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

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REPORT

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Summary

The MONIS 3 project reports the development of 22 species-specific eDNA-based test systems for monitoring and mapping of the occurrence of non-indigenous species in Danish marine waters. The development has been undertaken by the MONIS partnership and the species have been selected based on the outcome of the MONIS 2 project. It should be noted that the test systems are considered operational cf. the MONIS definition (see Chapter 1). It should also be noted that the 22 test systems have been applied in the MONIS 4 project, where monitoring of 16 Danish harbours have been carried out to map occurrence of non-indigenous marine species using both conventional methods and molecular methods (eDNA). Development of additional test systems is being planned and expected to be carried out in 2019 pending funding. The MONIS partnership encourages interested institutions, especially in neighbouring countries (Germany, Norway, Poland and Sweden) to make use of the test systems.

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

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

Preface

The MONIS 3 project has been funded by the Danish Environmental Protection Agency (formerly Agency for Water and Nature Management (SVANA)) with an overarching aim of developing species-specific eDNA-based test systems for more than 20 marine species.

Thus, the developments are anchored in the outcomes of MONSI 1 and MONIS 2 which have previously been reported:

- Andersen, J.H., S.A. Pedersen, J. Thaulow, F. Stuer-Lauridsen & S. Cochrane (2014): Monitoring of non-indigenous species in Danish marine waters. Background and proposals for a monitoring strategy and a monitoring network. Danish Nature Agency. 55 pp., and especially
- Andersen, J.H., E. Kallenbach, M. Hesselsøe, S.W. Knudsen, P.R. Møller, D. Bekkevold, B.K. Hansen & J. Thaulow (2016): Steps toward nation-wide monitoring of non-indigenous species in Danish marine waters under the Marine Strategy Framework Directive. NIVA Denmark. 123 pp.

This report represents a leap forward in terms of making the Danish nation-wide monitoring of non-indigenous species operational according to Andersen *et al.* (2014).

A fully documented 'proof of concept' is not included in this technical report as it will be reported by MONIS 4 project (Andersen et al. *in prep*.) in which non-indigenous species have been monitored in 16 Danish ports. However, the results of *in vivo* testing of the 22 test systems developed and presented in this report, are summarized in Chapter 3.

Copenhagen, 31 January 2018

Jesper H. Andersen

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1 Introduction

In 2014, the Danish Nature Agency (now: Danish Environmental Agency) initiated a strategic project on 'Monitoring of non-indigenous species in Danish marine waters', also known as MONIS 1, with three key objectives:

- Definition of the optimal monitoring strategy and to design a cost-effective Marine Strategy Framework Directive (MSFD) monitoring programme targeting non-indigenous species in Danish waters (MSFD Descriptor 2; or in short D2).
- Proposal on how to make best use of existing data and to make linkages to other monitoring activities (understood as other MSFD descriptors than D2) in order to fulfil obligations related to both the MSFD and the Regulation of the European Parliament and the Commission on the prevention and management of the introduction and spread of invasive alien species.
- A Danish proposal for a D2-targeted monitoring programme focusing on non-indigenous species is consistent with the MSFD cycle, e.g. production of MSFD Initial Assessment, design and implementation of monitoring programmes, design of Programmes of Measures, and implementation of Programmes of Measures, and ultimately also fulfilment of the overarching MSFD target of 'good environmental status'.

For detailed information about MONIS 1, please confer with Andersen *et al.* (2014).

In 2015, the Danish Nature Agency initiated and funded a follow-up project 'Steps toward nationwide monitoring of non-indigenous species in Danish marine water under the Marine Strategy Framework Directive', abbreviated to MONIS 2. The key objectives of MONIS 2 were:

- Development of a proposal for national MSFD D2-specific Target Species List,
- Production of a draft Technical Guidance Report describing in detail how to carry out sampling, storage and lab analyses, and
- *In silico* development and testing of as many species-specific primer-probe assays as possible with regard to the national MSFD D2 Target Species List.

For detailed information about MONIS 2, please confer with Andersen *et al.* (2016).

As a follow up on MONIS 2, the Danish Agency for Nature and Water Management (the successor of the Danish Nature Agency; now: the Danish Environmental Protection Agency) initiated and funded a MONIS 3 project, with the following key objectives:

- Revision and publication of a Technical Guidance report on how to carry out sampling, storage and lab analyses in relation to eDNA-based monitoring of non-indigenous species in Danish marine waters.
- Development of species-specific environmental DNA (eDNA)-based test systems.

The Technical Guidance report has been published as Knudsen *et al.* (2018a). The developed operational test systems, in total 22, are all described and documented in this report. Regarding the definition of what 'operational level' is for a species-specific assay, it should be stressed that each of the species-specific primer-probe assays that are to be applied in a quantitative PCR (qPCR) setup must have been tested and validated beforehand in two separate development stages: *in silico* development and *in vitro* development stage.

