hemigrapsus	*takanoi*	Pensel klippekrabbe	Yes	Yes	species	Yes

Table 4: *List of species targeted in previous MONIS3-4 project, which have been re-evaluated in this study. 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 2. Species-specific eDNA assays (primers and probes) have been developed and tested in laboratorial setup (in silico and in vitro testing) during the MONIS 3-4 project. 'Assay ready' indicates whether the assay can be considered ready for test at operational level - i.e. subsequent testing in an ensuing project. TS = Target Species; NTS = Non-Target Species. 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.*

Genus	Species	Danish commmon name	TS	NTS collected and tested(1)	Level of specificity	Assay ready
Eriocheir	*sinensis*	Kinesisk uldhåndskrabbe	Yes	Yes	species	Yes
Homarus	*americanus*	Amerikansk hummer	Yes	Yes	Species (3)	Yes (3)
Paralithodes	*camtschaticus*	Kamchatka-krabbe	Yes	Yes	Species (2)	Yes
Rhithropanopeus	*harrisii*	Østamerikansk brakvandskrabbe	Yes	Yes	species	Yes

(1) Whether non-target species have been collected refers to whether species from potentially co-occurring and evolutionary closely related species in Danish marine waters 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.
(2) The assay developed during the MONIS 3-4 project for detection of P. camtschaticus *was found in this study to also return false positive detection on DNA from hermit crab (*Pagurus *spp.). Instead a new assay is presented in this report, and this new assay can distinguish between hermit crabs and P. camtschaticus being present in the water sample.*
(3) A cross contamination between some of the positive control samples and negative controls in the laboratory gave rise to doubt as to whether this assay for detection of H. americanus *is specific. The in-silico test confirms the specificity, but a new in vitro and in vivo test is required before this assay can be used on water samples. To work around this a new species-specific assay was eveloped and tested for the present report. This makes the previous species-specific assay (Andersen et al., 2017) redundant.*

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 vR7 (Kearse *et al.* 2012). The *in-silico* design was based on initial primer suggestions inferred from using Primer3 v0.4.0 (Koressaar & 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 test protocol described by Knudsen *et al*. (2019) and was set up by comparing sequences from NCBI GenBank and from own prepared de novo sequencing from extractions. New extractions obtained from museum or aquarium samples are denoted with an 'E'-number here: *Eriocheir_sinensis*: AY274302. *Hemigrapsus_sanguineus*: Hemsan203_E52-04-01, Hemsan204_E52-05-01, Hemsan209_E52-10-01, Hemsan210_E52-11-01, Hemsan211_E52-12-01, Hemsan212_E52-13-01, Hemsan213_E52-14-01, Hemsan214_E52-15-01, Hemsan215_E52-16-01. *Hemigrapsus_takanoi*: Hemtak200_E52-01-01, Hemtak201_E52-01-01, Hemtak202_E52-03-01, Hemtak205_E52-06-01, Hemtak206_E52-07-01, Hemtak207_E52-08-01. *Callinectes amnicola*: Calamn238_E53-23, *Callinectes pallidus*: Calsap225_E53-10, MH801206-MH801210, NC_006281. *Homarus_americanus*: AF370853, FJ174944, HQ402925, NC_15607, FJ581693, DQ889104. *Homarus_gammarus*: KT208429, KT209166, KT208891, KC107810, NC_20020. *Lithodes_aequispinus*: KC196523. *Lithodes_confundens*: KC196536. *Lithodes_ferox*: HM020903. *Lithodes_formosae*: GU289678. *Lithodes_longispina*: AB476813, AB476817. *Lithodes_maja*: KT209429, KT208393, AF425-309. *Lithodes_murrayi*: HM020899. *Lithodes_nintokuae*: AB375131. *Lithodes_paulayi*: GU289677. *Lithodes_santolla*: HM020898. Lithodes_turkayi: KC196531. *Maja_squinado*: GQ153553, GQ153551. *Neolithodes_asperrimus*: HM020890, HM020891. *Neolithodes_brodiei*: EU493263, EU493263. *Neolithodes_diomedeae*: KC196528. *Neolithodes_duhameli*: HM020892. *Neolithodes_grimaldii*: JQ305972.

Nephrops_norvegicus: FJ174945, JQ623962. *Palinurus_barbarae*: FJ174960. *Palinurus_charlestoni*: FJ174959. *Palinurus_delagoae*: FJ174958. *Palinurus_elephas*: DQ062206, KC789347. *Palinurus_gilchristi*: FJ174961, EF546352. *Palinurus_mauritanicus*: EF546365, DQ062207. *Palinustus_unicornutus*: EF546344. *Panulirus_homarus*: KU523817. *Panulirus_ornatus*: KU523792, KU523815. *Panulirus_versicolor*: KT001513, KT001512. *Papilio_palinurus*: JQ982114, JQ982116, JQ982115. *Paralithodes_brevipes*: NC_21458. *Paralithodes_camtschaticus*: AB211435, JF738168. *Paralomis_aculeata*: HM020904. *Paralomis_africana*: HM020907. *Paralomis_anamerae*: HM020905, HM020906. *Paralomis_birsteini*: EU493261. *Paralomis_cristata*: HM020911. *Paralomis_cristulata*: HM020908. *Paralomis_dofleini*: HM020913. *Paralomis_elongata*: HM020914. *Paralomis_erinacea*: HM020916. *Paralomis_formosa*: KC196530. *Paralomis_granulosa*: AF425318. *Paralomis_multispina*: DQ882130. *Paralomis_pacifica*: AB476747. *Paralomis_spinosissima*: EU493258. *Paralomis_zealandica*: HM020936.

2.2 Laboratorial test of specificity – *in vitro* testing

The designed primers and probes were tested in laboratory setups to ensure that amplification was specific. Specificity was ensured only once the test returned positive amplification when applied on DNA extracted from a tissue sample stemming from the target species.

In addition to the sequence data from the mitochondrial cytochrome oxidase 1 (mtDNA-co1) region available on NCBI GenBank for the species of *Callinectes* and *Hemigrapsus*, *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' (Folmer et al. 1994) in a PCR set up 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), 2.5 µL buffer (x10), 2.5 µL dNTP (2 mM per dNTP) and 0.1 µL (5 U/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, with extracted DNA from tissue being 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 set up 01 described by Knudsen *et al*. (2019) and is in this report referred to as 'set up 01'. 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. Sanger sequencing was performed in both forward and reverse directions and resulting sequence reads were assembled and manually inspected in the software Geneious vR7 (Kearse *et al.* 2012).

DNA from reference tissue samples were either supplied from external sources (Table 5) 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), 2.5 µL buffer (x10), 2.5 µL dNTP (2 mM per dNTP) 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, with the extracted DNA from tissue being 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 set up 01', however this second version of the 'PCR set up 01' differed by using different specific primers in combination, instead of the HCO-LCO primers (Folmer et al., 1994). For each species the 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 (Table 5). For the primer

combinations that returned species-specific amplification species-specific FAM-BHQ1 modified Taq-Man hydrolysis probes were ordered, to allow for subsequent testing of specificity against the gene region in a qPCR setup. This qPCR set up 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.

Table 5: *Species and corresponding tissue samples used for in vitro test and validation of specificity of the individual specific primer probe assays. The tissue sample abbreviation is used in the tables below listing the individual results from each of the in vitro tests performed on the assays designed. 'TS abbrev.' is the abbreviation used for the tissue sample.*

Species	TS abbrev.	Collected by	Collection locality
Astacus leptodactylus	Astlep	S. Agersnap; W.B. Larsen, NHMD	Sjælland, Denmark
Cancer pagurus	Canpag	Øresundsakvariet, S.W. Knudsen	Øresund, outside Helsingør
Carcinus maenus	Carmae	Øresundsakvariet, S.W. Knudsen	Øresund, outside Helsingør
Eriocheir sinensis	Erisin	NHMD	Denmark
Homarus americanus	Homame	"Den Blå Planet", S.W. Knudsen	"Den Blå Planet"
Homarus gammarus	Homgam	Øresundsakvariet, S.W. Knudsen	Øresund, outside Helsingør
Hyas araneus	Hyaara	Øresundsakvariet, S.W. Knudsen	Øresund, outside Helsingør
Hyas coarctatus	Hyacoa	Øresundsakvariet, S.W. Knudsen	Øresund, outside Helsingør
Lithodes maja	Litmaj	NHMD	NHMD
Nephrops norvegicus	Nepnor	S.W. Knudsen	North Sea, NHMD
Pacifastacus leniusculus	Paclen	S. Agersnap; W.B. Larsen, NHMD	Sjælland, Denmark
Pagurus bernhardus	Pagber	Øresundsakvariet, S.W. Knudsen	Øresund, outside Helsingør
Paralithodes camtschaticus	Parcam	Hjelset, A.M., IMR, Norway	North Atlantic Sea, off Norway
Paralomis sp.	Parsp	Øresundsakvariet, S.W. Knudsen	Øresund, outside Helsingør
Paralomis spectabilis	Parspe	S.W. Knudsen	North Sea, NHMD
Rhithropanopeus harrisii	Rhihar	A.B Aagaard, S.W. Knudsen, NHMD	Køge Bugt, Denmark
Hemigrapsus sanguineus	Hemsan	K. Jensen, NHMD	W Jylland
Hemigrapsus takanoi	Hemtak	K. Jensen, NHMD	W Jylland
Calinectes sapidus	Calsap	NHMD Collection	NW Atlantic coast

All qPCR reactions were run on a Stratagene Mx3005P qPCR Machine (Agilent, Santa Clara, California, United States). Primer probe specificity test was performed 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_2O and 2 µL 1:10 diluted template 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 target controls (NTC). All data obtained from the qPCR setups were exported as excel files from the Mx3005P software, and analysed in R v3.3 (R Core Team, 2016) using the packages: "ggplot2" (Wickham 2016), "pdp" (Greenwell, 2017) and "readxl" (Wickham & Bryan 2017).

3 Results

The sections below list each of the individual specific primer probe assays and report the amplification success for each setup. The first three assays listed are the primer probe systems developed during the present phase of the MONIS project. The next four assays listed are the re-evaluated assays developed during earlier phases of the MONIS project (Andersen *et al*. 2018, 2020).

3.1 Development and testing of new assays

3.1.1 Species-specific assay for detection of *Callinectes sapidus*

The 'American blue crab' is indigenous to the North-west Atlantic coast but has been introduced in European seas (Tendal & Jensen 2017).

Binomial nomenclature and author: *Callinectes sapidus* Rathbun, 1896
English common name: American blue crab
Danish common name: blå svømmekrabbe

Figure 1: Callinectes sapidus. *Photo of specimen Calsap225 from NHMD collection, specimen was collected at Sapelo Island, Georgia on August 2, 1971. Photo by S.W. Knudsen. This specimen measures around 10 cm from the left and the right pointy tip on the width of the carapace.*

The genus *Callinectes* comprise 16 species (Adema, 1991; Stephenson, 1972; Türkay, 2001; WoRMS, 2020a). Currently only *Callinectes sapidus* is known as being introduced in NE Atlantic seas and from Danish marine waters (Tendal & Jensen 2017), the other 15 species of *Callinectes* are native in the central east Pacific Ocean, along the coast of North- and South America and in the Caribbean Sea, and along the western coast of Africa in the central eastern Atlantic ocean (Adema 1991, Stephenson 1972, Türkay 2001, WoRMS, 2020). This study focuses only on *Callinectes sapidus* as this species to our knowledge is the only species of *Callinectes* that have been reported as non-indigenous in the NE Atlantic Ocean. Species-specific assay targeting mitochondrial DNA cytochrome oxidase 1 (mtDNA-

CO1) from *Callinectes sapidus* was developed and tested in the present study (table 6-7). The assay targeting mtDNA-CO1 in *Callinectes sapidus* are comprised of the oligos:

Calsap_co1_F01	5'-GGGCCTCAGTTGATCTTGGT-3'
Calsap_co1_P01	5'-FAM-ATACCTCATTCTTCGACCCAGCTGGAG-BHQ1-3'
Calsap_co1_R01	5'-GTAGAGAACAGGGTCGCCTC-3'

Table 6: *Primers and probes specific for* Callinectes sapidus*, targeting a 275 basepair long fragment from the mitochondrial cytochrome oxidase 1 gene.*

Oligo name	oligo sequence in 5'->3' direction	Temp (°C)	Length (bp)	GC (%)
Calsap_co1_F01	5'-GGGCCTCAGTTGATCTTGGT-3',	59.7	20	55.0
Calsap_co1_P01	5'-FAM-ATACCTCATTCTTCGACCCAGCTGGAG-BHQ1-3'	59.5	20	60.0
Calsap_co1_R01	5'-GTAGAGAACAGGGTCGCCTC-3'	65.8	27	51.9

Table 7: *Nucleotide sequence for targeted fragment for* Callinectes sapidus *in the mitochondrial cytochrome oxidase 1 gene.*

Species	sequence in 5'->3' direction	Length (bp)	Molecular weight (Da)
Callinectes sapidus	GGGCCTCAGTTGATCTTGGTATTTTCTCTCTCCACTTAGCTGGTGTAT CATCAATTCTAGGGGCTGTTAACTTTATAACTACCGTTATTAATATAC GTTCATTTGGTATAAGAATAGACCAAATGCCTTTATTCGTTTGATCT GTATTTATTACCGCTATTCTTCTACTTCTTTCTCTACCTGTATTAGCAG GTGCTATTACTATACTTCTCACTGATCGAAACTTAAATACCTCATTCT TCGACCCAGCTGGAGGAGGCGACCCTGTTCTCTAC	275	169768.6

Table 8: *Extracted DNA from tissue samples from various other co-occurring species that potentially can lead to false positive detection. Not all species are necessarily closely related to the genus Callinectes, but these species are all species of the order Decapoda, and all are commonly encountered in North European seas, including the invasive species* Rhithropanopeus harrisii *and* Hemigrapsus sanguineus *and* H. takanoi*.*

Species	Tissue sample abbreviation	Tested	Amplification result in qPCR	Acc. number or sequence
Callinectes sapidus	Calsap	Yes	Yes	MH801206, Calsap224_E53-09, Calsap225_E53-10
Cancer pagurus	Canpag	Yes	No	Canpag021_E32.1, NHMD
Eriocheir sinensis	Erisin	Yes	No	AY274302, Erisin031_E36.1-1, NHMD
Hyas araneus	Hyaara	Yes	No	Hyaara019_E32.6, NHMD
Nephrops norvegicus	Nepnor	Yes	No	Nepnor027_E33.1-1, NHMD
Rhithropanopeus harrisii	Rhihar	Yes	No	NCRhihar_hotA_AZ01.05, NHMD
Hemigrapsus_sanguineus	Hemsan	Yes	No	Hemsan203_E52-04-01-Hemsan215_E52-16-01
Hemigrapsus_takanoi	Hemtak	Yes	No	Hemtak200_E52-01-01-Hemtak207_E52-08-01

Figure 2: *Alignment of mitochondrial DNA cytochrome oxidase 1 gene from various species of marine Decapoda occurring in Danish marine waters. Primers and probes mapped on sequences from* Callinectes sapidus*. Primers are marked with green annotations. The probe is indicated with a red annotation. Sequences were obtained from NCBI GenBank and aligned in Geneious vR7.*

Primers and probes tested

The primers were designed by aligning sequences available from mitochondrial cytochrome oxidase 1 from different common species of Decapoda available from NCBI GenBank. This included: *Callinectes sapidus*: AY363392, MH801206, MH801207, MH801208, MH801209, MH801210, NC_006281. *Eriocheir sinensis*: AY274302. *Homarus americanus*: AF370853, DQ889104, FJ174944, FJ581693, HQ402925, NC_015607. *Homarus gammarus*: KC107810, KT208429, KT208891, KT209166, NC_020020. *Lithodes aequispinus*: KC196523. *Lithodes confundens*: KC196536. *Lithodes ferox*: HM020903. *Lithodes formosae*: GU289678. *Lithodes longispina*: AB476813, AB476817. *Lithodes maja*: AF425309, KT208393, KT209429. *Lithodes murrayi*: HM020899. *Lithodes nintokuae*: AB375131. *Lithodes paulayi*: GU289677. *Lithodes santolla*: HM020898. *Lithodes turkayi*: KC196531. *Maja squinado*: GQ153551, GQ153553. *Neolithodes asperrimus*: HM020890, HM020891. *Neolithodes brodiei*: EU493263. *Neolithodes diomedeae*: KC196528. *Neolithodes duhameli*: HM020892. *Neolithodes grimaldii*: JQ305972. *Nephrops norvegicus*: FJ174945, JQ623962. *Palinurus barbarae*: FJ174960. *Palinurus charlestoni*: FJ174959. *Palinurus delagoae*: FJ174958. *Palinurus elephas*: DQ062206, KC789347. *Palinurus gilchristi*: EF546352, FJ174961. *Palinurus mauritanicus*: DQ062207, EF546365. *Palinustus unicornutus*: EF546344. *Panulirus homarus*: KU523817. *Panulirus ornatus*: KU523792, KU523815. *Panulirus versicolor*: KT001512, KT001513. *Papilio palinurus daedalus*: JQ982114. *Papilio palinurus palinurus*: JQ982116. *Papilio palinurus vega*: JQ982115. *Paralithodes brevipes*: NC_021458. *Paralithodes camtschaticus*: AB211435, JF738168. *Paralomis aculeata*: HM020904. *Paralomis africana*: HM020907. *Paralomis anamerae*: HM020905, HM020906. *Paralomis birsteini*: EU493261. *Paralomis cristata*: HM020911. *Paralomis cristulata*: HM020908. *Paralomis dofleini*: HM020913.

Paralomis elongata: HM020914. *Paralomis erinacea*: HM020916. *Paralomis formosa*: KC196530. *Paralomis granulosa*: AF425318. *Paralomis multispina*: DQ882130. *Paralomis pacifica*: AB476747. *Paralomis spinosissima*: EU493258. *Paralomis zealandica*: HM020936. Sequence alignment was performed using Geneious v. R7 (Kearse et al. 2012) and MAFFT 6.822 (Katoh and Toh, 2010) and primers matched against the target-species sequence with Primer3 v2.3.4 (Untergasser et al., 2012).

The following primers and probes were *in silico* designed and tested in vitro in a PCR reactions set up (PCR setup 01 as described in the protocol by Knudsen et al, 2019) to find a species-specific combination of primers and Calsap_co1_F01: 5'-GGGCCTCAGTTGATCTTGGT-3'; Calsap_co1_F02: 5'-ACTCAGACTACCCAGATGCCT-3'; Calsap_co1_F03: 5'-TGGTCGAAAGTGGAGTTGGT-3'; Calsap_co1_F04: 5'-CCATGGGTGCTGTATTCGGA-3'; Calsap_co1_F05: 5'-CAGGGGCCTCAGTTGATCTT-3'; Calsap_co1_P01: 5'-FAM-ATACCTCATTCTTCGACCCAGCTGGAG-BHQ1-3'; Calsap_co1_P02: 5'-FAM-TCTCCTTTCCTTCCATCATCCATTGAA-BHQ1-3'; Calsap_co1_P03: 5'-FAM-ATGAACTGTTTACCCTCCCCTTGCTGC-BHQ1-3'; Calsap_co1_P04: 5'-FAM-CCCCCAACACTTCTTAGGGCTTAACGG-BHQ1-3'; Calsap_co1_P05: 5'-FAM-TACCTCATTCTTCGACCCAGCTGGAGG-BHQ1-3'; Calsap_co1_R01: 5'-GTAGAGAACAGGGTCGCCTC-3'; Calsap_co1_R02: 5'-CAGCTGGTGGGTAAGAGTGG-3'; Calsap_co1_R03: 5'-ACCAAGATCAACTGAGGCCC-3'; Calsap_co1_R04: 5'-AGGCATCTGGGTAGTCTGAGT-3'; Calsap_co1_R05: 5'-TTGGTAGAGAACAGGGTCGC-3'. The initial PCR results from the test performed using these primers are not included in this report.