In silico development requires that the species-specific primer-probe assays have been tested on computer by comparison with known DNA sequences from gene-bank databases (such as National Center for Biotechnology Information, NCBI) from the same gene-fragment from both the invasive species (i.e. the targeted species) as well as co-occurring and possibly evolutionary closely related organisms (i.e. non-target species). If sequence data is unavailable for those species for the sought gene-fragment, *de novo* sequencing must be performed prior to comparison of sequence data. This requires that samples of tissue can be accessed from curated and taxonomically correct identified individuals, usually through the collection on the natural history museum, for both target-species as well as for non-target species.

In vitro development requires that the *in sillico* tested primers and probes have been tested in a qPCR setup on DNA extracted from target-species and non-target species. As a minimum for validation the geographically co-occurring non-target species must be included. This qPCR *in vitro* test serves to determine whether the developed primer- and probe pair is capable of giving rise to false positive amplification due to similarity in the DNA-fragment targeted. This could be due to non-target species that have less than 5 base pair differences in the *in silico* developed primers and or probes. It is strongly recommended that such tissue samples from target and non-target species are drawn from natural history museum collections as such samples can be taxonomically reidentified later on, and provide collection reference numbers for later evaluation of the filtered eDNA samples.

The species-specific primers and probes listed below do not cover species that were unknown or had no sequence data deposited on gene-databases at the inceptionof this project.

Once an *in vitro* test has been completed with a validated and positive result for the species-specific assay that only returns positive amplification in a qPCR setup for the sought species, this assay can be regarded as being species-specific at an operational level. It is, however, still a requirement that species-specific assays tested positive at operational level are further validated through *in vivo* or *in situ* development stages. *In vivo* or *in situ* development stages requires that the species-specific assay also is capable of returning positive amplification in a qPCR setup performed on filtered and extracted water samples collected from where the sought organism is known to occur. The test and validation of species-specificity in this report follows the detailed protocols presented in previous studies on development and test of species-specific assays (Agersnap *et al*., 2017; Knudsen *et al*., 2018b; Jensen *et al*., 2018).

Additional testing at *in vivo* or *in situ* development stages has already been carried out under the MONIS 4 project. A full documentation report is being prepared (Andersen et al. *in prep.*), but the results of the *in vivo* testing have been summarized in Chapter 4 of this report. All *in vivo* testing follows the protocols presented in previous studies on eDNA detection with species-specific primer-probe systems (Agersnap *et al*., 2017; Knudsen *et al*., 2018a,b; Jensen *et al*., 2018; Spens *et al*. 2016).

2 Materials and methods

The main objective for the present study was to validate the utility of previous *in silico* developed species-specific quantitative PCR (qPCR) assays for detection of invasive marine species in Danish waters. From an initial list of 50 prioritized species, 22 assays were validated *in vitro* by testing the specific assays on DNA from the target-species (i.e. ascertaining that target species can be detected) and on DNA from closely related co-occurring species (i.e. ascertaining that the assay does not erroneously detect DNA from non-targets). The *in vitro* tests serve as a verification step of the assay and thus represent the final control step required, in order to apply *in vivo* tests on eDNA samples and subsequently to use the assays for eDNA-based monitoring of invasive marine species in Denmark. The 22 assays were selected based on the following criteria:

- Their importance for the national monitoring program of marine invasive species.
- Invasive species exhibiting the largest total biomass were in general preferred.
- Whether development of primer-probe assays was expected to be straightforward, based on previous *in silico* tests.
- Whether target species showed marked genetic differentiation from other potentially co-existing and closely related species (generally preferred to decrease risk of assays not being specific).
- Whether detailed knowledge existed about the genomic variation within the species, as well as between co-existing and evolutionary closely related species (generally preferred to reduce the risk of assays not being specific).
- Whether additional collection of tissue or DNA, e.g. from genetic similar invasive or non-invasive organisms, was not required for robust *in vitro* testing (unless novel tissue samples could easily be obtained within the project period).

Assay specificity was validated by qPCR analysis using DNA from the target species and, when appropriate, from genetically closely related co-existing species and sister-species. Here, the target DNA served to test the overall function of the assay, i.e. that the assay successfully amplified DNA from the target species. DNA from closely related species was used to test the specificity of the assay in order to ensure that it did not amplify non-target DNA, which otherwise would lead to false-positive detections. In a few cases, DNA from all closely related sister-species could not be obtained for testing assay specificity. However, in most of these cases these un-sampled species do not exist in Danish marine waters. Thus, assays can in all cases still be considered to be specific when used on eDNA samples from Danish waters, and if they were to immigrate in future, they would likely be considered to be invasive.

In general, assays were tested using the same reagents and qPCR programmes. All assays were analysed using the Applied Biosystems TaqMan Environmental Mastermix 2.0 (Thermo Fisher Scientific, Waltham, Massachusetts, United States) and were in most cases run using a standard qPCR program: using 10 minutes initial denaturation at 95°C, followed by 50 cycles of 95°C for 15 seconds and 60°C for 1 minute. Target and non-target DNA samples were always analysed in replicates and included negative controls.

An in-depth description of each specific assay can be found in the following species-specific Chapters of the report, which include descriptions of the specifically utilized primer and probes, results of the cross-amplification tests and recommendations for the utility of each assay.

3 Results

We report and document the development of species-specific test systems for eDNA-based monitoring of 22 non-indigenous species in Danish marine waters. All 22 species-specific primer-probe systems are considered operational cf. the definition in Chapter 1. An overview is given in Table 3.1.

Table 3.1 *List of non-indigenous marine species in Danish seas targeted for eDNA monitoring in the MONIS 3 project. 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 project. 'Assay ready' indicates whether the assay can be considered ready for test at operational level - i.e. subsequent testing in the MONIS 4 project. TS = Target Species; NTS = Non-Target Species. 'At gl' indicates the assay is ready for use with specificity at genus level – i.e. the assay cannot discriminate between eDNA from different species within the listed genus.*

No*	Genus	Species	Danish commmon name	Assay testing responsible	TS collected	NTS collected and tested**	Level of specificity	Assay ready
01	*Bonnemaisonia*	*hamifera*	Rødtot	NIVA	Yes	Yes	Species	Yes
02	*Prorocentrum*	*minimum*	Dinoflagelat	NIVA	Yes	Yes	Genus	At gl
03	*Pseudochattonella*	*farcimen*	Heterokont flagelat	NIVA	Yes	Yes	Species	Yes
04	*Pseudochattonella*	*verriculosum*	Heterokont flagelat	NIVA	Yes	Yes	Species	Yes
05	*Karenia*	*mikimotoi*	Dinoflagelat	NIVA	Yes	Yes	Genus	At gl
06	*Carassius*	*auratus*	Sølvkarusse	DTU Aqua	Yes	Yes	Species	Yes
07	*Cyprinus*	*carpio*	Karpe	DTU Aqua	Yes	Yes	Species	Yes
08	*Colpomenia*	*peregrina*	Østerstyv	DTU Aqua	Yes	NA	Genus§	At gl
09	*Neogobius*	*melanostomus*	Sortmundet kutling	DTU Aqua	Yes	Yes	Species	Yes
10	*Oncorhynchus*	*mykiss*	Regnbueørred	DTU Aqua	Yes	Yes	Species	Yes
11	*Oncorhynchus*	*gorbuscha*	Pukkellaks	NHMD/Amphi	Yes	Yes	Species	Yes
12	*Crassostrea*	*gigas*	Stillehavsøsters	NHMD/Amphi	Yes	Yes	Species	Yes
13	*Mya*	*arenaria*	Almindelig sandmusling	NHMD/Amphi	Yes	Yes	Species	Yes
14	*Rhithropanopeus*	*harrisii*	Østamerikansk brakvandskrabbe	NHMD/Amphi	Yes	Yes	Species	Yes
15	*Paralithodes*	*camtschaticus*	Kamtjatka-krabbe	NHMD/Amphi	Yes	Yes	Species	Yes
16	*Eriocheir*	*sinensis*	Kinesisk uld-Håndskrabbe	NHMD/Amphi	Yes	Yes	Species	Yes
17	*Homarus*	*americanus*	Amerikansk hummer	NHMD/Amphi	Yes	Yes	Species	Yes
18	*Cordylophora*	*caspia*	Brakvands-kølle-polyp	DTU Aqua	Yes	NA	Genus§	At gl
19	*Mnemiopsis*	*leidyi*	Amerikansk ribbegople	NHMD/Amphi	Yes	Yes	Species	Yes
20	*Acipenser*	*baerii*	Sibirisk stør	NHMD/Amphi	Yes	Yes	Species	Yes
21	*Acipenser*	*gueldenstaedtii*	Diamant stør	NHMD/Amphi	Yes	Yes	Genus#	At gl
22	*Acipenser*	*ruthenus*	Sterlet	NHMD/Amphi	Yes	Yes	Genus#	At gl

**) The species number is an arbitrary number assigned through this report.*
***) Whether non-target species have been collected refers to whether species from potentially co-occurring and evolutionary closely related species in Danish Seas have been collected, and if the assay has been tested on the Non-Target-Species. The 'NA' indicates that the species was unavailable for testing.*
#) The primers and probes developed for non-indigenous species of Acipenser *(i.e.* A. gueldenstaedtii *and A.* ruthneus*) in Danish is unable to differentiate between eDNA at species-level (i.e. not able to discern between species), but is able to differentiate between indigenous species and non-indigenous species of* Acipenser *in Danish waters.*
§) The eDNA assays developed for Colpomenia peregrine *and* Cordylophora caspia *were not tested on DNA from sister-species as it could not be obtained. Hence, whether these assays are species-specific is uncertain.*

Table 3.2 *Table of primer and probe qPCR detection systems developed by MONIS 3 with a summary of the final product. 'PM' indicates a probe modification. All oligos are written in a 5' -> 3' direction.*