Assay specificity results

The assay designed and tested in this study (Calsap_COI_F01, Calsap_COI_R01, Calsa _COI_P01) amplified for the four replicate reactions containing genomic DNA from *Callinectes sapidus* at a Cq of 35 (Figure 3A). The new F01-R01-P01-assay tested in this study was found to be species-specific only against the targeted species (Figure 3A) when tested on DNA extracted from other congeners (Table 7).

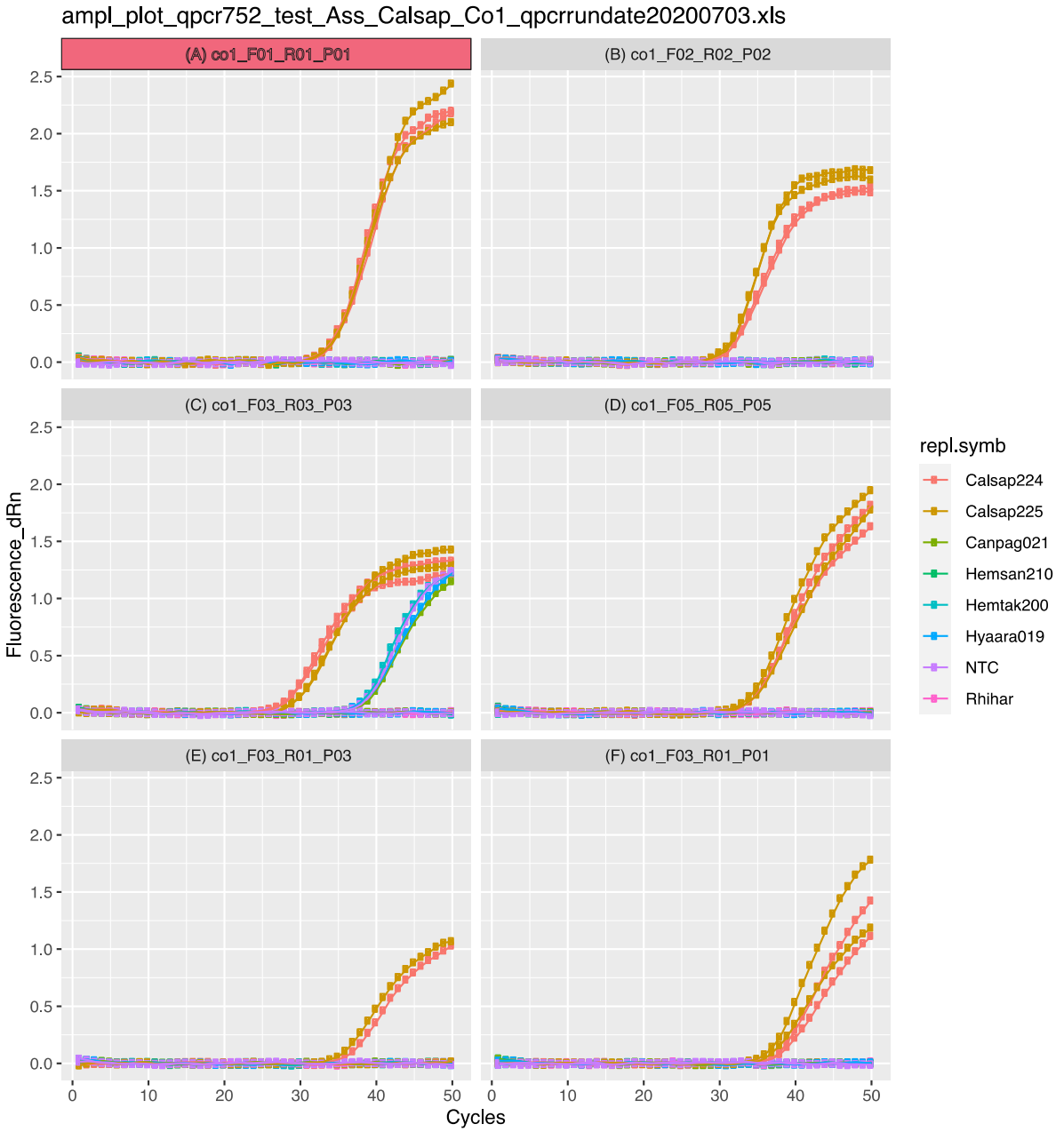

Figure 3: *Amplification of* Callinectes sapidus *using six species-specific assays developed in the present study. Amplification of DNA for the target species* Callinectes sapidus *is shown in red (Calsap224), and orange (Calsap225) and other non-target species of Decapoda are in other colours. The six assays show the different combinations of primer and probe tested. The assay using Calsap_co1_F01+ Calsap_co1_R01+ Calsap_co1_P01 (A) returns species-specific detection with the highest relative fluorescence level. This assay (A) was preferred among the six assays tested. The other combinations of primers (B-F) either amplified at a later cycle threshold for quantification or also amplified on other non target species or returned a lower difference in relative fluorescence. The assays that performed inefficiently (B-F) and returned unspecific amplification, should not be used in future projects.*

3.1.2 Species-specific assay for detection of *Hemigrapsus sanguineus*

The 'Japanese shore crab' is indigenous to the Pacific Ocean but has been introduced in European seas (WoRMS 2020b, Tendal & Jensen 2017).

Binomial nomenclature and author:	*Hemigrapsus sanguineus* Asakura and T. Watanabe 2005
English common name:	Japanese shore crab
Danish common name:	Stribet klippekrabbe

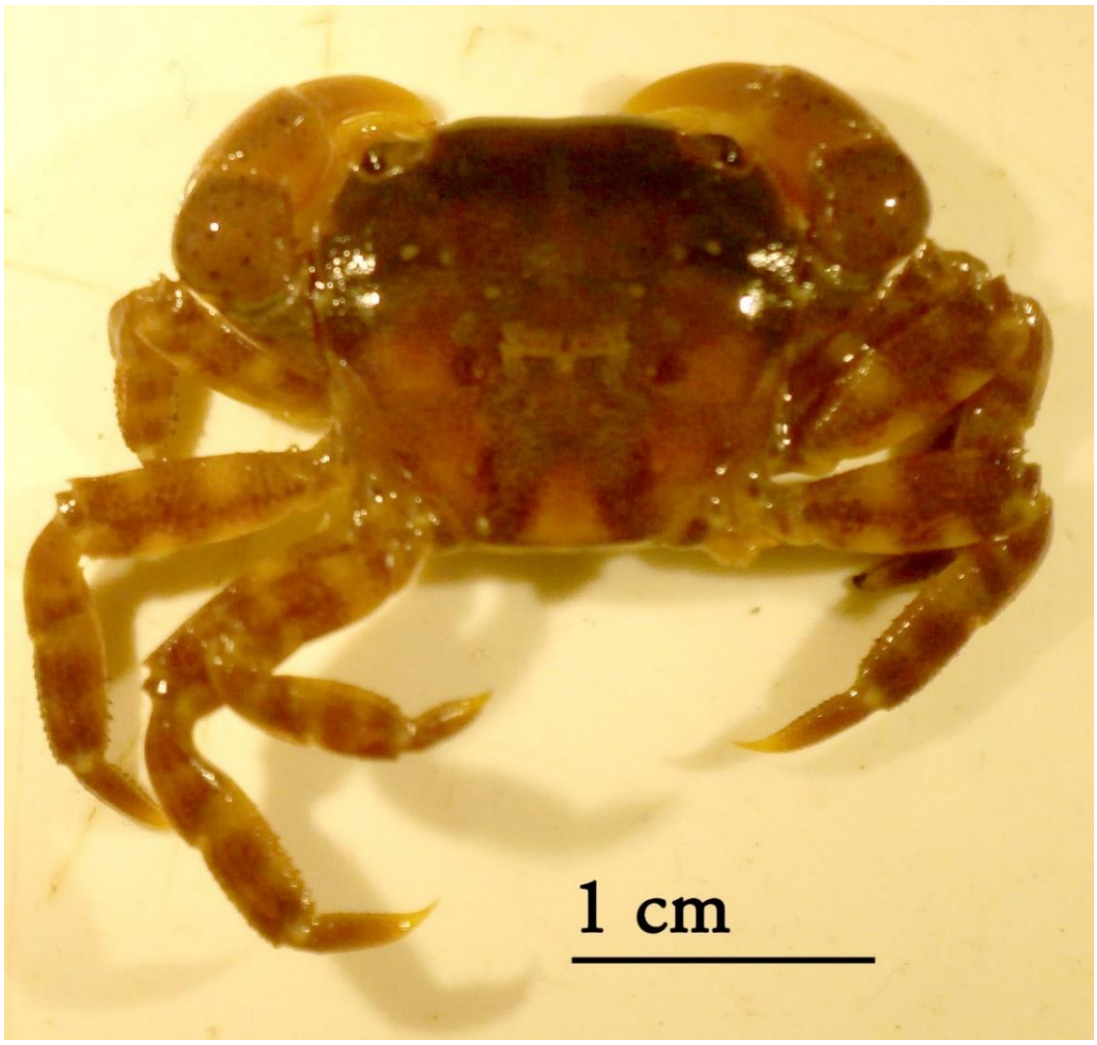

Figure 4: Hemigrapsus sanguineus*. Photo of specimen Hemsan210 from NHMD's collection. Specimen was collected from the west coast of Denmark by 'Fiskeri- og Søfartsmuseet' in February 2019. Photo by S.W. Knudsen.*

Two species of the genus *Hemigrapsus* have been recorded as non-indigenous in Danish marine waters (Tendal & Jensen 2017). Among the 15 known species of *Hemigrapsus* (Türkay 2001, WoRMS 2020b) the native distribution covers the south western, north western and north eastern Pacific Ocean. One species was native to saline caves in northern Texas but is considered extinct. *Hemigrapsus sanguineus* and *Hemigrapsus takanoi* have been caught along the western coast of Denmark (Tendal & Jensen 2017). *Hemigrapsus penicillatus* is native to the north western Pacific Ocean but have been recorded from the Bay of Biscay (WoRMS 2020b).

Both *H. sanguineus* and *H. takanoi* have been caught on the western coast of Denmark and are considered non indeginous in the North East Atlantic. Tissue samples from *H. sanguineus* and *H. takanoi* were obtained from both species from individuals caught on the western coast of Denmark. DNA was then extracted from these two species (table 5). Using this extracted DNA, species-specific assays targeting DNA in the mitochondrial cytochrome oxidase 1 region (mtDNA-CO1) (table 9-10) in these two species were developed and tested in the present study. The assay targeting mtDNA-CO1 in *H. sanguineus* are comprise of the oligos:

Hemsan_COI_F01 5'- CCTGGGCCGGTATAGTAGGT-3'
Hemsan_COI_R01 5'- GGGGCTCCGAGTATAAGTGG-3'
Hemsan_COI_P01 5'-FAM CGAGCAGAATTAAGACAACCAGGAAGC-BHQ1-3'

Table 9: *Primers and probes specific for* Hemigrapsus sanguineus*, targeting a 204 base-pair long fragment from the mitochondrial cytochrome oxidase 1 gene.*

Oligo name	*oligo sequence in 5'->3' direction*	Temp (°C)	Length (bp)	GC (%)
Hemsan_COI_F01	5'- CCTGGGCCGGTATAGTAGGT-3'	60.2	20	60.2
Hemsan_COI_R01	5'- GGGGCTCCGAGTATAAGTGG -3'	59.3	20	60.0
Hemsan_COI_P01	5'-FAM- CGAGCAGAATTAAGACAACCAGGAAGC-BHQ1-3'	64.2	27	48.1

Table 10: *Nucleotide sequence for targeted fragment for* Hemigrapsus sanguineus *in the mitochondrial cytochrome oxidase 1 gene.*

Species	*sequence in 5'->3' direction*	Length (bp)	Molecular weight (Da)
Hemigrapsus sanguineus	TCGGAGCCCCAGATATAGCCTTTCCCCGTATAAATAATA TAAGATTTTGACTTCTTCCTCCTTCTCTATCCCTCCTTTTA ACAAGAAGAATAGTAGAAAGAGGTGTAGGCACCGGAT GAACCGTTTATCCGCCACT	136	83900.7

Table 11: *Extracted DNA from tissue samples from various other co-occurring species that potentially can lead to false positive detection. Not all species are necessarily closely related to the genus* Hemigrapsus*, but these species are all species of the order Decapoda, and all are commonly encountered in Northern European seas, including* Rhithropanopeus harrisii *and* Hemigrapsus takanoi*.*

Species	Tissue sample abbreviation	Tested	Amplification result in qPCR	Acc. number or sequence
Cancer pagurus	Canpag	Yes	No	Canpag021_NHMD
Calinectes sapidus	Calsap	Yes	No	Calsap225_NHMD
Hyas araneus	Hyaara	Yes	No	Hyaara019_NHMD
Rhithropanopeus harrisii	Rhihar	Yes	No	Rhihar_hotA_AZ01.05_NHMD
Eriocheir_sinensis	Erisin	Yes	No	Erisin031_E36.1-1, NHMD
Nephrops_norvegicus	Nepnor	Yes	No	Nepnor027_E33.1-1, NHMD
Hemigrapsus_sanguineus	Hemsan	Yes	Yes	Hemsan203_E52-04-01-Hemsan215_E52-16-01
Hemigrapsus_takanoi	Hemtak	Yes	No	Hemtak200_E52-01-01-Hemtak207_E52-08-01

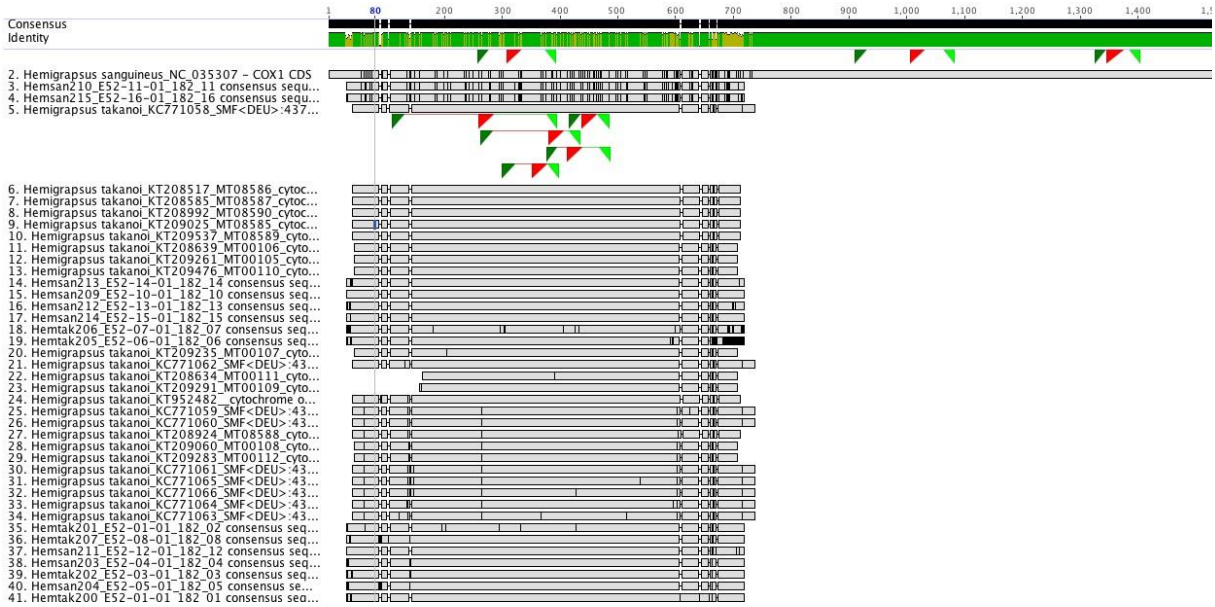

Figure 5*: Alignment of mitochondrial DNA cytochrome oxidase 1 gene from various species of marine Decapoda occurring in Danish marine waters. Primers and probes mapped on sequences from* Hemigrapsus sanguineus*. Primers are marked with green annotations. The probe is indicated with a red annotation. Sequences were obtained from NCBI GenBank and aligned in Geneious vR7.*

Primers and probes tested

The primers were designed by aligning sequences available from mitochondrial cytochrome oxidase 1 from different common species of Decapoda available from NCBI GenBank. This included: *Eriocheir sinensis*: AY274302. *Hemigrapsus sanguineus*: KX456205, NC_035307, Hemsan203 E52-04-01, Hemsan204 E52-05-01, Hemsan209 E52-10-01, Hemsan210 E52-11-01, Hemsan211 E52-12-01, Hemsan212 E52-13-01, Hemsan213 E52-14-01, Hemsan214 E52-15-01, Hemsan215 E52-16-01. *Hemigrapsus takanoi*: KC771058, KC771059, KC771060, KC771061, KC771062, KC771063, KC771064, KC771065, KC771066, KT208517, MT08586, KT208585, MT08587, KT208634, MT00111, KT208639, MT00106, KT208924, MT08588, KT208992, MT08590, KT209025, MT08585, KT209060, MT00108, KT209235, MT00107, KT209261, MT00105, KT209283, MT00112, KT209291, MT00109, KT209476, MT00110, KT209537, MT08589, KT952482, Hemtak200 E52-01-01, Hemtak201 E52-01-01, Hemtak202 E52-03-01, Hemtak205 E52-06-01, Hemtak206 E52-07-01, Hemtak207 E52-08-01. *Homarus americanus*: AF370853, DQ889104, FJ174944, FJ581693, HQ402925, NC_015607. *Homarus gammarus*: KC107810, KT208429, KT208891, KT209166, NC_020020. *Lithodes aequispinus*: KC196523. *Lithodes confundens*: KC196536. *Lithodes ferox*: HM020903. *Lithodes formosae*: GU289678. *Lithodes longispina*: AB476813, AB476817. *Lithodes maja*: AF425309, KT208393, KT209429. *Lithodes murrayi*: HM020899. *Lithodes nintokuae*: AB375131. *Lithodes paulayi*: GU289677. *Lithodes santolla*: HM020898. *Lithodes turkayi*: KC196531. *Maja squinado*: GQ153551, GQ153553. *Neolithodes asperrimus*: HM020890, HM020891. *Neolithodes brodiei*: EU493263, EU493263. *Neolithodes diomedeae*: KC196528. *Neolithodes duhameli*: HM020892. *Neolithodes grimaldii*: JQ305972. *Nephrops norvegicus*: FJ174945. *Nephrops norvegicus*: JQ623962. *Palinurus barbarae*: FJ174960. *Palinurus charlestoni*: FJ174959. *Palinurus delagoae*: FJ174958. *Palinurus elephas*: DQ062206, KC789347. *Palinurus gilchristi*: EF546352, FJ174961. *Palinurus mauritanicus*: DQ062207, EF546365. *Palinustus unicornutus*: EF546344. *Panulirus homarus*: KU523817. *Panulirus ornatus*: KU523792, KU523815. *Panulirus versicolor*: KT001512, KT001513. *Papilio palinurus daedalus*: JQ982114. *Papilio palinurus palinurus*: JQ982116. *Papilio palinurus vega*: JQ982115. *Paralithodes brevipes*: NC_021458. *Paralithodes camtschaticus*: AB211435, JF738168. *Paralomis aculeata*:

HM020904. *Paralomis africana*: HM020907. *Paralomis anamerae*: HM020905, HM020906. *Paralomis birsteini*: EU493261. *Paralomis cristata*: HM020911. *Paralomis cristulata*: HM020908. *Paralomis dofleini*: HM020913. *Paralomis elongata*: HM020914. *Paralomis erinacea*: HM020916. *Paralomis formosa*: KC196530. *Paralomis granulosa*: AF425318. *Paralomis multispina*: DQ882130. *Paralomis pacifica*: AB476747. *Paralomis spinosissima*: EU493258. Paralomis zealandica: HM020936. Sequence alignment was performed using Geneious v. R7 (Kearse et al. 2012) and MAFFT 6.822 (Katoh and Toh, 2010) and primers matched against the target-species sequence with Primer3 v2.3.4 (Untergasser et al., 2012).