No	Species	Primer and probe name (R and F)	Sequence, primer and probe	PM 5'-end:	PM 3'-end:
01	*Bonnemaisonia hamifera*	Bon_ham_rbcL_F02	CAATTACTAGATTACCTGGGCAAT		
		Bon_ham_rbcL_R02	CTTCTTTTACAAAGTCCCGACCT		
		Bon_ham_rbcL_P01	TCGTGCCATAACCATAGACTCTAAAGCC	FAM	BHQ-1
02	*Prorocentrum minimum*	Pro_min_28S_F03	CTTGGCAAGATTGTCGGGT		
		Pro_min_28S_R03	TATTCACTCACCCATAGACGA		
		Pro_min_28S_P03	ACACACAAGGCAAGAGACGATCAAGC	FAM	BHQ-1
03	*Pseudochattonella farcimen*	PsefarF	GGGAGAAATTCTTTGGAACAAGG		
		PsefarR	GCAACTCGACTCCACTAGG		
		PseP	TCAGAGAGGGTGACAATCCCGTCT	FAM	BHQ-1
04	*Pseudochattonella verruculata*	PseverF	GGGAGAAGTCCTTTGGAACAAGG		
		PseverR	GCAACTCGACTCCATTAGC		
		PseP	TCAGAGAGGGTGACAATCCCGTCT	FAM	BHQ-1
05	*Karenia mikimotoi*	KarmikF3	CCGAGTGACTGAATGTCCTC		
		KarmikR3	GATCGCAGGCAAGCACATGA		
		KarmikP3	GCAGTGCTACCAGACACACAGAG	FAM	BHQ-1
06	*Carassius auratus*	Caraur_COI_F01	TTCTTCCCCCATCATTCCTGT		
		Caraur_COI_R01	GTATACTGTCCATCCGGAGG		
		Caraur_COI_P02	TAGCTTCCTCTGGTGTTGAAGCCGGAG	FAM	BHQ-1
07	*Cyprinus carpio*	CCcytbF	CTAGCACTATTCTCCCCTAACTTAC		
		CCcytbR	ACACCTCCGAGTTTGTTTGGA		
		CCcytbP	CCCTCTAGTTACACCACC	FAM	TAMRA
08	*Colpomenia peregrine*	Col_per_COX_3_F01	GCAAGCTTTTGAATATGCTAATG		
		Col_per_COX_3_R01	CAGCTAAAAATATTGTACCGATT		
		Col_per_COX_3_P01	TTCAGTTTTTTACATGGCTACAGGCTTC	FAM	TAMRA
09	*Neogobius melanostomus*	Neo_Mel_COI_F01	CTTCTRGCCTCCTCTGGWGTTG		
		Neo_Mel_COI_R01	CCCWAGAATTGASGARATKCCGG		
		Neo_Mel_COI_P01	CAGGCAACTTRGCACATGCAG	FAM	BHQ-1
10	*Oncorhynchus mykiss*	Onc_myk_CytB_F01	ACCTCCAGCCATCTCTCAGT		
		Onc_myk_CytB_R01	AGGACGGGGAGGGAAAGTAA		
		Onc_myk_CytB_P01	TGAGCCGTGCTAGTTACTGCTGTCCTT	FAM	BHQ-1
11	*Oncorhyncus gorbuscha*	Oncgor_CO1_F09	TCCTTCCTCCTCCTCTTTC		
		Oncgor_CO1_R06	TGGCCCCTAAAATTGATGAG		
		Oncgor_CO1_P06	CAGGGGCATCCGTCGACTTAACTAT	FAM	BHQ-1
12	*Magallana gigas*	Cragig_CO1_F07	TTGAGTTTTGCCAGGGTCTC		
		Cragig_CO1_R09	ACCAGCAAGGTGAAGGCTTA		
		Cragig_CO1_P06	AACATTGTAGAAAACGGAGTTGGGGC	FAM	BHQ-1
13	*Mya arenaria*	Mya_are_CO1_F01	CCCTCCGTTGTCGAGAAATA		
		Mya_are_CO1_R02	ACGCATGTTACCCCAAGTTC		
		Mya_are_CO1_P06	TATCCCTTCATATTGGAGGGGCTTCAT	FAM	BHQ-1
14	*Rhithropanopeus harrisii*	Rhihar_co1_F03	GTCAACCTGGTACTCTCATTGGT		
		Rhihar_co1_R03	ACGAGGAAATGCTATATCAGGGG		
		Rhihar_co1_P03	TGTTGTAGTAACAGCTCACGCCTTTGT	FAM	BHQ-1
15	*Paralithodes camtschaticus*	Parcam_co1_F02	GGGCTTGAGCTGGAATAGTG		
		Parcam_co1_F02	GGGCTTGAGCTGGAATAGTG		
		Parcam_co1_R05	CAATTTCCAAACCCTCCAAT		
		Parcam_co1_P02	ATTCGAGCTGAACTAGGACAACCAGGT	FAM	BHQ-1
16	*Eriocheir sinensis*	Erisin_cytb_F02	ACCCCTCCTCATATCCAACCA		
		Erisin_cytb_R02	AAGAATGGCCACTGAAGCGG		
		Erisin_cytb_P02	TTTGCTTACGCTATTTTACGATCAATTCCT	Fam	BHQ-1
17	*Homarus americanus*	Homame_co1_F06	TTACAGCAGTTCTTTTACTACTCTCG		
		Homame_co1_R08	ACTGGGTCTCCACCTCCAG		
		Homame_co1_P08	TCGAAATTTAAATACTTCATTCTTCGATCCA	FAM	BHQ-1
18	*Cordylophora caspia*	Cor_cas_COI_F01	TCATCTGTACAAGCACATTCTGG		
		Cor_cas_COI_R01	TTGAAGAAGCTCCTGCACAGT		
		Cor_cas_COI_P01	CCTTCTGTAGACATGGCTATATTTAGTC	FAM	BHQ-1
19	*Mnemiopsis leidyi*	Mnelei_its2_F04	ACGGTCCCTTGAAGTAGAGC		
		Mnelei_its2_R06	TCTGAGAAGGCTTCGGACAT		
		Mnelei_its2_P06	GTGCCTCTCGGTGTGGTAGCAATATCT	FAM	BHQ-1
20	*Acipenser baerii*	Acibae_CR_F02	CAGTTGTATCCCCATAATCAGCC		
		Acibae_CR_R03	TTATTCATTATCTCTGAGCAGTCGTGA		
		Acibae_CR_P01	ATGCCGAGAACCCCATCAACATTTGGT	FAM	BHQ-1
21 & 22	*Acipenser spp.**	Acibae_cytb_F11	TTCCACCCGTACTTCTCATAC		
		Acibae_cytb_P11	CCTAATGCTAGTCGGACTCACCTCCGT		
		Acibae_cytb_R16	GGCGTAGGCGAAGAGAAAGTA	FAM	BHQ-1

**) The primers and probes developed for detection of eDNA from* Acipenser gueldenstaedtii *and* A. ruthenus *are unable to distinguish between DNA from* A. gueldenstaedtii, A. ruthenus, A. baerii *and A*. stellatus*. In Danish Seas all four species (i.e.* A. gueldenstaedtii, A. ruthenus, A. baerii *and* A. stellatus*) are considered non-indigenous. Whereas* Acipenser sturio *and* A. oxyrhynchus *are indigenous to Danish Seas. The primers and probes developed here are able to distinguish between indigenous and non-indigenous species of* Acipenser *in Danish waters.*

3.1 Species no. 01: *Bonnemaisonia hamifera*

Binomial nomenclature and author: *Bonnemaisonia hamifera* Hariot 1891.
English common name: Bonnemaison's Hook weed.
Danish common name: Rødtot.

Figure 1.1. Bonnemaisonia hamifera. *Photo by Ignacio M. Bárbara (UCD, Spain).*

In the genus *Bonnemaisonia* there are six accepted species. Among the sequenced genes available in the genus *Bonnemaisonia* the *rbc*L gene showed the best potential for assay design (Table 1.1):

- Bon_ham_*rbc*L_F02: 5'-CAATTACTAGATTACCTGGGCAAT-3'.
- Bon_ham_*rbc*L_R02: 5'-CTTCTTTTACAAAGTCCCGACCT-3'.
- Bon_ham_*rbc*L_P01: 5'-FAM-TCGTGCCATAACCATAGACTCTAAAGCC-BHQ-1-3'.

Table 1.1 *Species specific primer/probe assay for Bonnemaisonia hamifera 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.*

Species	Gene	Size	Temp	Length	GC
Bonnemaisonia hamifera	*rbc*L	157 base pair (bp)			
Bon_ham_rbcL_F02	CAATTACTAGATTACCTGGGCAAT		60.3	24	38
Bon_ham_rbcL_R02	CTTCTTTTACAAAGTCCCGACCT		60.9	23	43
Bon_ham_rbcL_P01	TCGTGCCATAACCATAGACTCTAAAGCC		68.5	28	46
Related species	**Tested**	**Amplification**		Accession nr*	
Bonnemaisonia hamifera	Yes	Yes		KC130209	
Bonnemaisonia asparagoides	Yes	No		AF212188	
Bonnemaisonia clavata	Maybe**	No		GQ337067	
Bonnemaisonia geniculata	No	-		KC174794	
*Bonnemaisonia californica****	Yes	No			
*Bonnemaisonia australis****	No	-			
*Bonnemaisonia spinescens****	No	-			

** Additional sequences from each species and additional not listed species were used in the alignment for increased accuracy and diversity coverage within and among* Bonnemaisonia *species.*
**** Bonnemaisonia clavata *could not be taxonomically separated from* B. asparagoides*, however COI sequences different between the two species (pers. comm. Line Le Gall, MNHM, France).*
**** Reference sequence information not available via GeneBank.*

The primers were designed by aligning sequences of B. *hamifera*: GQ252543, FJ195604, GQ337066, KC130209, *B. asparagoides*: AF212188, U26813, GQ337065, *B. clavata*: GQ337067, and *B. geniculate*: KC174794. Sequence alignment was performed using Geneious v. R10 (Kearse *et al.* 2012). It was not possible to acquire reference material from *B. geniculata*, *B. australis* and *B. spinescens* for the specificity test. However, from the sequence comparison of the two other non-target species *B. geniculate* and *B. californica* and lack of amplification of the latter, *B. geniculata* is not expected to amplify with the developed assay. Since no sequence information was available and reference material could not be acquired it is not known if *B. australis* and *B. spinescens* will amplify. According to the AlgaeBase.org (Guiry & Guiry 2017), these two species should however only occur in the Oceans around Australia and New Zealand.