The following primers and probes were *in silico* designed and tested in vitro in the PCR reactions "set up 01" (see PCR set up numbering in Knudsen *et al.*, 2019) to find a species-specific combination of primers and probes Hemsan_co1_F01: 5'-CCTGGGCCGGTATAGTAGGT-3'; Hemsan_co1_F02: 5'-TCGGAGCCCCAGATATAGCC-3'; Hemsan_co1_F03: 5'-GGCTTGACCGGGGTAGTTC-3'; Hemsan_co1_F04: 5'-CTGATTACCCCGACGCCTAC-3'; Hemsan_co1_F05: 5'-GCTTACTTTACCTCCGCCACT-3'; Hemsan_co1_P01: 5'-FAM-CGAGCAGAATTAAGACAACCAGGAAGC-BHQ1-3'; Hemsan_co1_P02: 5'-FAM-ACTTCTTCCTCCTTCTCTATCCCTCCT-BHQ1-3'; Hemsan_co1_P03: 5'-FAM-TATTTGGAATTTTT-GCGGGGGTTGCCC-BHQ1-3'; Hemsan_co1_P04: 5'-FAM-GCAACATGAAATATTATCTCATCCTTAGGCTC-BHQ1-3'; Hemsan_co1_P05: 5'-FAM-TCTCCTTCACTTTTATGAGCCCTAGGA-BHQ1-3'; Hemsan_co1_R01: 5'-GGGGCTCCGAGTATAAGTGG-3'; Hemsan_co1_R02: 5'-AGTGGCGGATAAACGGTTCA-3'; Hemsan_co1_R03: 5'-GTAGGCGTCGGGGTAATCAG-3'; Hemsan_co1_R04: 5'-CCTAGAGCGGCTACAAAGGA-3'; Hemsan_co1_R05: 5'-TTGGCTAGAACTACCCCGGT-3'. The initial PCR results from the test performed using these primers are not included in this report

Assay specificity results

The assay designed and tested in this study (Hemsan_COI_F01, Hemsan_COI_R01, Hemsan_COI_P01) amplified for the two replicate reactions in a qCPR containing genomic DNA from *Hemigrapsus sanguineus* at a Cq of 25 (Figure 6). The new F01-R01-P01-assay tested in this study was found to be species-specific only against the targeted species (Figure 6A) when tested on DNA extracted from other congeners (table 11).

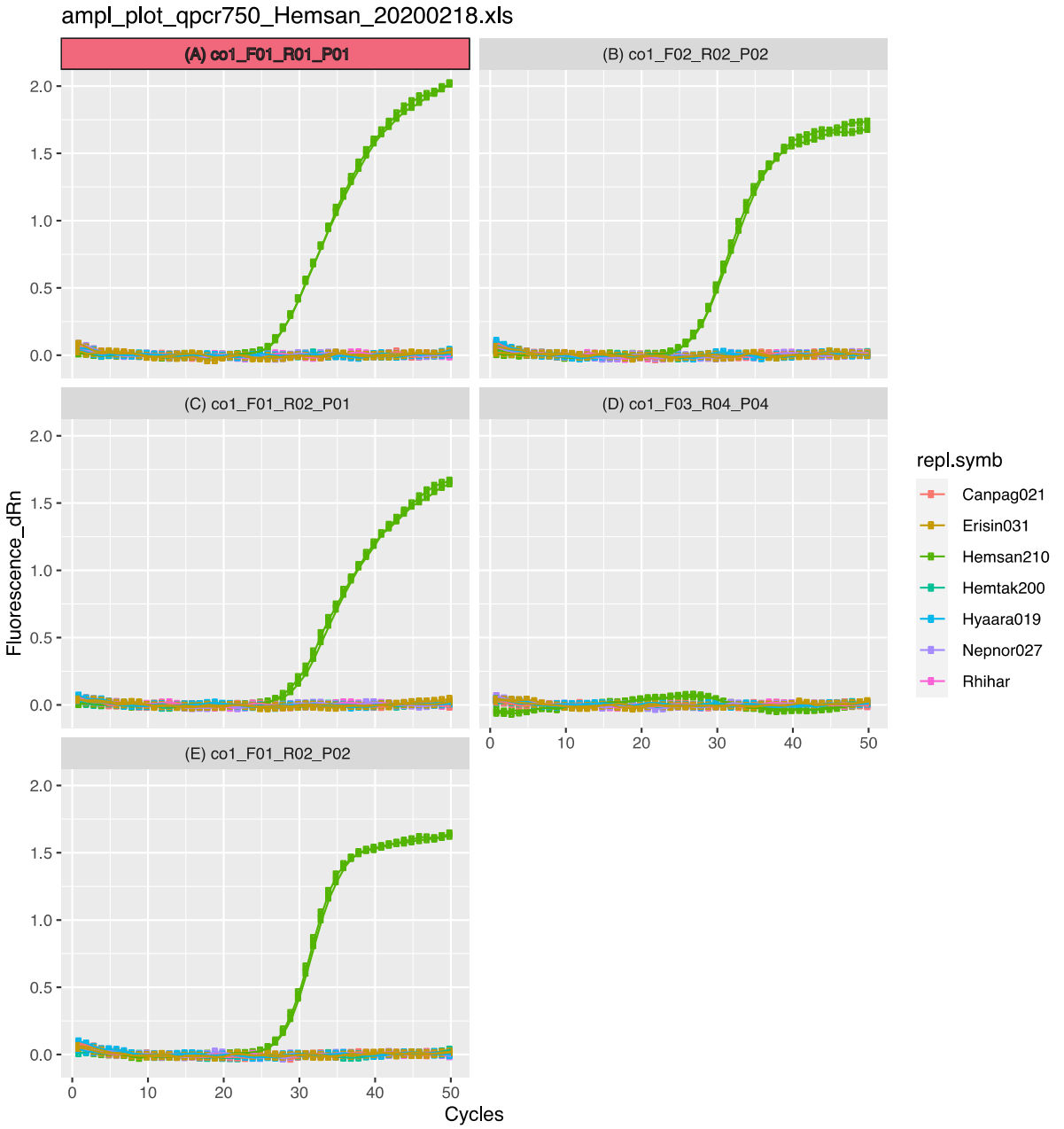

Figure 6: *Amplification of Hemigrapsus sanguineus species using four new assays developed in the present study. Target species Hemigrapsus sanguineus is shown in light green (Hemsan210) and non-target sister species (Hemigrapsus takanoi, Hemtak200) in bluish-green (not amplified). Other non-target species of Decapoda are in other colours (not amplified in any assay test). The five assays show the different combinations of primer and probe tested. The assay using Hemsan_co1_F01+ Hemsan_co1_R01+ Hemsan_co1_P01 (A) returns species-specific detection with the highest relative fluorescence level and lowest Cq. This assay (A) was preferred among the five assays tested. The other combinations of primers (B-F) either amplified at a later cycle threshold for quantification or also amplified on other non target species or returned a lower difference in relative fluorescence. The assays that performed inefficiently (B-F) and returned unspecific amplification, should not be used in future projects.*

3.1.3 Species-specific assay for detection of *Hemigrapsus takanoi*

The 'brush-clawed shore crab' is indigenous to the Pacific Ocean but has been introduced in European seas (Türkay, 2001; WoRMS, 2020b; Tendal and Jensen, 2017).

Binomial nomenclature and author:	*Hemigrapsus takanoi* Asakura & Watanabe, 2005
English common name:	Brushclawed crab or Asian brush crab
Danish common name:	Pensel-klippekrabbe

Figure 7: Hemigrapsus takanoi. *Photo of specimen Hemtak200 from NHMD collection. Specimen was collected from Dybsø Fjord near Enø in Denmark in July 2018. Photo by S.W. Knudsen.*

Two species of genus *Hemigrapsus* have been introduced in Danish marine waters. *Hemigrapsus sanguineus* and *Hemigrapsus takanoi*. A species-specific assay targeting DNA from the mitochondrial cytochrome oxidase 1 (mtDNA-CO1) region (table 12-13) in *Hemigrapsus takanoi* was developed and tested in the present study. The assay specific for mtDNA-CO1 in *Hemigrapsus takanoi* is comprised of the oligos:

Hemtak_co1_F05	5'-AGGTTTTGACTTCTTCCTCCTTCT-3'
Hemtak_co1_R05	5'-CTGCGAGTGGAGGGTAAACG-3'
Hemtak_co1_P05	5'-FAM-TAGAAAGAGGTGTAGGTACAGGATGGA-BHQ1-3'

Table 12: *Primers and probes specific for* Hemigrapsus takanoi*, targeting a 100 basepair long fragment from the mitochondrial cytochrome oxidase 1 gene.*

Oligo name	*oligo sequence in 5'->3' direction*	Temp (°C)	Length (bp)	GC (%)
Hemtak_COI_F05	5'- AGGTTTTGACTTCTTCCTCCTTCT -3'	59.6	24	41.7
Hemtak_COI_R05	5'- CTGCGAGTGGAGGGTAAACG -3'	60.7	20	60.0
Hemtak_COI_P05	5'-FAM- TAGAAAGAGGTGTAGGTACAGGATGGA -BHQ1-3'	62.2	27	44.4

Table 13: *Nucleotide sequence for targeted fragment for* Hemigrapsus takanoi *in the mitochondrial cytochrome oxidase 1 gene.*

Species	*sequence in 5'->3' direction*	Length (bp)	Molecular weight (Da)
Hemigrapsus takanoi	TCGGAGCCCCAGATATAGCCTTTCCCCGTATAAATAATA TAAGATTTTGACTTCTTCCTCCTTCTCTATCCCTCCTTTTA ACAAGAAGAATAGTAGAAAGAGGTGTAGGCACCGGAT GAACCGTTTATCCGCCACT	100	61658.5

Table 14: *Extracted DNA from tissue samples from various other co-occurring species that potentially can lead to false positive detection used for in vitro testing of the species-specific assay. Not all species are necessarily closely related to* Hemigrapsus*, but these species are all species of the order Decapoda, and all are commonly encountered in North European seas, including the invasive species* Rhithropanopeus harrisii *and* Hemigrapsus sangineus*.*

Species	Tissue sample abbreviation	Tested	Amplification result in qPCR	Acc. number or sequence
Cancer pagurus	Canpag	Yes	No	Canpag021, NHMD
Eriocheir sinensis	Erisin	Yes	Yes	Erisin031_E36.1-1, NHMD
Hyas araneus	Hyaara	Yes	No	Hyaara019_E32.6, NHMD
Nephrops norvegicus	Nepnor	Yes	No	Nepnor027_E33.1-1, NHMD
Rhithropanopeus harrisii	Rhihar	yes	No	Rhihar_hotA_AZ01.05, NHMD
Hemigrapsus_sanguineus	Hemsan	Yes	No	Hemsan203_E52-04-01-Hemsan215_E52-16-01
Hemigrapsus_takanoi	Hemtak	yes	yes	Hemtak200_E52-01-01-Hemtak207_E52-08-01

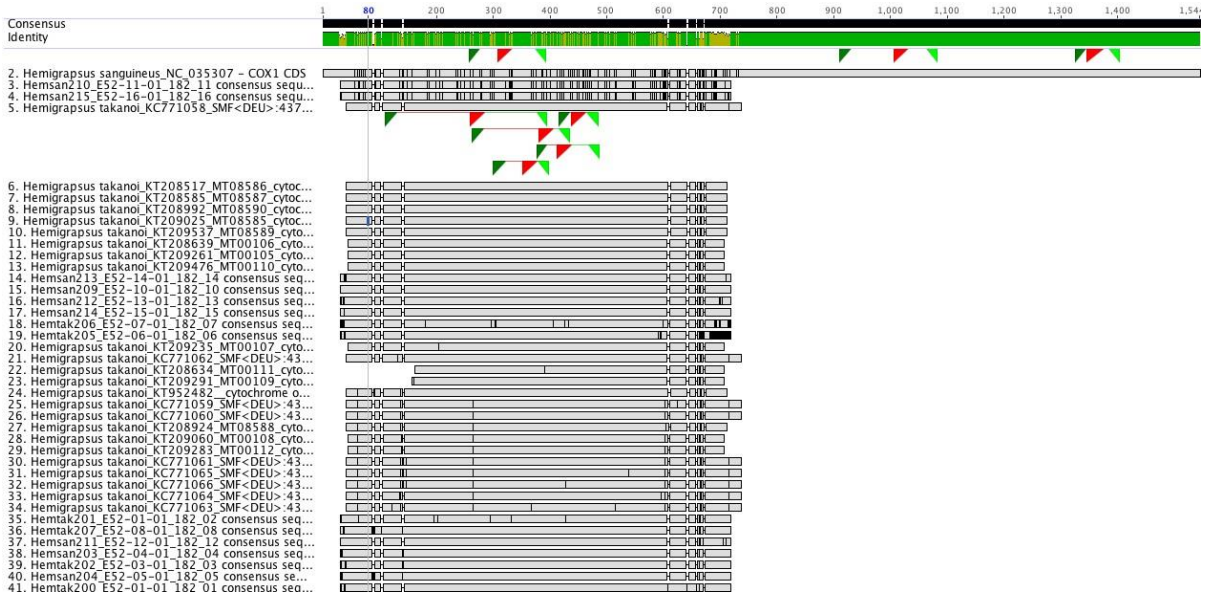

Figure 8: *Alignment of mitochondrial DNA cytochrome oxidase 1 gene from various species of marine Decapoda occurring in Danish marine waters. Primers and probes mapped on sequences from* Hemigrapsus takanoi*. Primers are marked with green annotations. The probe is indicated with a red annotation. Sequences were obtained acquired from NCBI GenBank and aligned in Geneious vR7.*

Primers and probes tested

The primers were designed by aligning sequences available from mitochondrial cytochrome oxidase 1 from different common species of Decapoda available from NCBI GenBank, which included the same sequences as listed under the assay development for *Hemigrapsus sangeineus*. The sequences were aligned in Geneious v. R7 (Kearse et al. 2012) using MAFFT 6.822 (Katoh and Toh, 2010) and primers matched against the target-species sequence with Primer3 v2.3.4 (Untergasser et al., 2012).

The following primers and probes were *in silico* designed and tested in vitro in a PCR reactions set up (PCR setup 01 as described in the protocol by Knudsen et al, 2019) to find a species-specific combination of primers and probes: Hemtak_co1_F05: 5'-AGGTTTTGACTTCTTCCTCCTTCT-3'; Hemtak_co1_F-04: 5'-CGAGCAGAATTAAGACAACCAGG-3'; Hemtak_co1_F03: 5'-GACCGTTTACCCTCCACTCG-3'; Hemtak_co1_F02: 5'-GCACCAGATATAGCTTTCCCC-3'; Hemtak_co1_F01: 5'-GGGGCTTCCGTAGATCTTGG-3'; Hemtak_co1_R05: 5'-CTGCGAGTGGAGGGTAAACG-3'; Hemtak_co1_R04: 5'-CGAGTGGAGGGTAAACG-GTC-3'; Hemtak_co1_R03: 5'-ATTGACAGCCCCTAAGATCG-3'; Hemtak_co1_R02: 5'-CCAAGATCTAC-GGAAGCCCC-3'; Hemtak_co1_R01: 5'-TGACAGCCCCTAAGATCGAAG-3'; Hemtak_co1_P05: 5'-FAM-TAGAAAGAGGTGTAGGTACAGGATGGA-BHQ1-3'; Hemtak_co1_P04: 5'-FAM-TGGAGCACCAGATAT-AGCTTTCCCCCG-BHQ1-3'; Hemtak_co1_P03: 5'-FAM-TGCTGGGGCTTCCGTAGATCTTGGTAT-BHQ1-3'; Hemtak_co1_P02: 5'-FAM-GTTTACCCTCCACTCGCAGCAGCTATT-BHQ1-3'; Hemtak_co1_P01: 5'-FAM-TCTTTTCTCTTCACCTTGCAGGAGTTT-BHQ1-3'. The initial PCR results from the test performed using these primers are not included in this report.

Assay specificity results

The assay designed and tested in this study (Hemtak_COI_F05, Hemtak_COI_R05, Hemtak_COI_P05) amplified for the two replicates of *Hemigrapsus takanoi* at a Cq of 25 (Figure 9). The F05-R05-P05-assay tested in this study was found to be species-specific only against the targeted species (Figure 9A) when tested on DNA extracted from other congeners (table 14).

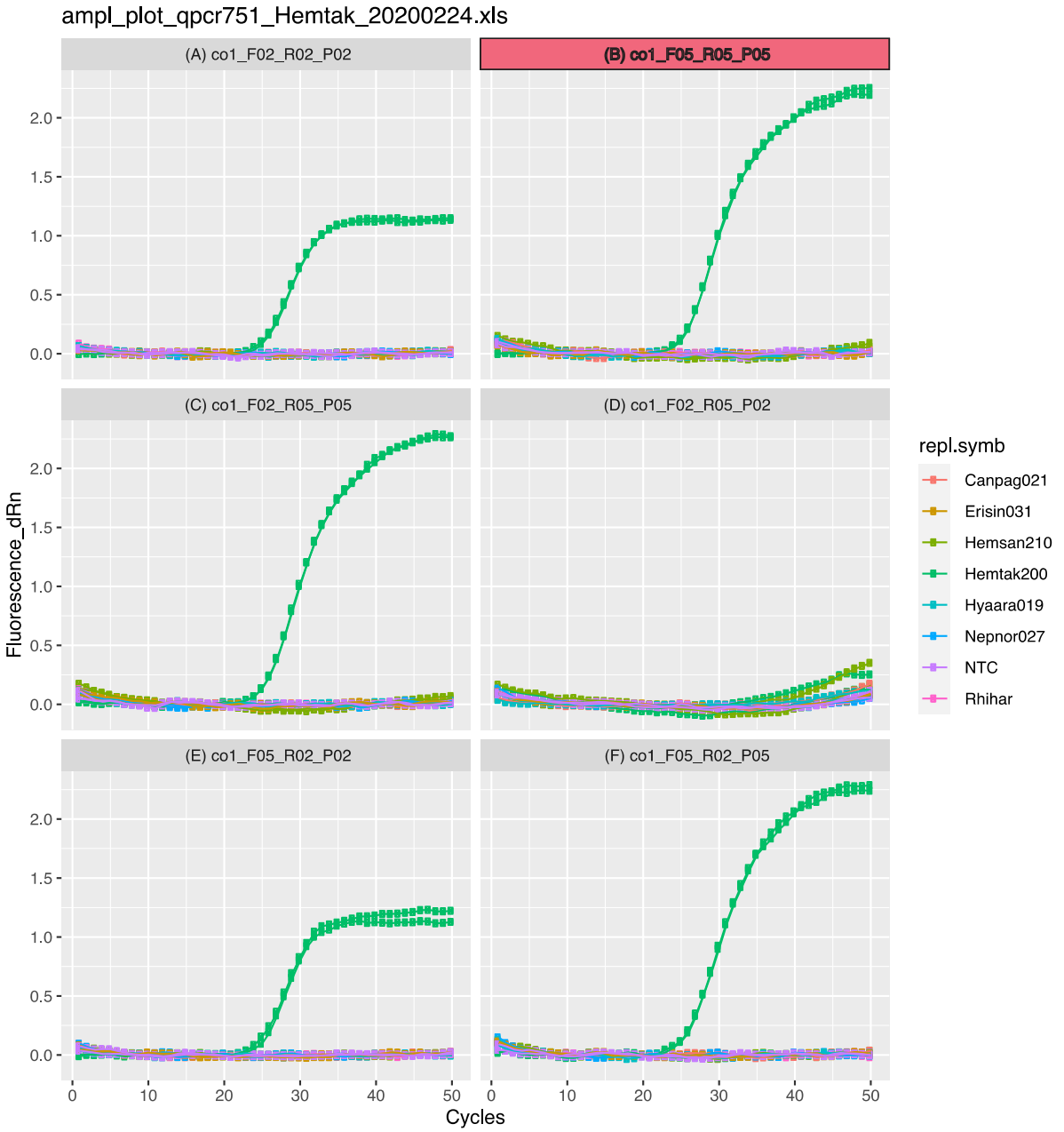

Figure 9: *Amplification of* Hemigrapsus takanoi *species using six new assays developed in the present study. Target species* Hemigrapsus takanoi *is shown in dark green (represented by the sample Hemtak200) and non-target sister species in other colours. The evolutionary closest related species in the genus* Hemigrapsus *(*Hemigrapsus sangineus*, represented by the Hemsan210 sample) did not amplify with this assay. The six amplification plots show the qPCR results from the different combinations of primer and probe tested. The assay using Hemtak_co1_F05 + Hemtak_co1_R05 + Hemtak_co1_P05 (B) returns species-specific detection with the highest relative fluorescence level and lowest Cq. This assay (B) was preferred among the six assays tested. The other combinations of primers (A and C-F) either amplified at a later cycle threshold for quantification or also amplified on other non target species or returned a lower difference in relative fluorescence. The assays that performed inefficiently (A and C-F) and returned unspecific amplification, should not be used in future projects.*

3.2 Further development and testing of existing assays

The next four assays listed present the primer probe systems developed during the MONIS 3-4 projects (Andersen et al., 2018; 2020), but here tested against DNA extracted from a broader representation of co-occurring non- target species.

3.2.1 Species-specific assay for detection of *Eriocheir sinensis*

The 'Chinese mitten crab' is indigenous to the Pacific Ocean but has been introduced in European seas.

Binomial nomenclature and author:	*Eriocheir sinensis* H. Milne Edwards 1853
English common name:	Chinese mitten crab
Danish common name:	Kinesisk uldhåndskrabbe

Figure 10: Eriocheir sinensis*. Photo by Henrik Carl and Peter R. Møller at the Natural History Museum of Denmark. The legs are about twice as long as the carapace width. The carapace width can grow up to 10 cm in width.*

The genus *Eriocheir* comprises two valid species, *Eriocheir japonicus* and *E. sinensis*, in the family Varunidae. Both *E. sinensis* and *E. japonicus* are non-native in European seas, and no other genera in the family Varunidae occurs natively in European seas (WoRMS, 2020c). Among the sequences for *E. sinensis* deposited in NCBI GenBank the mtDNA-cytochrome b (mtDNA-cytb) gene showed potential for assay design (table 15-16). The assay specific for mtDNA-cytb in *E. sinensis* is comprised of the oligos:

- Erisin_*cytb*_F02: 5'- ACCCCTCCTCATATCCAACCA -3'
- Erisin_*cytb*_R02: 5'- AAGAATGGCCACTGAAGCGG -3'
- Erisin_*cytb*_P02: 5'-FAM- TTTGCTTACGCTATTTTACGATCAATTCCT -BHQ-1-3'

Table 15: *Primers and probes specific for* Eriocheir sinensis*, targeting a 114 basepair long fragment from the mitochondrial cytochrome b gene. Species-specific primer/probe assay for* Eriocheir sinensis *with, target gene, product size, melting temperature, primer/probe length, GC ratio (%), and number of mismatches between primer and probe region in closely related non-target species.*

Oligo name	Oligo sequence in 5'->3' direction	Temp (°C)	Length (bp)	GC (%)
Erisin_cytb_F02	ACCCCTCCTCATATCCAACCA	62.73	21	52.38
Erisin_cytb_R02	AAGAATGGCCACTGAAGCGG	64.73	20	55.00
Erisin_cytb_P02	TTTGCTTACGCTATTTTACGATCAATTCCT	66.32	30	33.33

Table 16: *Nucleotide sequence for targeted fragment for* Eriocheir sinensis *in the mitochondrial cytochrome b gene.*

Species	*sequence in 5'->3' direction*	**Length (bp)**	**Molecular weight (Da)**
Eriocheir sinensis	ACCCCTCCTCATATCCAACCAGAATGGTATTTTCTTTTTG CTTACGCTATTTTACGATCAATTCCTAATAAATTAGGAGG AGTTGTAGCATTAGCCGCTTCAGTGGCCATTCTT	100	61658.5

Table 17: *Extracted DNA from tissue samples from various other co-occurring species that potentially can lead to false positive detection used for in vitro testing of the specificity of the assay. Not all species are necessarily closely related to* Eriocheir*, but these species are all species of the order Decapoda, and all are commonly encountered in North European seas, including the invasive* Rhithropanopeus harrisii*.*

Species	**Tissue sample abbreviation**	**Tested**	**Amplification result in qPCR**	**Tissue sample number used for in vitro test**
Cancer pagurus	Canpag	Yes	No	Canpag021_E32.1-1, NHMD
Carcinus maenus	Carmae	Yes	No	Carmac020_E32.5-1, NHMD
Calinectes_sapidus	Calsap	Yes	No	Calsap224:E53.09-2, Calsap225:E53.10-2, NHMD
Eriocheir sinensis	Erisin	Yes	Yes	E36.1-1:Erisin, NHMD
Homarus americanus	Homame	Yes	No	Homame01, NHMD
Homarus gammarus	Homgam	Yes	No	E32.2_Homgam024, NHMD
Hyas araneus	Hyaara	Yes	No	Hyaara019_E32.6-1, NHMD
Hyas coarctatus	Hyacoa	Yes	No	Hyacor023_E32.3-1, NHMD
Lithodes maja	Litmaj	Yes	No	Litmaj043_E33.5-1, NHMD
Nephrops norvegicus	Nepnor	Yes	No	Nepnor027_E33.1-1, NHMD
Pacifastacus leniusculus	Paclen	Yes	No	SW_DE_E01_07, NHMD
Pagurus bernhardus	Pagber	Yes	No	Pagber022_E32.4-1, NHMD
Paralithodes camtschaticus	Parcam	Yes	No	Parcam055_E35.1-1, NHMD
Paralomis sp.	Parsp	Yes	No	Parspp028_E33.2-1, NHMD
Paralomis spectabilis	Parspe	Yes	No	Parsp047_E33.3-1, NHMD
Rhithropanopeus harrisii	Rhihar	Yes	No	R_harrisii_hpt_C_AZ_01_04, NHMD
Hemigrapsus_sanguineus	Hemsan	Yes	No	Hemsan210:E52.11, NHMD
Hemigrapsus_takanoi	Hemtak	Yes	No	Hemtak200:E52.01, NHMD

The primers were designed by aligning sequences available from mitochondrial cytochrome b from North European species of Decapoda available from NCBI GenBank. This included: *Homarus gammarus*: KC107810, NC_020020; *Homarus americanus*: HQ402925; *Nephrops norvegicus*: NC_025958; *Eriocheir sinensis*: DQ779886, AY274302, KY041629; *Astacus astacus*: KX279347; *Astacus leptodactylus*: KX279349; *Pacifastacus leniusculus*: NC_033509; *Lithodes nintokuae*: AB769476; *Paralithodes camtschaticus*: NC_020029; *Paralithodes brevipes*: AB735677; *Paralithodes brevipes*: NC_021458; *Panulirus homarus*: KF738903. Sequence alignment was performed using Geneious v. R7 (Kearse et al. 2012) and MAFFT 6.822 (Katoh and Toh, 2010) and primers matched against target-species sequence with Primer3 v2.3.4 (Untergasser et al., 2012). The sequence alignment indicated that only the target-species: *Eriocheir sinensis*, would be amplified by the F02_R02_P02 system in a qPCR setup. In addition the following primers and probes were considered and tested in both initial PCR and qPCR setups, but found less suitable than the F02_R02_P02 system: Erisin_cytb_F03: 5'-CAAACAGGAGCTAATAACCCCT-3', Erisin_cytb_F04: 5'-CCGCTATCCCATTTATCGGT-3', Erisin_cytb_F05: 5'-ACCCTTTAGTAACCCCTCCTCA-3', Erisin_cytb_F06: 5'-CCCCTTAGGTATTTCAAGACAAAC-3', Erisin_cytb_P03: 5'-FAM-AGCCCCATATTTTCTAGGAGATCCAGABHQ1-3', Erisin_cytb_P04: 5'-FAM-ACCGACCTAGTACAATGAATCTGAGGGGGBHQ1-3', Erisin_cytb_P05: 5'-FAM-GGAGGAGTTGTAGCATTAGCCGCTTCABHQ1-3', Erisin_cytb_R03: 5'-TGAGGAGGGGTTACTAAAGGGT-3', Erisin_cytb_R04: 5'-CCTAAGGGGTTATTAGCTCCTGT-3', Erisin_cytb_R05: 5'-TGGGGTAAAATGCTAGTCTTTGA-3', Erisin_cytb_R06: 5'-TGGTTGGATATGAGGAGGGGT-3', Eri_sin_CytB_F01: 5'-TCGGTACCGACCTAGTACAA-3', Eri_sin_CytB_R01: 5'-AGAAAATGCTGATGCTACTAAAGGT-3', Eri_sin_CytB_P01: 5'-FAM-TGAGGAGGGTTTTCTGTTGATAATGCCAC-3'.

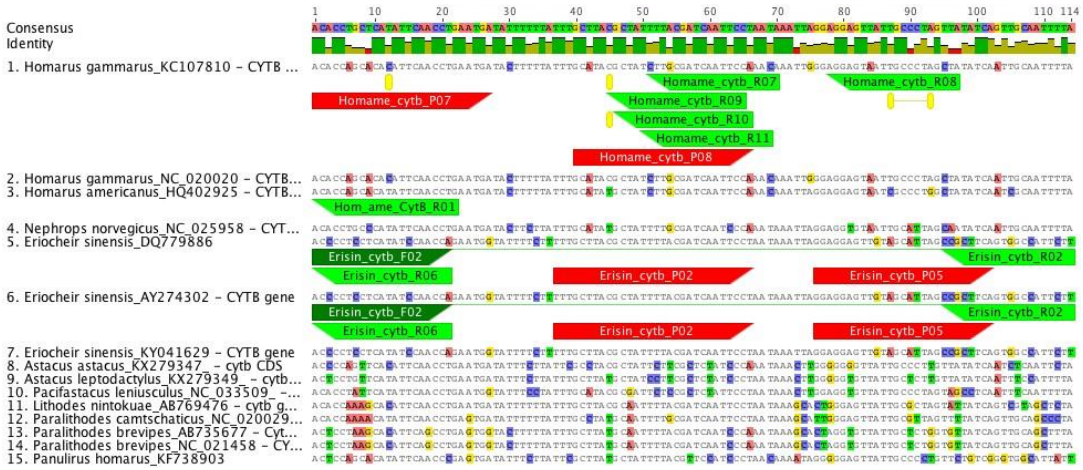

Figure 11: *Alignment of* Eriocheir sinensis *and other native species of the order Decapoda occurring in North European seas for the mtDNA-cytochrome b gene. All sequences were acquired from NCBI GenBank.*

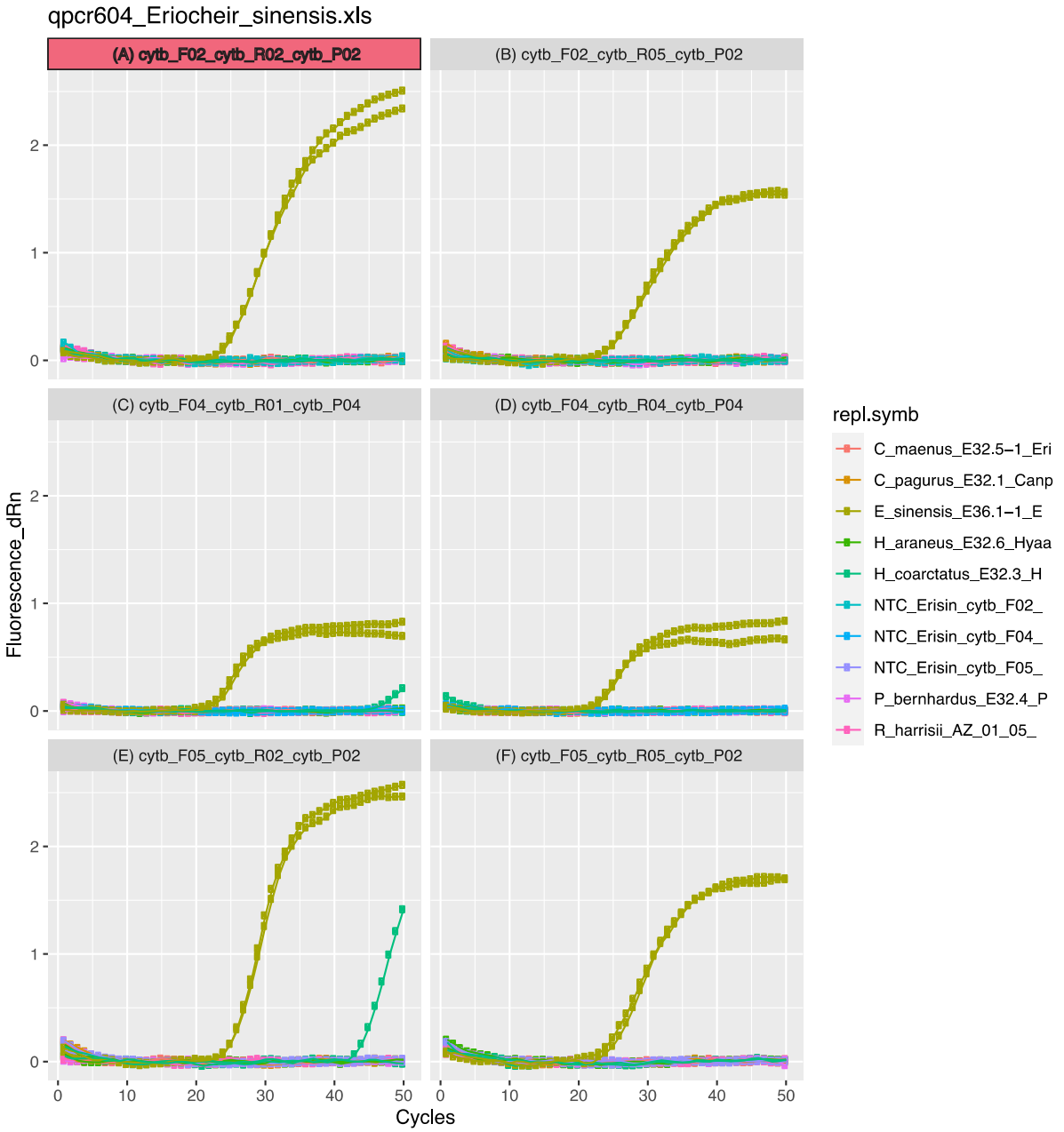

Figure 12: *Amplification of* Eriocheir sinensis *using the F02_R02_P02 assay (A) and other primer and probe combinations. Amplification signal for the target species* Eriocheir sinensis *is shown in brown-green colour and non-target species:* Cancer pagurus*,* Carcinus maenus*,* Hyas araneus*,* Hyas coarctatus*,* Pagurus bernhardus*,* Rhithropanopeus harrisii *and non-target controls (NTC) in other colours. The other combinations of primers (B-F) either amplified at a later cycle threshold for quantification or also amplified on other non target species or returned a lower difference in relative fluorescence. The assays that performed inefficiently (B-F) and returned unspecific amplification, should not be used in future projects.*

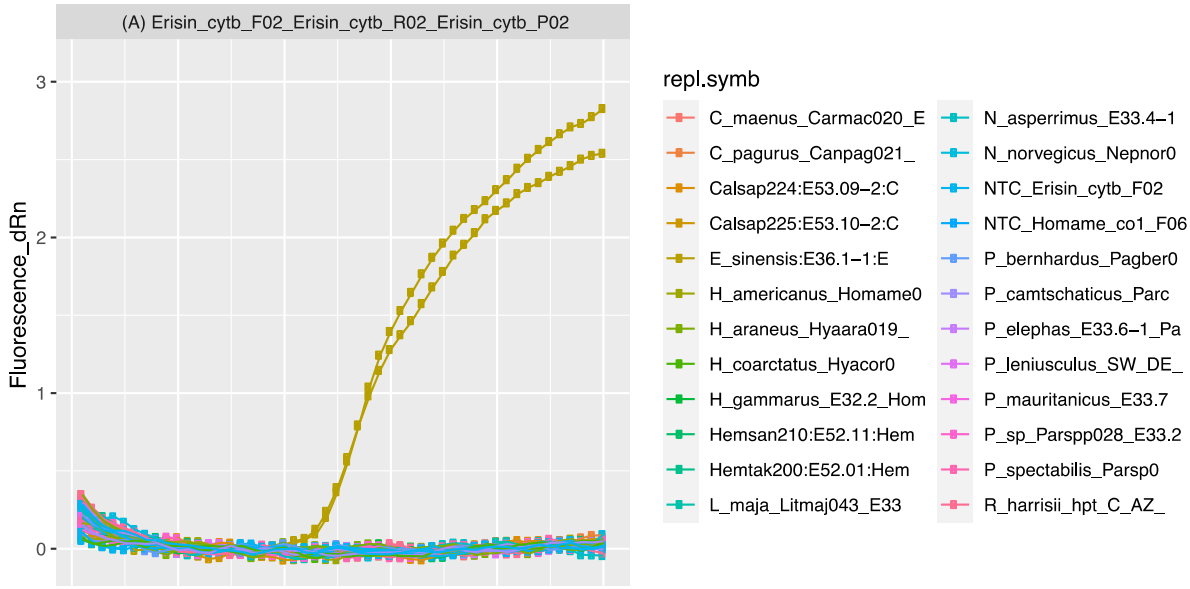

Figure 13: *Specificity for DNA from* Eriocheir sinensis *using the F02_R02_P02 assay. This additional quantitative polymerase chain reaction test included more non target species to ensure the previously developed assay combination only was specific towards the targeted species.*

Assay specificity results

The two replicates of *E. sinensis* amplified at a Cq of 24 (Figure 12). None of the non-target species (table 17) amplified with the F02_R02_P02 assay (Figure 13).