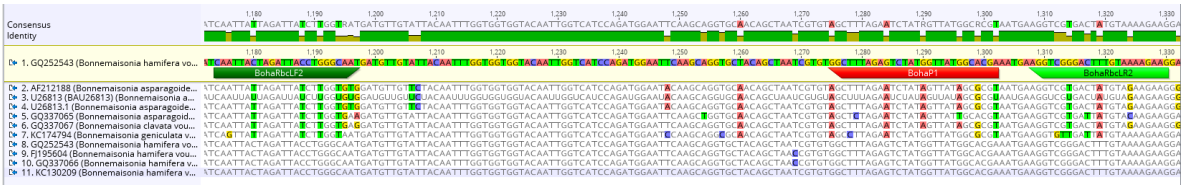

Figure 1.2 *Alignment of* Bonnemaisonia *species for the rbcL gene acquired from GenBank.*

Conditions of the specificity test

DNA from reference tissue samples were either supplied from external sources (Table 1.2) or extracted from tissue samples using the DNeasy PowerPlant Pro Kit (Qiagen, Hilden, Germany) according to manufacturer's specifications. Initial primer specificity, optimal annealing temperature and primer concentration was performed in a 25 µL reaction volume comprising forward and reverse primers (Eurofins Genomics, Ebersberg, Germany), SsoFast EvaGreen master Mix (BioRad, Hercules, California, Unites States) and 2.5 µL template DNA that was adjusted to a concentration around 1 ng/µL. QPCR reactions were run on a CFX96 Touch Real-Time PCR Detection Systems (BioRad, Hercules, California, Unites States). Primer probe specificity test was run using 500 µM of each primer and 50 µM probe in a 20 µL reaction volume of Applied Biosystems TaqMan Environmental Mastermix 2.0 (Thermo Fisher Scientific, Waltham, Massachusetts, United States) and 2.5 µL template DNA. Target- and non-target species were run in duplicate reactions and four negative controls.

Table 1.2 *List of tested species with information regarding collector and origin*

Species	Abbreviation	Collector	Sampling location
Bonnemaisonia hamifera	Bonham	NIVA, Norway	Launes, Flekkefjord, Norway
Bonnemaisonia hamifera	Bonham	Ignacio M. Bárbara Criado, UCD, Spain	Peinzás, Fazouro, Foz, Spain
Bonnemaisonia californica	Boncal	Sandra Lindstrom, UBC, CA	British Columbia, Canada
Bonnemaisonia asparagoides	Bonasp	Line Le Gall, MNHN, France	Manche, Normandy, France
Bonnemaisonia clavata	Bonasp*	Line Le Gall, MNHN, France	Manche, Normandy, France

* Bonnemaisonia clavata *is also abbreviated as* B. asparagoides *since taxonomic confirmation for the three specimens used uncertain. All of these three specimens* B. asparagoides *was obtained through from Line Le Gall, NHMN, France.*

Assay specificity results

The two replicated of *B. hamifera* amplified at a Cq of 24.53 and 23.41, respectively (Figure 1.3). None of the non-target sister species amplified with the F2R2P1 assay. The designed eDNA target assay for *Bonnemaisonia hamifera* is expected to only amplify the target species when tested on laboratory or environmental water samples.

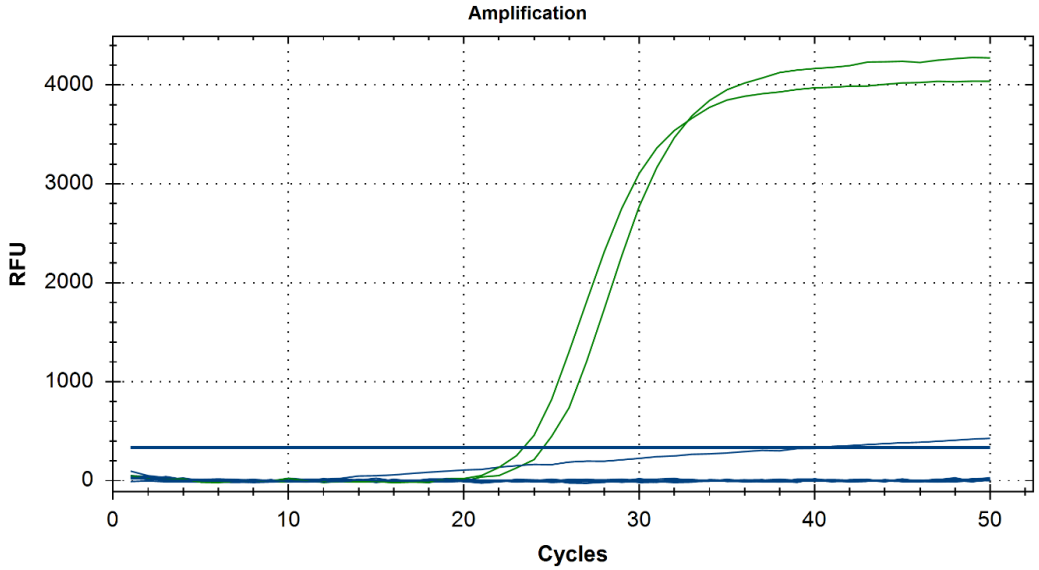

Figure 1.3 *Amplification of* Bonnemaisonia *species using the F2R2P1 assay. Target species* B. hamifera *is shown in green and non-target sister species in blue.*