The designed eDNA target assay for *Eriocheir sinensis* is expected to only amplify DNA from the target species when tested on environmental water samples.

3.2.2 Species-specific assay for detection of *Homarus americanus*

The 'American lobster' is indigenous to the North Western Atlantic coast but have been introduced in European seas.

Binomial nomenclature and author:	*Homarus americanus* H. Milne Edwards, 1837
English common name:	American lobster
Danish common name:	Amerikansk hummer

Figure 14: Homarus americanus*. Photo of specimen on display at NHMD's collection, specimen was collected from 'Danmarks Akvarium'. Photo by S.W. Knudsen. They can grow to a considerable size measuring up to more than 60 cm in length and weighing more than 20 kg. Colouration is usually red body with green legs, but the body can vary from bluish, to yellow, red and orange.*

The genus *Homarus* comprises two extant valid species, *Homarus americanus* (American lobster) and *H. gammarus* (European lobster), in the family Nephropidae. In North-European seas the three species *Homarus gammarus*, *Nephropsis atlantica* and *Nephrops norvegicus* are natively occurring and are evolutionary closely related to *H. americanus*. The family Nephropidae comprise 14 genera found worldwide, but the North-east Atlantic is only inhabited by *Homarus*, *Nephrops*, *Nephropsis* and *Thymopides*, where the latter two are considered deep-sea species (>500 m depth), and rare. A broad representation of species of Decapoda occurring in Danish marine waters were selected for mitochondrial DNA cytochrome b sequences and mitochondrial DNA cytochrome oxidase 1 deposited on

NCBI GenBank, and these two gene regions appeared to have sufficient genetic variation to warrant assay design (Figure 15). Initial tests performed on mitochondrial DNA cytochrome oxidase 1 (Andersen et al., 2018) allowed for inferring a species-specific primer set (Hoame_co1 F06+R08+P08 assay) (Fig.16). Unfortunately, a qPCR test performed in the laboratory at the University of Copenhagen (Jan-2020) leaked the target amplicon from the PCR tubes that were not sealed properly. A consequence of this was that the laboratory had the mtDNA co1 amplicon in every reaction set up afterwards (Fig. 17), and that this amplicon only could be removed by adding Uracil-DNA glycosylase (UNG) enzyme to all tubes. To avoid the addition of this enzyme in future qPCR tests for detection of eDNA from *H. americanus*, a new primer and probe combination was teted (Fig. 18). Different combinations of primers and probes targeting the mitochondrial cytochrome b region was tested (Fig. 18) The subsequent tests showed that primers and probes designed for mitochondrial DNA cytochrome b (mtDNA-cytb) sequences (table 18-19) were optimal for distinguishing between *Homarus americanus* and other species of marine Decapoda occuring in Danish seas (table 20). The assay specific for mtDNA-cytb in *Homarus americanus* is comprised of the oligos:

- Homame_cytb_F02: 5'- TTTTAGTAGCAGCAGCGACTCTT -3'
- Homame_cytb_R14: 5'- CCAAGAAGGTAGGGATTTAGAAGA -3'
- Homame_cytb_P12: 5'-FAM- TGCAAGACATATTGATAAAGTTCCATTCCA -BHQ-1-3'

Table 18: Primers and probes specific for *Homarus americanus*, targeting a 193 basepair long fragment from the mitochondrial cytochrome b gene, and also listing melting temperature, primer/probe length and GC ratio (%).

Oligo name	*oligo sequence in 5'->3' direction*	Temp (°C)	Length (bp)	GC (%)
Homame_cytb_F02	TTTTAGTAGCAGCAGCGACTCTT	60.3	23	43.5
Homame_cytb_R14	CCAAGAAGGTAGGGATTTAGAAGA	57.4	24	41.7
Homame_cytb_P12	TGCAAGACATATTGATAAAGTTCCATTCCA	61.8	30	33.3

Table 19: *Nucleotide sequence for targeted fragment for* Homarus americanus *in the mitochondrial cytochrome b gene.*

Species	*sequence in 5'->3' direction*	Length (bp)	Molecular weight (Da)
Homarus americanus	TTTTAGTAGCAGCAGCGACTCTTATCCATATTTTATTTAT TCATCAAACTGGAGCTAACAACCCACTTGGAATTGCAAG ACATATTGATAAAGTTCCATTCCATCCTTATTTCACTTTTA AAGATGTTGTTGGATTTATAGTTATACTAACCGCATTAAT TTTATTGACTCTTCTAAATCCCTACCTTCTTGG	193	119098

Table 20: *Extracted DNA from tissue samples from various other co-occurring species that potentially can lead to false positive detection. These species are not necessarily closely related to* Homarus*, but all are species of the order Decapoda and all are commonly encountered in North European seas and could potentially give rise to false positive detection.*

Related species	Abbreviation	Tested	Amplification	Tissue sample number used for in vitro test
Cancer pagurus	Canpag	Yes	No	Canpag021_E32.1-1, NHMD
Carcinus maenus	Carmae	Yes	No	Carmac020_E32.5-1, NHMD
Calinectes_sapidus	Calsap	Yes	No	Calsap224:E53.09-2, Calsap225:E53.10-2, NHMD
Eriocheir sinensis	Erisin	Yes	No	E36.1-1:Erisin, NHMD
Homarus americanus	Homame	Yes	Yes	Homame01, NHMD

Homarus gammarus	Homgam	yes	No	E32.2_Homgam024, NHMD
Hyas araneus	Hyaara	yes	No	Hyaara019_E32.6-1, NHMD
Hyas coarctatus	Hyacoa	yes	No	Hyacor023_E32.3-1, NHMD
Lithodes maja	Litmaj	yes	No	Litmaj043_E33.5-1, NHMD
Nephrops norvegicus	Nepnor	yes	No	Nepnor027_E33.1-1, NHMD
Pacifastacus leniusculus	Paclen	yes	No	SW_DE_E01_07, NHMD
Pagurus bernhardus	Pagber	yes	No	Pagber022_E32.4-1, NHMD
Paralithodes camtschaticus	Parcam	yes	No	Parcam055_E35.1-1, NHMD
Paralomis sp.	Parsp	yes	No	Parspp028_E33.2-1, NHMD
Paralomis spectabilis	Parspe	yes	No	Parsp047_E33.3-1, NHMD
Rhithropanopeus harrisii	Rhihar	yes	No	R_harrisii_hpt_C_AZ_01_04, NHMD
Hemigrapsus_sanguineus	Hemsan	yes	No	Hemsan210:E52.11, NHMD
Hemigrapsus_takanoi	Hemtak	yes	No	Hemtak200:E52.01, NHMD

The primers were designed by aligning sequences available from mitochondrial cytochrome oxidase 1 and from the mitochondrial cytochrome b gene region from North European species of Decapoda available from NCBI GenBank. This included: *Eriocheir sinensis*: AY274302; *Homarus americanus*: AF370853, FJ174944, HQ402925, NC_015607, FJ581693, DQ889104; *Homarus gammarus*: KT208429, KT209166, KT208891, KC107810, NC_020020; *Lithodes aequispinus*: KC196523; *Lithodes confundens*: KC196536; *Lithodes ferox*: HM020903; *Lithodes formosae*: GU289678; *Lithodes longispina*: AB476813, AB476817; *Lithodes maja*: AF425309, KT209429, KT208393; *Lithodes murrayi*: HM020899; *Lithodes nintokuae*: AB375131; *Lithodes paulayi*: GU289677; *Lithodes santolla*: HM020898; *Lithodes turkayi*: KC196531; *Maja squinado*: GQ153553, GQ153551; *Neolithodes asperrimus*: HM020890, HM020891; *Neolithodes brodiei*: EU493263; *Neolithodes diomedeae*: KC196528; *Neolithodes duhameli*: HM020892; *Neolithodes grimaldii*: JQ305972; *Nephrops norvegicus*: FJ174945, JQ623962; *Palinurus barbarae*: FJ174960; *Palinurus charlestoni*: FJ174959; *Palinurus delagoae*: FJ174958; *Palinurus elephas*: DQ062206, KC789347; *Palinurus gilchristi*: FJ174961, EF546352; *Palinurus mauritanicus*: EF546365, DQ062207; *Palinustus unicornutus*: EF546344; *Panulirus homarus*: KU523817; *Panulirus ornatus*: KU523792, KU523815; *Panulirus versicolor*: KT001513, KT001512; *Papilio palinurus*: JQ982114, JQ982116, JQ982115; *Paralithodes brevipes*: NC_021458; *Paralithodes camtschaticus*: AB211435, JF738168; *Paralomis aculeata*: HM020904; *Paralomis africana*: HM020907; *Paralomis anamerae*: HM020905, HM020906; *Paralomis birsteini*: EU493261; *Paralomis cristata*: HM020911; *Paralomis cristulata*: HM020908; *Paralomis dofleini*: HM020913; *Paralomis elongata*: HM020914; *Paralomis erinacea*: HM020916; *Paralomis formosa*: KC196530; *Paralomis granulosa*: AF425318; *Paralomis multispina*: DQ882130; *Paralomis pacifica*: AB476747; *Paralomis spinosissima*: EU493258; *Paralomis zealandica*: HM020936. *Homarus americanus*: HQ402925, NC_015607; *Homarus gammarus*: KC107810, NC_020020; *Nephrops norvegicus*: NC 025958. Sequence alignment was performed using Geneious v. R7 (Kearse et al. 2012) and MAFFT 6.822 (Katoh and Toh, 2010) and primers matched against target-species sequence with Primer3 v2.3.4 (Untergasser et al., 2012). The sequence alignment indicated that only the target-species: *Homarus americanus*, would be amplified by the co1-F06_R08_P08 system in a qPCR setup. In addition the following primers and probes were considered and tested in both initial PCR and qPCR setups, but found less suitable than the co1-F06_R08_P08 system: Homame_co1_F01: 5'-CAGATATAGCATTTCCCCGTATG-3', Homame_co1_F11: 5'-AGTCCATCACTTCTCTGAGCTCTT-3', Homame_co1_P01: 5'-FAM-GGAGTAGGAACTGGATGAACTGTCTACCC-BHQ-1-3', Homame_co1_P02: 5'-FAM-GAAAGTGGAGTAGGAACTGGATGAACTG-BHQ-1-3', Homame_co1_P05: 5'-FAM-AGAAAGTGGAGTAGGAACTGGATGAAC-BHQ-1-3', Homame_co1_P06: 5'-FAM-GCAGGAGCTATTACTATACTCTTAACAGATCG-BHQ-1-3', Homame_co1_P11: 5'-FAM-TGGTGGTCTTACAGGAGTAGTTCTTGC-BHQ-1-3', Homame_co1_R01: 5'-CAATTGCTGCTGAGAGTGGA-3', Homame_co1_R02: 5'-

GCTGCTGAGAGTGGAGGGTA-3', Homame_co1_R03: 5'-CTGCTGAGAGTGGAGGGTAGA-3', Homame_co1_R04: 5'-TGCTGAGAGTGGAGGGTAGA-3', Homame_co1_R05: 5'-GCTGAGAGTGGAGGGTAGACA-3', Homame_co1_R06: 5'-CAGCTGGATCGAAGAATGAAG-3', Homame_co1_R07: 5'-CAGCTGGATCGAAGAATGAA-3', Homame_co1_R09: 5'-CCAGCTGGATCGAAGAATGA-3', Homame_co1_R10: 5'-AACTGGGTCTCCACCTCCAG-3', Homame_co1_R11: 5'-CGTAATGAAAGTGAGCAACAACA-3', Homame_co1_R12: 5'-GAACGTAATGAAAGTGAGCAACA-3', Homame_co1_R13: 5'-AACGTAATGAAAGTGAGCAACAA-3', Homame_co1_R14: 5'-AACGTAATGAAAGTGAGCAACAAC-3', Homame_co1_R15: 5'-CGTAATGAAAGTGAGCAACAACAT-3', Homame_cytb_F02: 5'-TTTTAGTAGCAGCAGCGACTCTT-3', Homame_cytb_F07: 5'-CCGGCTAATCCACTCGTT-3', Homame_cytb_F12: 5'-GGAGCTAACAACCCACTTGGA-3', Homame_cytb_P02: 5'-FAM-TCCATATTTTATTTATTCATCAAACTGGAGC-BHQ-1-3', Homame_cytb_P07: 5'-FAM-ACACCAGCACATATTCAACCTGAATGA-BHQ-1-3', Homame_cytb_P08: 5'-FAM-GCATATGCTATCTTGCGATCAATTCCA-BHQ-1-3', Homame_cytb_P12: 5'-FAM-TGCAAGACATATTGATAAAGTTCCATTCCA-BHQ-1-3', Homame_cytb_R02: 5'-GCAATTCCAAGTGGGTTGTT-3', Homame_cytb_R03: 5'-GCAATTCCAAGTGGGTTGTTA-3', Homame_cytb_R04: 5'-TGCAATTCCAAGTGGGTTGT-3', Homame_cytb_R05: 5'-GCAATTCCAAGTGGGTTGT-3', Homame_cytb_R06: 5'-TGCAATTCCAAGTGGGTTGTT-3', Homame_cytb_R07: 5'-TGTTTGGAATTGATCGCAAG-3', Homame_cytb_R08: 5'-TAGCCAGGGCGATTACTCCT-3', Homame_cytb_R09: 5'-GGAATTGATCGCAAGATAGCA-3', Homame_cytb_R10: 5'-TGGAATTGATCGCAAGATAGC-3', Homame_cytb_R11: 5'-GTTTGGAATTGATCGCAAGA-3', Homame_cytb_R12: 5'-TCTCCAAGAAGGTAGGGATTTAGA-3', Homame_cytb_R13: 5'-TCCAAGAAGGTAGGGATTTAGAA-3', Homame_cytb_R14: 5'-CCAAGAAGGTAGGGATTTAGAAGA-3', Homame_cytb_R15: 5'-TCCAAGAAGGTAGGGATTTAGAAG-3', Homame_cytb_R16: 5'-CTCCAAGAAGGTAGGGATTTAGAA-3'.

Figure 15: *Alignment of* Homarus americanus *and other species of the order Decapoda for the mtDNA-cytochrome b gene. All sequences were acquired from NCBI GenBank.*

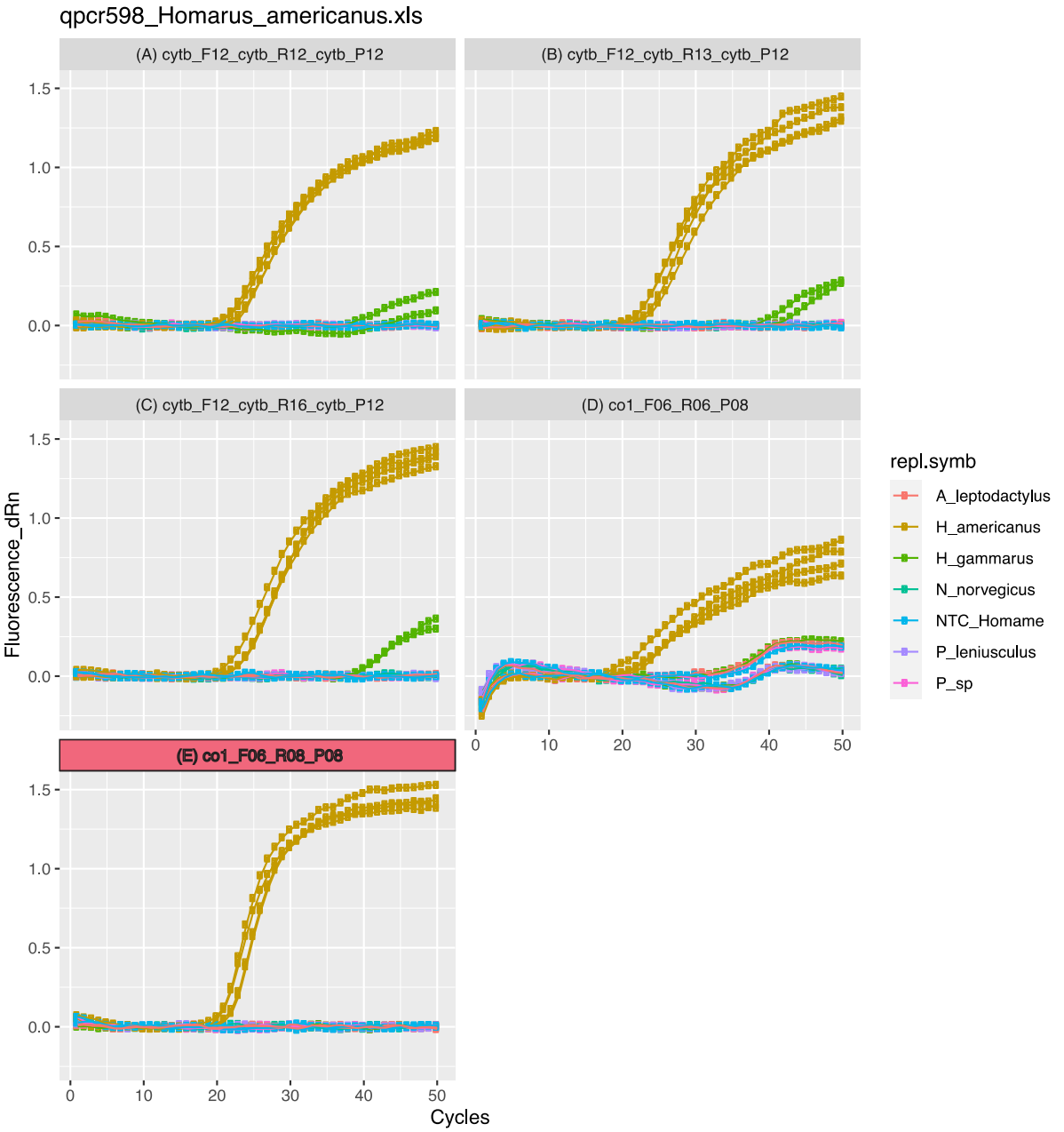

Figure 16: *Amplification of* Homarus americanus *using the co1-F06_R08_P08 assay (E) (Andersen et al., 2017). Target species* Homarus americanus *is shown in yellow-brown colour. Non-target species:* Homarus gammarus*,* Nephrops norvegicus*,* Paralomis *sp,* Astacus leptodactylus*, and* Pacifastacus leniusculus*, in other colours. The other combinations of primers (A-D) either amplified at a later cycle threshold for quantification or also amplified on other non target species or returned a lower difference in relative fluorescence. The assays that performed inefficiently (A-D) are therefore not recommended for future projects.*

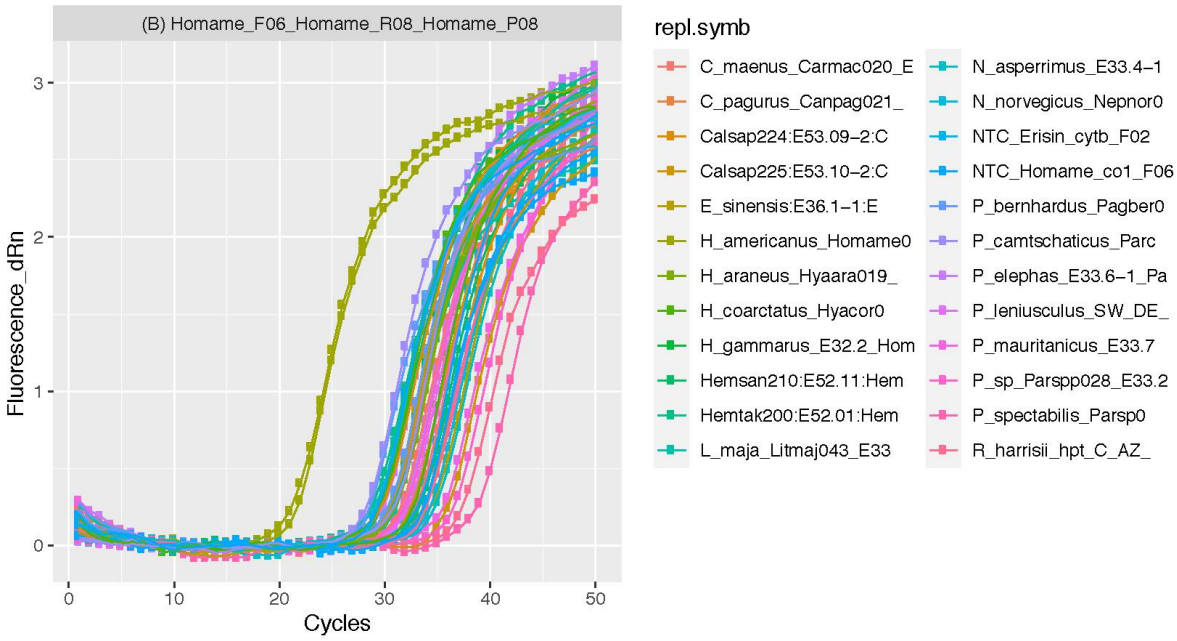

Figure 17: *A second test of the specificity for DNA from* Homarus americanus *using the F06_R08_P08 assay. This additional quantitative polymerase chain reaction test included more non target species to check whether the previously developed assay combination only was specific towards the targeted species. Unfortunately, this test showed that the laboratory has been contaminated with fragments from the F06_R08_P08 assay. This F06+R08+P08 assay should not be used at the University of Copenhagen, as this contamination likely will persist. Instead a new assay was tested.*

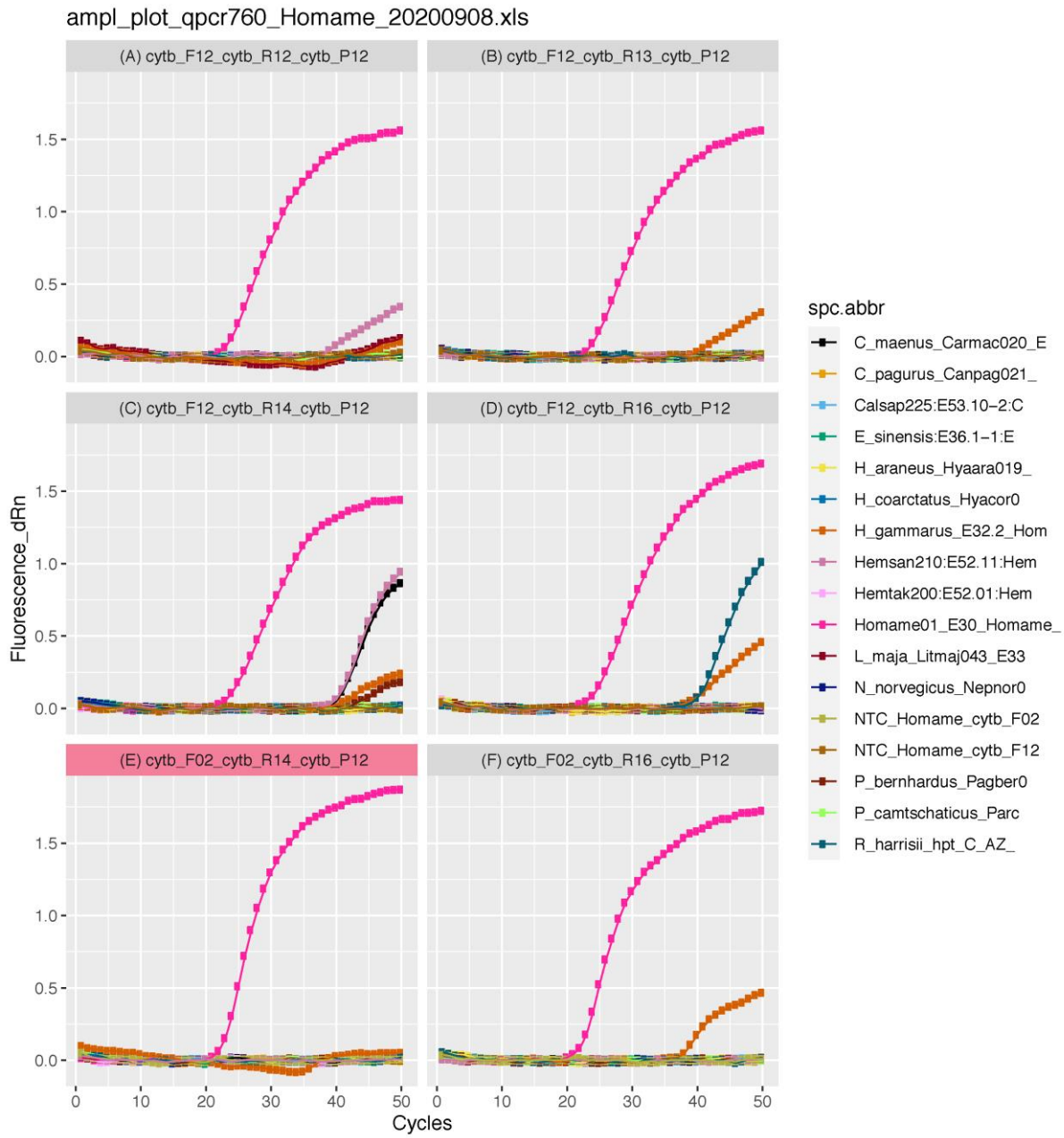

Figure 18: *A test of the specificity for DNA from* Homarus americanus *using six new combinations of primers and probe targeting the mtDNA-cytchrome b region, instead of the mtDNA-cytochrome oxidase 1 region. The combination of primer F02, R14 and P12 (E) (highlighted in red) only return positive amplification on DNA from* H. americanus. *The other primer combinations (A-D, F) return positive amplification when tested on DNA extracted from several additional non-target species. These other primer and probe combinations (A-D, F) are thus not recommended for future species-specific tests on water samples.*

Assay specificity results

The sample with DNA extracted from *Homarus americanus* amplified at a Cq of 21 (Figure 18). None of the non-target species (table 20) amplified with the cytb-F02_R14_P12 assay (Figure 18). The designed eDNA assay for *Homarus americanus* is expected to only amplify DNA from the target species when tested on laboratory extracted DNA from tissue or when used on environmental water samples.

3.2.3 Species-specific assay for detection of *Paralithodes camtschaticus*

The 'Kamchatka crab' is indigenous to the Pacific Ocean but has been introduced in European seas.

Binomial nomenclature and author:	*Paralithodes camtschaticus* (Tilesius 1815)
English common name:	Red king crab
Danish common name:	Japan-krabbe

Figure 18: Paralithodes camtschaticus. *Photo of specimen from NHMD collection, specimen number ZMUC META042756, collected from Barents Sea, May-2001. Photo by S.W. Knudsen. Specimens can grow to a considerable size. The carapace width can grow up to 28 cm, and the legs can span 180 cm. Individuals can weigh more 12 kg, with males being larger than females. They are usually found in deep waters between 20 m and 200 m depth, and prefer a temperature range around 3 °C to 5 °C.*

The genus *Paralithodes* comprises five extant valid species, *Paralithodes brevipes; P. californiensis; P. camtschaticus; P. platypus and P. rathbuni* in the family Lithodidae (WoRMS, 2020e). In North-European seas all species of *Paralithodes* are considered non-native. The family Lithodidae comprise the genera: *Acantholithus*, *Cryptolithodes*, *Ctenorhinus*, *Echinocerus*, *Glyptolithodes*, *Leptolithodes*, *Lithodes*, *Lopholithodes*, *Neolithodes*, *Paralithodes*, *Paralomis*, *Petaloceras*, *Phyllolithodes*, *Pristopus*, *Pseudolithodes*, *Rhinolithodes* and *Sculptolithodes*, where the genera *Lithodes*, *Neolithodes*, *Paralomis* and *Paralithodes* occurs in North European seas. In the order Decapoda a broad representation of species occurring in Danish marine waters were selected for mitochondrial DNA cytochrome b sequences and mitochondrial DNA cytochrome oxidase 1 deposited on NCBI GenBank. The mitochondrial DNA cytochrome oxidase 1 showed potential for assay design (Figure 19). Previous tests in MONIS3 suggested a combination of primers and probes (Parcam_co1_F02, Parcam_co1_R05, Parcam_co1_P02) to be used for detection of *P. camschaticus*. However, in the present study this primer and probe combination was found to be unspecific, by also returning false positive amplification on

DNA from tissue from hermit crab (*Pagurus* spp.). Instead a new combination of primers and probes were tested against DNA extracted from a broader diversity of marine Decapoda. The tests performed showed that a new set of primers and a probe designed for mitochondrial DNA cytochrome oxidase 1 (mtDNA-CO1) sequences (table 21-22) were optimal for detecting mtDNA-CO1 from *P. camschaticus* and not amplifying on mtDNA-CO1 from other Decapoda. The assay specific for mtDNA-CO1 in *P. camschaticus* is comprised of the oligos:

- Parcam_co1_F12: 5'- CGTCCACAAGGAATAACCTTAGAC-3'
- Parcam_co1_R12: 5'- AACTGGGTCTCCTCCTCCTG-3'
- Parcam_co1_P12: 5'-FAM- TTTGTGTGATCCGTATTTATTACTGCAA-BHQ-1-3'

Table 21: *Primers and probes specific for* Paralithodes camtschaticus *targeting a 174 basepair long fragment from the mitochondrial cytochrome oxidase 1 gene. Species-specific primer/probe assay for* Paralithodes camtschaticus *with, target gene, product size, melting temperature, primer/probe length, GC ratio (%).*

Oligo name	*oligo sequence in 5'->3' direction*	Temp (°C)	Length (bp)	GC (%)
Parcam_co1_F12	CGTCCACAAGGAATAACCTTAGAC	59.1	24	45.8
Parcam_co1_R12	CAATTTCCAAACCCTCCAAT	60.3	20	60.0
Parcam_co1_P12	FAM- TTTGTGTGATCCGTATTTATTACTGCAA-BHQ1	60.1	28	32.1

Table 22: *Nucleotide sequence for targeted fragment for* Paralithodes camtschaticus *in the mitochondrial cytochrome oxidase 1 gene.*

Species	*sequence in 5'->3' direction*	Length (bp)	Molecular weight (Da)
Paralithodes camtschaticus	CGTCCACAAGGAATAACCTTAGACCGTATACCTTTATTTG TGTGATCCGTATTTATTACTGCAATTCTACTTTTATTATCA CTACCAGTTTTAGCAGGAGCTATTACTATATTACTTACAG ATCGAAATTTAAACACCTCTTTTTTTGACCCTGCAGGAG GAGGAGACCCAGTT	174	107368.2

Table 23: *Extracted DNA from tissue samples from various other co-occurring species that potentially can lead to false positive detection. Not all species are necessarily closely related to* Paralithodes camtschaticus*, but these species are all species of the order Decapoda, and all are commonly encountered in North European seas, including the invasive* Rhithropanopeus harrisii*.*

Related species*	Abbreviation	Tested	Amplification	Tissue sample number used for in vitro test
Cancer pagurus	Canpag	Yes	No	Canpag021_E32.1-1, NHMD
Carcinus maenus	Carmae	Yes	No	Carmac020_E32.5-1, NHMD
Calinectes_sapidus	Calsap	Yes	No	Calsap224:E53.09-2, Calsap225:E53.10-2, NHMD
Eriocheir sinensis	Erisin	Yes	No	E36.1-1:Erisin, NHMD
Homarus americanus	Homame	Yes	No	Homame01, NHMD
Homarus gammarus	Homgam	Yes	No	E32.2_Homgam024, NHMD
Hyas araneus	Hyaara	Yes	No	Hyaara019_E32.6-1, NHMD
Hyas coarctatus	Hyacoa	Yes	No	Hyacor023_E32.3-1, NHMD
Lithodes maja	Litmaj	Yes	No	Litmaj043_E33.5-1, NHMD
Nephrops norvegicus	Nepnor	Yes	No	Nepnor027_E33.1-1, NHMD
Pacifastacus leniusculus	Paclen	yes	No	SW_DE_E01_07, NHMD
Pagurus bernhardus	Pagber	yes	Yes**/No	Pagber022_E32.4-1, NHMD
Paralithodes camtschaticus	Parcam	yes	Yes	Parcam055_E35.1-1, NHMD
Paralomis sp.	Parsp	yes	No	Parspp028_E33.2-1, NHMD
Paralomis spectabilis	Parspe	yes	No	Parsp047_E33.3-1, NHMD
Rhithropanopeus harrisii	Rhihar	yes	No	R_harrisii_hpt_C_AZ_01_04, NHMD
Hemigrapsus_sanguineus	Hemsan	yes	No	Hemsan210:E52.11, NHMD
Hemigrapsus_takanoi	Hemtak	yes	No	Hemtak200:E52.01, NHMD

** Not necessarily closely related to Paralithodes, but these species are all species of the order Decapoda, and evolutionary closely related to the family Lithodidae, and all are commonly encountered in North European seas.*
***) Unintended amplification was registered for the assay developed during MONIS3 (Andersen et al., 2017). But no amplification was registered using the newly designed and tested F12+R12+P12 assay presented in this report.*

The primers were designed by aligning sequences available from mitochondrial cytochrome oxidase 1 from North European species of Decapoda available from NCBI GenBank. This included: *Eriocheir sinensis*: AY274302, NC_006992, KP126617, KY041629, KP064329; *Homarus americanus*: HQ402925, NC_015607; *Homarus gammarus*: KC107810, NC_020020; *Nephrops norvegicus*: LN681403, NC_025958; *Lithodes nintokuae*: NC_024202; *Paralithodes camtschaticus*: JX944381, NC_020029; *Paralithodes brevipes*: NC_021458; *Lithodes aequispinus*: AF425308; *Lithodes maja*: AF425309; *Paralithodes camtschaticus*: AF425317; *Lithodes santolla*: AF425310; *Paralomis granulosa*: AF425318; *Hyas araneus*: EU682834,FJ581699, FJ581701, FJ581702, FJ581706, KT073232, FJ581704, JQ305959, KT209456, JQ305960, KT208691, KT209382, KT209560, FJ581703, KT208460, FJ581700, KT208661, KT209003, KT209502, FJ581705, KT208612, KT209353, KT208434; *Hyas coarctatus*: FJ581707, FJ581708, FJ581712, FJ581709, FJ581710, FJ581711, FJ581713, JQ306008, JQ306009, KT208545, KT209008, KT208498, KT208590, KT208863, KT208982, KT208987, KT209122, KT209369, KT208565, AB244632, EU682835. Sequence alignment was performed using Geneious v. R7 (Kearse et al. 2012) and MAFFT 6.822 (Katoh and Toh, 2010) and primers matched against target-species sequence with Primer3 v2.3.4 (Untergasser et al., 2012). The sequence alignment indicated that only the target-species: *Homarus americanus*, would be amplified by the co1-F02_R05_P02 system in a qPCR setup. In addition the following primers and probes were considered and tested in both initial PCR and qPCR

setups, but found less suitable than the co1- F02_R05_P02 system: Parcam_co1_F07: 5'-AGGAG-CATCAGTGGATTTAGGT-3', Parcam_co1_F12: 5'-CGTCCACAAGGAATAACCTTAGAC-3', Par-cam_co1_P12: 5'-FAM-TTTGTGTGATCCGTATTTATTACTGCAA-BHQ-1-3', Parcam_co1_R02: 5'-GTCA-ATTTCCAAACCCTCCA-3', Parcam_co1_R03: 5'-TCAATTTCCAAACCCTCCAA-3', Parcam_co1_R06: 5'-TCAATTTCCAAACCCTCCAAT-3', Parcam_co1_R07: 5'-CGGTCTAAGGTTATTCCTTGTGG-3', Par-cam_co1_R09: 5'-AAGGTTATTCCTTGTGGACGTA-3', Parcam_co1_R10: 5'-ACGGTCTAAGGTTATTCC-TTGTG-3', Parcam_co1_R11: 5'-GGTCTAAGGTTATTCCTTGTGGA-3', Parcam_co1_R12: 5'-AACTGGG-TCTCCTCCTCCTG-3', Parcam_co1_R14: 5'-AAAACTGGGTCTCCTCCTCCT-3', Parcam_co1_R16: 5'-CTCCTCCTGCAGGGTCAA-3'.