3.2 Species no. 02: *Prorocentrum minimum*

Binomial nomenclature and author: *Prorocentrum minimum* J. Schiller 1933.
English common name: -
Danish common name: -

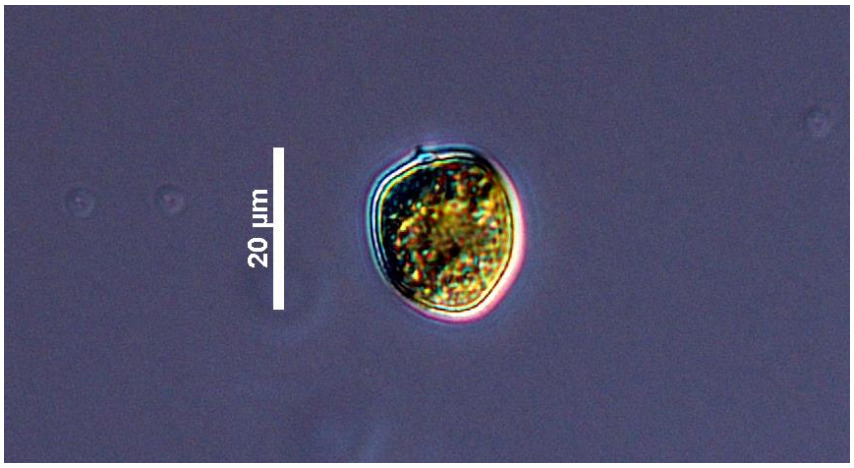

Figure 2.1 Prorocentrum minimum*. Photo from www.eoas.ubs.ca.*

Prorocentrum minimum belongs to the genus *Prorocentrum* that comprises 85 species. Initially primer/probe assay was designed after Scorzetti *et al.* (2009). The authors had selected the D1D2 region of the 28S sequence, which commonly selected for assay design for algae species (e.g. Dittami *et al.* 2013). The assay that was finally used was positioned with an overlap just upstream in the sequence to the assay designed by Scorzetti *et al.* (2009):

- Pro_min_28S_F03: 5'-CTTGGCAAGATTGTCGGGT-3'.
- Pro_min_28S_R03: 5'-TATTCACTCACCCATAGACGA-3'.
- Pro_min_28S_P03: 5'-FAM-ACACACAAGGCAAGAGACGATCAAGC-BHQ1-3'.

In total 16 species within the genus *Prorocentrum* were acquired for the specificity test of the designed assay (Table 2.1).

Table 2.1 *Species specific primer/probe assay for Prorocentrum minimum with, target gene, product size, melting temperature, primer/probe length, GC ratio, tested target and non-target species with source and strain information, and GenBank accession number used for sequence alignment (not linked to strain).*

Species	Gene	Size		Temp	Length	GC
Prorocentrum minimum	28S	118 bp				
Pro_min_28S_F03	CTTGGCAAGATTGTCGGGT			57.5	19	53
Pro_min_28S_R03	TATTCACTCACCCATAGACGA			57.5	21	43
Pro_min_28S_P03	ACACACAAGGCAAGAGACGATCAAGC			67.9	26	50
Related species	**Tested**	**Amplification**	**Source**	**Strain**	**Accession no.***	
Prorocentrum minimum	Yes	Yes	NIVA CCA[1]	K-1569	AY863005	
Prorocentrum micans	Yes	No	NIVA CCA[1]	K-1350	AF260377	
Prorocentrum lima	Yes	No	NIVA CCA[1]	K-1648	DQ336182.1	
Prorocentrum donghaiense	Yes	No	NIVA CCA[1]	K-1446	KF998562	
Prorocentrum triestinum	Yes	Yes	NIVA CCA[1]	K-1811	See Table 2.2	
Prorocentrum sp.	Yes	No	NIVA CCA[1]	K-1804	-	
Prorocentrum rhathymum	Yes	No	UNCW[2]	Prrh1209-1	KR230012	
Prorocentrum belizeanum	Yes	No	UNCW[2]	Prbe0607-1	JQ638946.1	
Prorocentrum elegans	Yes	No	UNCW[2]	Prel0702-1	KT275813	
Prorocentrum hoffmannianum	Yes	No	UNCW[2]	Peho0808-1	EU196415.1	
Prorocentrum texanum	Yes	No	UNCW[2]	Prte0903-1	JQ390505	
Prorocentrum compressum	Yes	No	CICCM[3]	CAWD30	EF517256	
Prorocentrum cf. balticum	Yes	No	CICCM[3]	CAWD38	AF042816	
Prorocentrum sigmoides	Yes	No	CICCM[3]	CAWD120	EF566746	
Prorocentrum cf. maculosum	Yes	No	CICCM[3]	CAWD158	-	
Prorocentrum rhathymum	Yes	No	CICCM[3]	CAWD159	KR230012	
Oslo fjord June 2017, *Procentrum* sp. unknown	Yes	No**	Environmental Lugol fixated sample, Wenche Eikrem, NIVA			