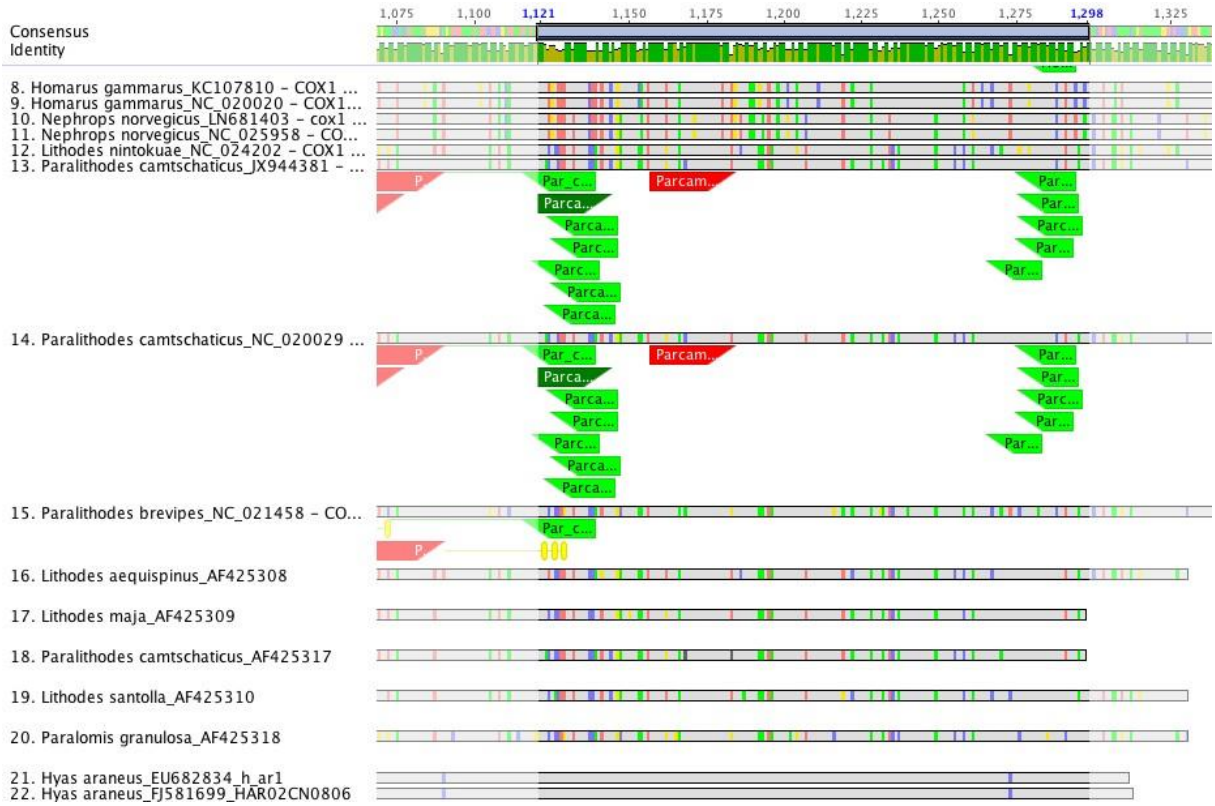

Figure 19: Alignment of *Paralithodes camtschaticus* and other species of the order Decapoda for the mtDNA-*cytochrome oxidase 1* gene. All sequences were acquired from NCBI GenBank. This alignment shows the positioning of the F12-R12-P12 primer and probe assay specific for only mtDNA –CO1 from *P. camschaticus*.

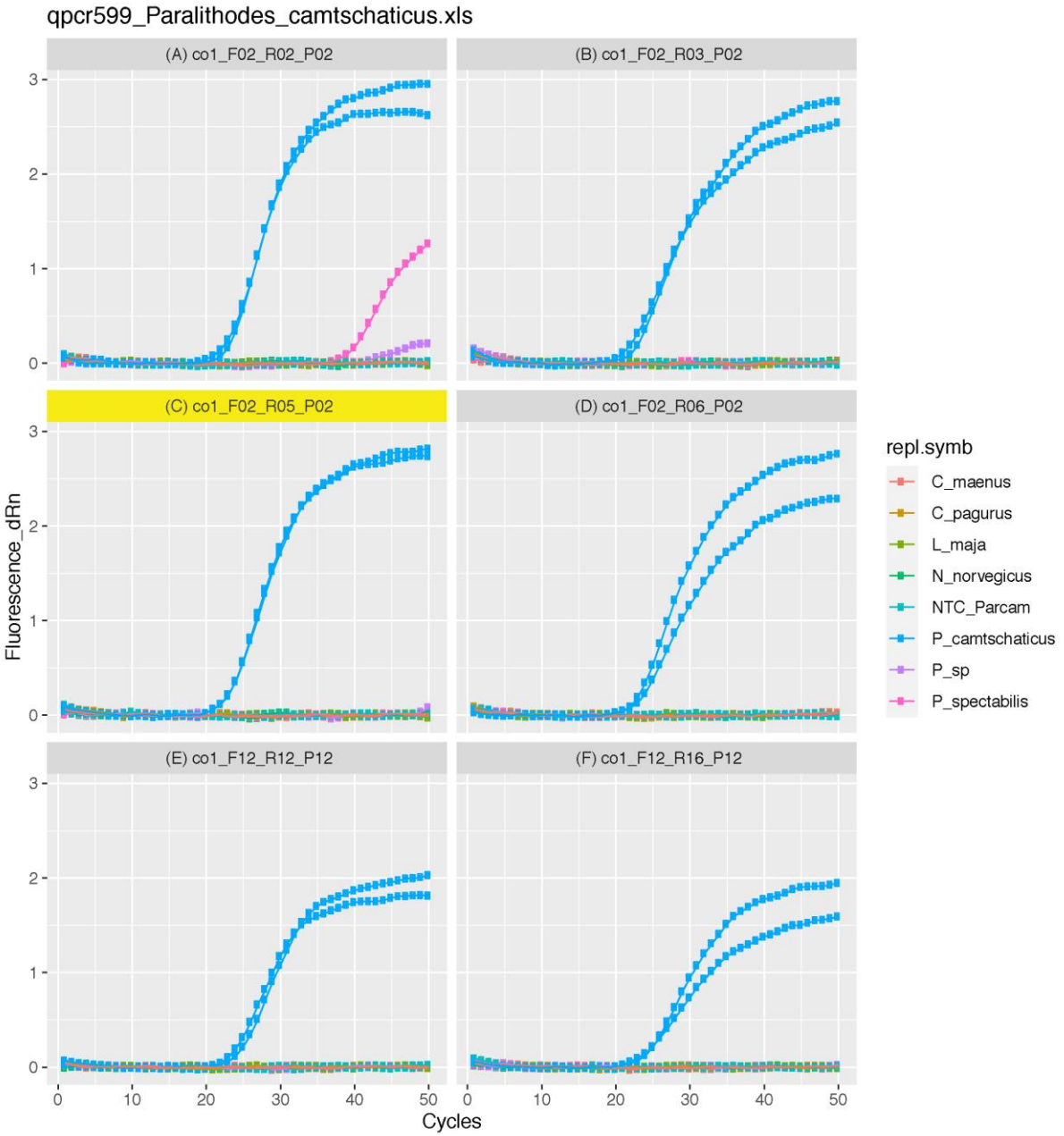

Figure 20: *Amplification of* Paralithodes camtschaticus *using the co1-F02_R05_P02 assay (C). Target species* Paralithodes camtschaticus *is shown in blue. Non-target species: Nephrops norvegicus,* Paralomis sp.*,* Cancer pagurus*,* Carcinus maenus*,* Paralomis spectabilis *and* Lithodes maja*, in other colours. The Negative target control (NTC) is not returning amplification, indicating that reagents were not contaminated with DNA from* Paralithodes camtschaticus*. The other combinations of primers (A-B, and D-F) either amplified at a later cycle threshold for quantification or also amplified on other non target species, or returned a lower difference in relative fluorescence, and these assays are therefore not recommended for future projects. However, the co1-F02_R05_P02 assay (with yellow heading) was later found to also amplify on DNA from hermit crab (*Pagurus *spp.). None of the assays in this figure can therefore be used for specific detection of DNA from* Paralithodes camtschaticus.

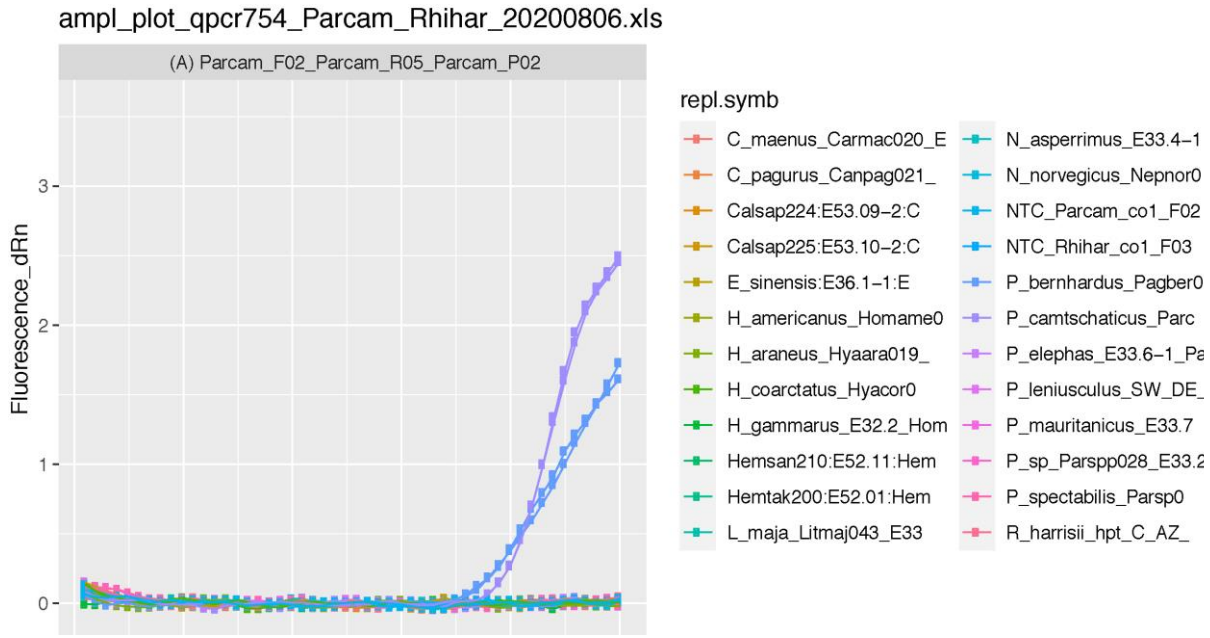

Figure 21: *Specificity for DNA from* Paralithodes camtschaticus (purple line) *using the F02_R05_P02 assay. This additional quantitative polymerase chain reaction test included more non target species. Unfortunately, this showed that the otherwise species-specific assay developed during the MONIS3 project (Andersen et al., 2018) was unspecific, and also returned amplification on DNA from* Pagurus *spp. (light blue line) A new assay was instead designed and tested for* P. camschaticus.

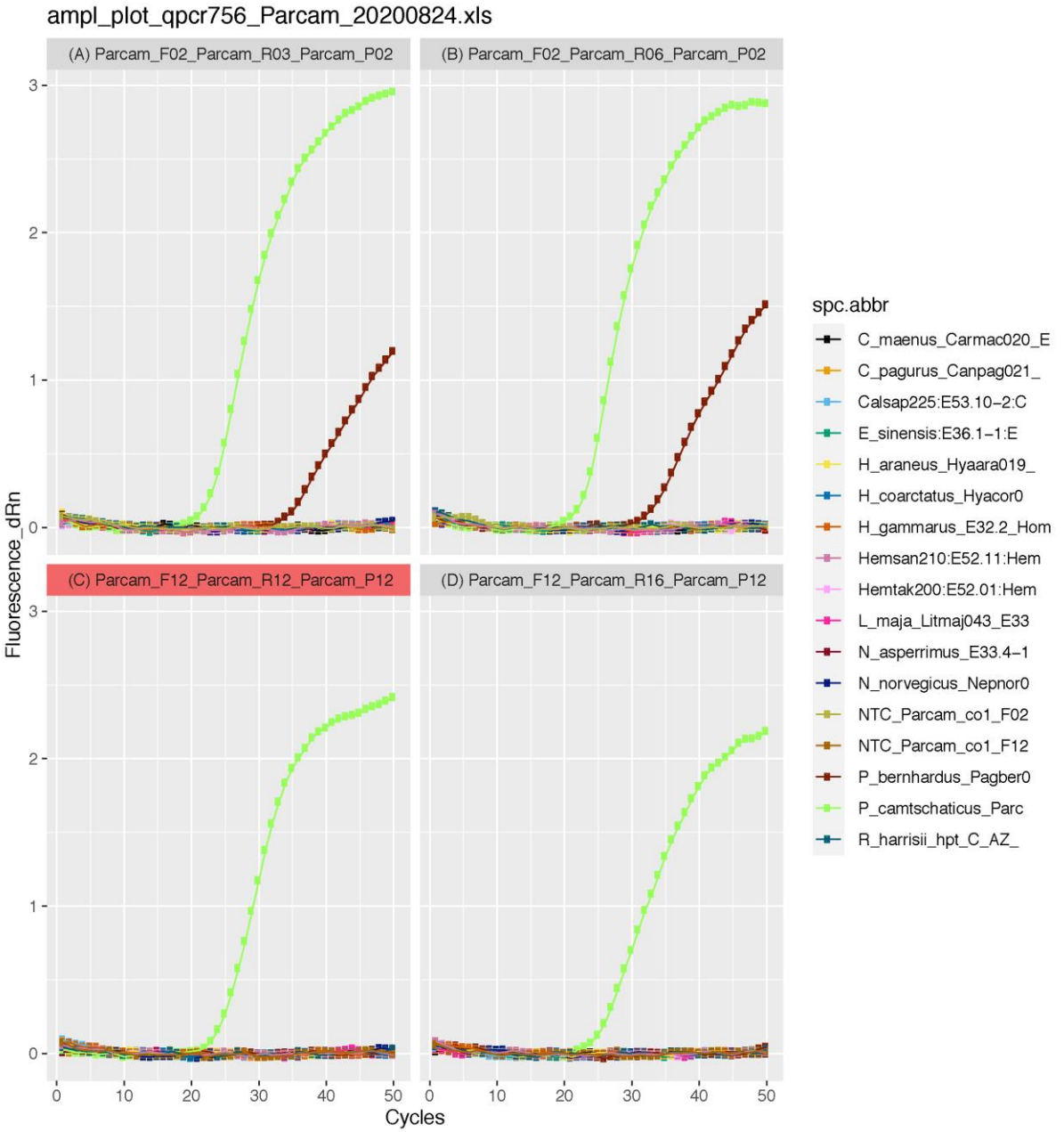

Figure 22: *Amplification of* Paralithodes camtschaticus *using the co1-F12_R12_P12 assay (C). Target species* Paralithodes camtschaticus *is shown in green. Non-target species in other colours. The Negative target control (NTC) is not returning amplification, indicating that reagents were not contaminated with DNA from* Paralithodes camtschaticus*. The other combinations of primers (A-B, and D) either amplified at a later cycle threshold for quantification or also amplified on other non target species, or returned a lower difference in relative fluorescence, and these assays are therefore not recommended for future projects. The co1-F12_R12_P12 assay (red heading) was found to be specific for detection of DNA from* Paralithodes camtschaticus and to return the optimal flourescence and earliest amplification onset. *The co1-F12_R12_P12 assay is thus recommended here to be used on water samples for testing for the presence of eDNA from* P. camschaticus*.*

Assay specificity results

In the initial MONIS3 test setup (Andersen et al., 2018) no other species tested (table 23) amplified with the co1-F02_R05_P02 assay (Figure 21), but this test did not include DNA from hermit crab

(*Pagurus* spp.). When this past assay was tested on DNA from a greater diversity of Decapoda, this old assay also returns positive amplification on DNA from *Pagurus* spp. (Figure 20 and 21). This unfortunately makes the previously developed assay unable to distinguish between eDNA from *Paralithodes camtschaticus* and from *Pagurus* spp. To work around this, a new assay with different primers and different probes (Figure 22) were tested for the present study. In this test the assay F12+R12+P12 targeting mtDNA-CO1 was found to be specific towards DNA from *Paralithodes camtschaticus* (Figure 22). The recommended assay in the present report is therefore the F12+R12+P12 assay.

3.2.4 Species-specific assay for detection of *Rhithropanopeus harrisii*

The 'Zuiderzee crab, estuarine mud crab' is indigenous to the western Atlantic Ocean but has been introduced in European seas.

Binomial nomenclature and author:	*Rhithropanopeus harrisii* (Gould, 1841)
English common names:	Zuiderzee crab, dwarf crab, estuarine mud crab, Harris mud crab, white-tipped mud crab
Danish common name:	Østamerikansk brakvandskrabbe

Figure 23: Rhithropanopeus harrisii*. Photo by Henrik Carl and Peter R. Møller at the Natural History Museum of Denmark. Individuals are relatively small with carapace width reaching up to 20 mm in width. They inhabit estuarine waters and live near stones and oyster beds or hidden in sandy bottom.*

The genus *Rhithropanopeus* comprise one valid species: *Rhithropanopeus harrisii*, in the family Panopeidae. *Rhithropanopeus* is non-native in European seas, and no other genera in the family Panopeidae occurs natively in North European seas (WoRMS, 2020d). Among the sequences for *Rhithropanopeus harrisii* deposited in NCBI GenBank, and the sequences obtained in a bachelor project performed at the Natural History Museum of Denmark (Aagaard 2015), the mtDNA-cytochrome oxidase 1 (mtDNA-CO1) gene showed potential for assay design (Figure 23; Table 25-26). The assay specific for mtDNA-CO1 in *Rhithropanopeus harrisii* is comprised of the oligos:

- Rhihar_*co1*_F03: 5'- GTCAACCTGGTACTCTCATTGGT -3'
- Rhihar_*co1*_R03: 5'- ACGAGGAAATGCTATATCAGGGG -3'
- Rhihar_*co1*_P03: 5'-FAM- TGTTGTAGTAACAGCTCACGCCTTTGT -BHQ-1-3'

Table 25: *Primers and probes specific for* Rhithropanopeus harrisii *a 164 basepair long fragment from the mitochondrial cytochrome oxidase 1 gene.*

Oligo name	*oligo sequence in 5'->3' direction*	Temp (°C)	Length (bp)	GC (%)
Rhihar_co1_F03	GTCAACCTGGTACTCTCATTGGT	63	23	48
Rhihar_co1_R03	ACGAGGAAATGCTATATCAGGGG	63	23	48
Rhihar_co1_P03	FAM-TGTTGTAGTAACAGCTCACGCCTTTGT-BHQ1	67	27	44

Table 26: *Nucleotide sequence for targeted fragment for* Rhithropanopeus harrisii *in the mitochondrial cytochrome oxidase 1 gene.*

Species	*sequence in 5'->3' direction*	Length (bp)	Molecular weight (Da)
Rhithropanopeus harrisii	GTCAACCTGGTACCCTCATTGGTAATGACCAAATTTACA ATGTTGTAGTAACAGCTCACGCCTTTGTAATAATCTTTTT TATAGTTATACCCATTATAATTGGAGGATTTGGTAATTG ACTAGTTCCATTAATATTAGGAGCCCCTGATATAGCATTT CCTCGT	164	101188.2

Table 27: *Tissue samples from species and DNA extracted from tissue and used for the in vitro testing of the specificity of the assay targeting DNA from* R. harrisii*. These species are not necessarily closely related to* Rhithropanopeus*, but these species are all representatives of the order Decapoda, and all are occurring in Northern Europe.*

Related species	Abbreviation	Tested	Amplification	Tissue sample number used for in vitro test
Cancer pagurus	Canpag	yes	No	Canpag021_E32.1-1, NHMD
Carcinus maenus	Carmae	yes	No	Carmac020_E32.5-1, NHMD
Calinectes_sapidus	Calsap	yes	No	Calsap224:E53.09-2, Calsap225:E53.10-2, NHMD
Eriocheir sinensis	Erisin	yes	No	E36.1-1:Erisin, NHMD
Homarus americanus	Homame	yes	No	Homame01, NHMD
Homarus gammarus	Homgam	yes	No	E32.2_Homgam024, NHMD
Hyas araneus	Hyaara	yes	No	Hyaara019_E32.6-1, NHMD
Hyas coarctatus	Hyacoa	yes	No	Hyacor023_E32.3-1, NHMD
Lithodes maja	Litmaj	yes	No	Litmaj043_E33.5-1, NHMD
Nephrops norvegicus	Nepnor	yes	No	Nepnor027_E33.1-1, NHMD
Pacifastacus leniusculus	Paclen	yes	No	SW_DE_E01_07, NHMD
Pagurus bernhardus	Pagber	yes	No	Pagber022_E32.4-1, NHMD
Paralithodes camtschaticus	Parcam	yes	No	Parcam055_E35.1-1, NHMD
Paralomis sp.	Parsp	yes	No	Parspp028_E33.2-1, NHMD
Paralomis spectabilis	Parspe	yes	No	Parsp047_E33.3-1, NHMD
Rhithropanopeus harrisii	Rhihar	yes	Yes	R_harrisii_hpt_C_AZ_01_04, NHMD
Hemigrapsus_sanguineus	Hemsan	yes	No	Hemsan210:E52.11, NHMD
Hemigrapsus_takanoi	Hemtak	yes	No	Hemtak200:E52.01, NHMD

The primers were designed by aligning sequences available from mitochondrial cytochrome oxidase 1 genes from North European species of Decapoda available from NCBI GenBank. In addition, more than six different haplotypes for mitochondrial cytochrome oxidase 1 for *R. harrisii* were included.

This covers all the haplotypes known in North European Seas (Projecto-Garcia, et al., 2009). The alignment was prepared using the following sequences from NCBI GenBank and *de novo* sequencing performed at the Natural History Museum of Denmark: *Ashtoret lunaris*: NC_024435; *Austinograea alayseae*: NC_020314; *Austinograea rodriguezensis*: NC_020312; *Callinectes sapidus*: NC_006281; *Chaceon granulatus*: NC_023476; *Charybdis feriata*: NC_024632; *Charybdis japonica*: NC_013246; *Cyclograpsus granulosus*: NC_025571; *Damithrax spinosissimus*: NC_025518; *Eriocheir hepuensis*: NC_011598; *Gandalfus yunohana*: NC_013713; *Halocaridina rubra*: CO1; *Homarus americanus*: HQ402925; *Homarus gammarus*: KC107810; *Homologenus malayensis*: NC_026080; *Rhithropanopeus harrisii* hpt: A, NHMD; *Rhithropanopeus harrisii* hpt: B, NHMD; *Rhithropanopeus harrisii* hpt: C, NHMD; *Rhithropanopeus harrisii* hpt: R, NHMD; *Rhithropanopeus harrisii* hpt: U, NHMD; *Rhithropanopeus harrisii* hpt: unkn, NHMD; *Rhithropanopeus harrisii*: DQ882140; *Hyas araneus*: EU682834; *Ilyoplax deschampsi*: NC_020040; *Lithodes aequispinus*: AF425308; *Lithodes confundens*: KM887493; *Lithodes couesi*: DQ882086; *Lithodes ferox*: HM020903; *Lithodes formosae*: GU289678; *Lithodes longispina*: AB476817; *Lithodes maja*: KT209538; *Lithodes murrayi*: HM020899; *Lithodes nintokuae*: AB375131; *Lithodes paulayi*: GU289677; *Lithodes santolla*: AF425310; *Lithodes turkayi*: KC196540; *Maja squinado*: KC789212; *Mictyris longicarpus*: NC_025325; *Myomenippe fornasinii*: NC_024437; *Neolithodes asperrimus*: HM020891; *Neolithodes brodiei*: EU493263; *Neolithodes duhameli*: HM020896; *Neolithodes grimaldii*: JQ305973; *Nephrops norvegicus*: KT209472; *Ocypode ceratophthalmus*: NC_025324; *Pachygrapsus crassipes*: NC_021754; *Palinurus delagoae*: FJ174958; *Palinurus elephas*: AJ889577; *Palinurus gilchristi*: FJ174961; *Palinurus mauritanicus*: FJ174957; *Palinustus unicornutus*: EF546344; *Panulirus ornatus*: KU523814; *Panulirus versicolor*: KT001513; *Paralithodes brevipes*: NC_021458; *Paralithodes camtschaticus*: JX944381; *Paralomis africana*: HM020907; *Paralomis anamerae*: HM020906; *Paralomis birsteini*: HM020909; *Paralomis cristulata*: HM020908; *Paralomis dofleini*: HM020913; *Paralomis erinacea*: HM020916; *Paralomis formosa*: KC196533; *Paralomis granulosa*: HM020926; *Paralomis multispina*: AB211296; *Paralomis pacifica*: AB476750; *Paralomis spinosissima*: KC196534; *Paralomis zealandica*: HM020936; *Portunus pelagicus*: NC_026209; *Portunus trituberculatus*: NC_005037; *Pseudocarcinus gigas*: NC_006891; *Ranina ranina*: NC_023474; *Rhithropanopeus harrisii*: DQ882141, DQ882142, DQ882143; *Scylla olivacea*: NC_012569; *Scylla paramamosain*: NC_012572; *Scylla serrata*: NC_012565; *Scylla tranquebarica*: NC_012567; *Thalamita crenata*: NC_024438; *Umalia orientalis*: NC_026688; *Xenograpsus testudinatus*: NC_013480. Sequence alignment was performed using Geneious v. R7 (Kearse et al. 2012) and MAFFT 6.822 (Katoh and Toh, 2010) and primers matched against target-species sequence with Primer3 v2.3.4 (Untergasser et al., 2012). The sequence alignment indicated that only the target-species: *Rhithropanopeus harrisii*, would be amplified by the F03_R03_P03 system in a qPCR setup. In addition the following primers and probes were considered and tested in both initial PCR and qPCR setups, but found less suitable than the F03_R03_P03 system: Rhihar_co1_F01: 5'-CCACCATCACTTACACTCCTCC-3', Rhihar_co1_F02: 5'-CCCCTGATATAGCATTTCCTCGT-3', Rhihar_co1_F04: 5'-AGCCCCTGATATAGCATTTCCT-3', Rhihar_co1_F05: 5'-GGAGCCCCTGATATAGCATTT-3', Rhihar_co1_P01: 5'-AAAGAGGAGTTGGAACAGGATGAACTG-3', Rhihar_co1_P02: 5'-FAM-TTTACCACCATCACTTACACTCCTCCTBHQ1-3', Rhihar_co1_R01: 5'-TCCTATATCAACGGAGGCTCC-3', Rhihar_co1_R02: 5'-TCATCCTGTTCCAACTCCTCT-3', Rhihar_co1_R04: 5'-CAGTTCATCCTGTTCCAACTCC-3', Rhihar_co1_R05: 5'-TCCTGTTCCAACTCCTCTTTCT-3'.

The species-specific primers designed by Forstrom & Vasemagi (2016) for detection of *R. harrisii* were also tested in an initial pilot study and were found to be unable to distinguish between *Rhithropanopeus* and *Hyas* (not shown). Since two species in the genus *Hyas* (i.e. 'Hyas araneus' common Danish name is 'sandkrabbe', and '*Hyas coarctatus'* common Danish name is 'pyntekrabbe') are widely distributed, native and common in the Skagerak, Kattegat and North Sea the primers recommended by Forstrom & Vasemagi (2016) were found unsuitable for species-specific detection of eDNA from *R. harrisii* in the Danish marine waters.

Assay specificity results

The two replicate reactions with genomic DNA from *Rhithropanopeus harrisii* amplified at a Cq of below 30 (Figure 24). None of the non-target species tested (Table 27) amplified with the F03R03P03 assay (Figure 25). Because the designed eDNA assay targeting *Rhithropanopeus harrisii* did not return amplification when applied on relatively high concentrations of genomic DNA extracted from tissue from other closely related and/or co-occurring species, it is expected that this primer and probe combination will amplify mtDNA from the target species only (*Rhithropanopeus harrisii*) when tested on environmental water samples.

Figure 24: *Alignment of* Rhithropanopeus harrisii *and other species of the order* Decapoda *occurring in North European seas for the mtDNA-cytochrome oxidase 1 gene. Sequences were acquired from NCBI GenBank, and from a bachelor project (Aagaard 2015) focusing on mitochondrial population genetic variation among* R. harrisii *in Øresund (the Sound, the strait between Denmark and Sweden).*

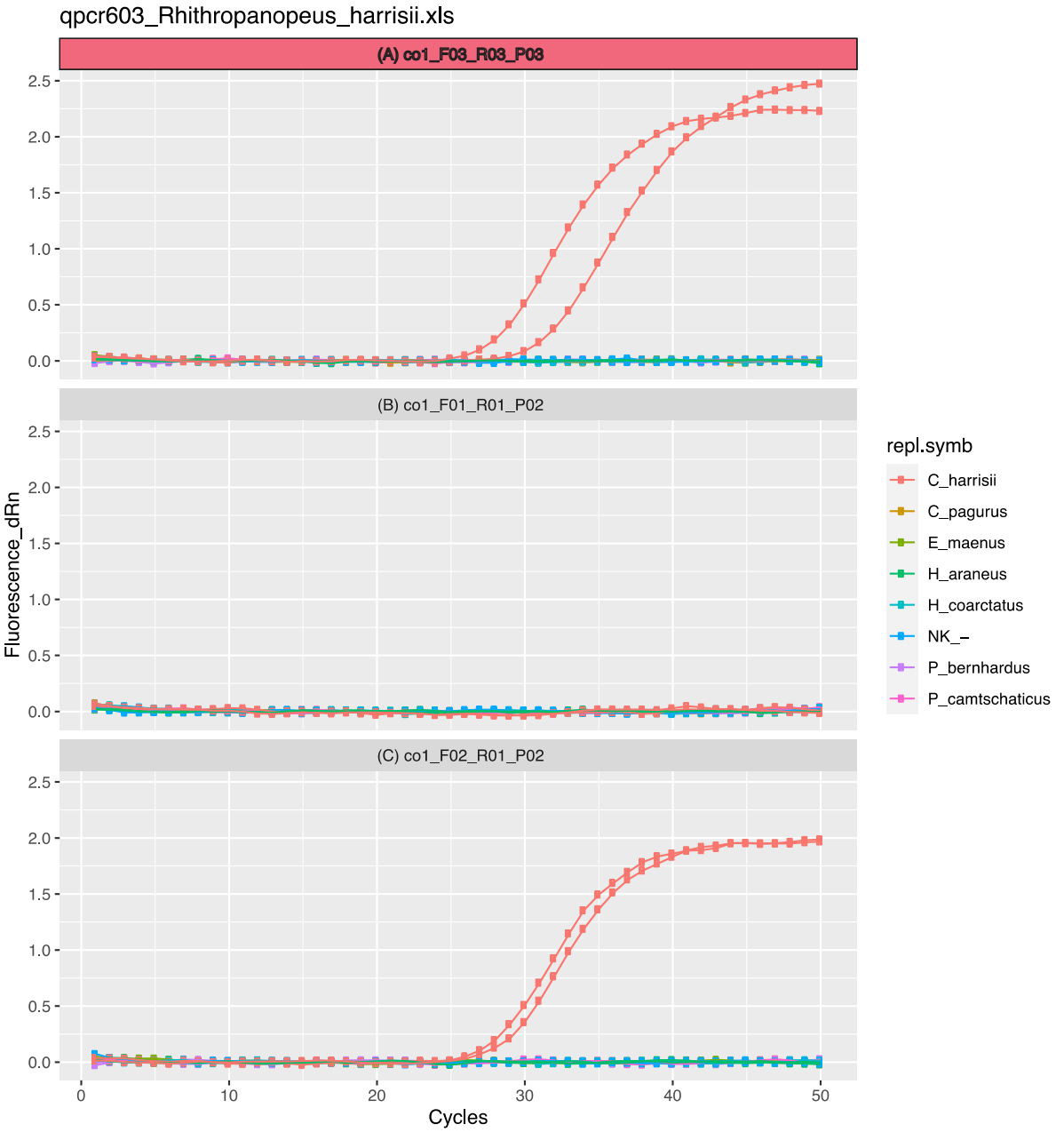

Figure 25: *Amplification of* Rhithropanopeus harrisii *using the F03R03P03 assay (A) targeting mtDNA-cytochrome oxidase 1 performed best among the three combinations of primers and probes tested. Target species* Rhithropanopeus harrisii *is shown in red and non-target species:* Cancer pagurus*,* Carcinus maenus*,* Paralithodes camtschaticus*,* Hyas araneus*,* Pagurus bernhardus*,* Hyas coarctatus *in other colours. The negative target control (NK) is not returning amplification, indicating that reagents were not contaminated with DNA from* Paralithodes camtschaticus*. The other combinations of primers (B-C) either amplified at a later cycle threshold for quantification or also amplified on other non target species or returned a lower difference in relative fluorescence. The assays that performed inefficiently (B-C) and returned unspecific amplification, should not be used in future projects.*

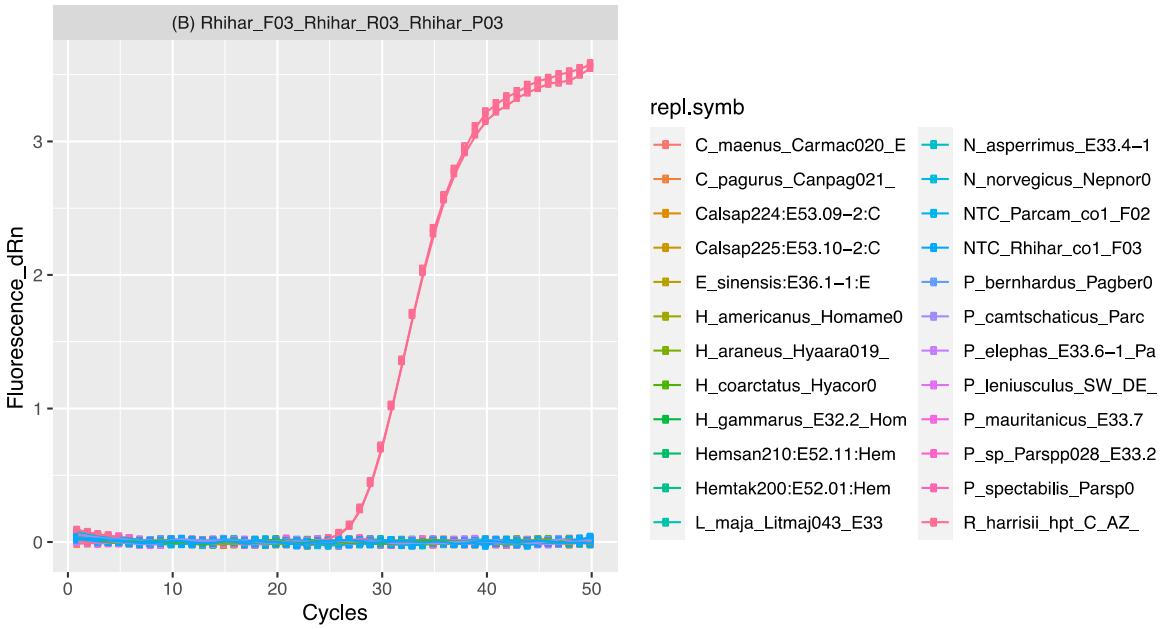

Figure 26: *Specificity for DNA from* Rhithropanopeus harrisii *using the F03_R03_P03 assay. This additional quantitative polymerase chain reaction test included more non target species to ensure the previously developed assay combination was specific towards only the targeted species. The samples named (NTC) are non target controls containing only reagents and double distilled water. The other samples have reagents plus genomic extracted DNA stemming from tissue samples.*

4 Conclusions

Three new specific assays were developed and tested *in vitro* for detection of eDNA from *Callinectes sapidus*, *Hemigrapsus sanguineus* and *Hemigrapsus takanoi*. All three assays were found to be specific for a mitochondrial co1 gene fragment, when applied on genomic DNA extractions obtained from other North European marine congeners of Decapoda. The assays presented, however, were not tested against a full coverage of the species diversity in *Callinectes* and *Hemigrapsus*, as it was not possible to obtain a full collection of the broad diversity of species in these genera. Because all species of *Callinectes* and *Hemigrapsus* are considered non-indigenous in northern European marine waters, the primer-probe assays presented in this report can be considered specific against non-indigenous species in these two genera when applied on water samples obtained in northern Europe.

The specific assays presented might not be able to distinguish between other species of *Callinectes* and other species of *Hemigrapsus* that otherwise are native to the Pacific and Western Atlantic Ocean. If the assays presented in this report were to be used on samples from these oceanic regions it would require some additional initial tests with PCR and qPCR setups as described in this report. Such tests would have to confirm that the assays indeed are species-specific even though the more closely related sister species are present at the sampled habitat, before these assays can be used for detection of the same species in Pacific and Western Atlantic Ocean.

The assays presented here in this report are presented as only being useful for monitoring non-indigenous species in Northern Europe. The assays targeting the four species of Decapoda (i.e. *Eriocheir sinensis*, *Homarus americanus*, *Paralithodes camtschaticus*, and *Rhithropanopeus harrisii*) developed during previous phases of the MONIS project (Andersen *et al*., 2016, 2018) are included again in this report, but were this time tested against additional species of marine Decapoda that were not included in the first MONIS report (Andersen et al., 2018). These additional tests were performed to underline the specificity for two of these previously developed assays (i.e the assays for detection of eDNA from *Eriocheir sinensis* and *Rhithropanopeus harrisii)*. For *Eriocheir sinensis* and *Rhithropanopeus harrisii* the previous published assays were found to be specific towards mitochondrial DNA from only the targeted species. The assay designed during the MONIS3 project (Andersen et al., 2018) for targeting eDNA from *Paralithodes camtschaticus* was found to also return positive amplification on DNA from hermit crabs (*Pagurus* spp.). This makes the previously published assay for *Paralithodes camtschaticus* inadequate, as *Pagurus* spp. is commonly occuring along the coast of North Western Europe. Instead a different primer- and probe combination was tested (F12-R12-P12 for mtDNA-CO1) (Figure 22) and found to be specific towards mtDNA-CO1 from *P. camschaticus*, with the diversity of marine Decapoda available for this study. Because of a better precision with this F12-R12-P12 assay for mtDNA-CO1, the previously published assay (Andersen et al., 2018) should be disregarded, and instead the new assay presented in this report is recommended for detection of eDNA from *P. camschaticus*. A test of the previously published F06+R08+P08 assay developed during the MONIS3 project (Andersen et al., 2018) targeting the mitochondrial cytochrome oxidase 1 region in *Homarus americanus*, was found to amplify on cross contamination in the laboratory (Fig. 17) at the University of Copenhagen. A new assay targeting the mitochondrial cytochrome b region in *Homarus americanus* (F02+R14+P12) was instead developed and tested (Fig. 18).

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