** Additional sequences from each species and additional not listed species were used in the alignment for increased accuracy and diversity coverage within and among* Prorocentrum *species.*
*** One in six reactions amplified, [1] Norwegian Institute for Water Research Culture Collection of Algae, [2] University of North Carolina Wilmington, United States, [3] Cawthron Institute's Culture Collection of Micro-algae, New Zealand.*

The primers were designed by aligning sequences of as high a number of *Prorocentrum* species as possible from GenBank. Besides the perfect sequence similarity to *P. balticum* (AF042816) mismatches were found for all other aligned species (data not shown).

Conditions of the specificity test

DNA from reference tissue samples were either supplied from external sources (Table 2.1) or extracted from tissue samples using the DNeasy PowerPlant Pro Kit (Qiagen, Hilden, Germany) according to manufacturer's specifications. Initial primer specificity, optimal annealing temperature and primer concentration was performed in a 25 µL reaction volume comprising forward and reverse primers (Eurofins Genomics, Ebersberg, Germany), SsoFast EvaGreen master Mix (BioRad, Hercules, California, Unites States) and 2.5 µL template DNA that was adjusted to a concentration around 1 ng/µL. QPCR reactions were run on a CFX96 Touch Real-Time PCR Detection Systems (BioRad, Hercules, California, Unites States). Conditions for the thermos cycling were run with an initial warming at 95°C for 10 minutes followed by 50 cycles of 95°C for 15 s and 60°C for 1 minute. Primer probe specificity test was run using 500 µM of each primer and 50 µM probe (TAG Copenhagen, Denmark) in a 20 µL reaction volume of Applied Biosystems TaqMan Environmental Mastermix 2.0 (Thermo Fisher Scientific, Waltham, Massachusetts, United States) and 2.5 µL template DNA. Target- and non-target species were run in duplicate reactions and four negative controls.

Assay specificity results

The designed assay in the 28S gene showed high specificity towards *Prorocentrum minimum* (Cq: 19.35 and 19.51; Figure 2.2).

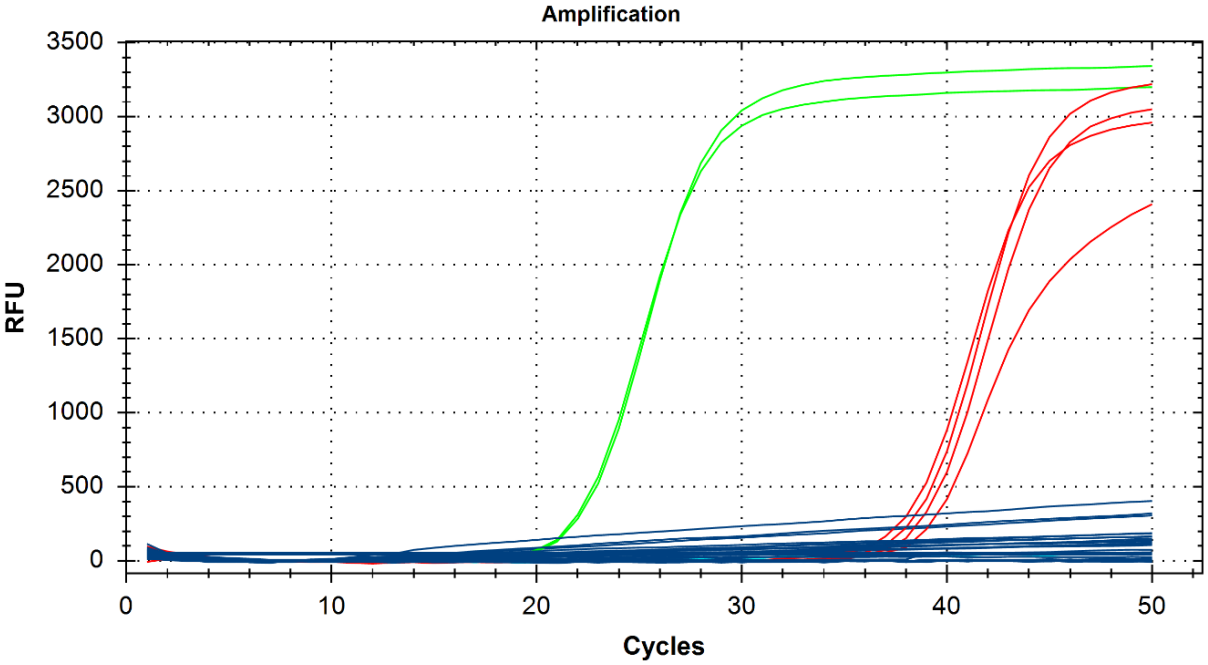

Figure 2.2 *Amplification of* Prorocentrum *species using the F3R3P3 assay. Target species* P. minimum *is shown in green and non-target sister species in blue.* P. triestinum *showed amplification with assay (marked in red).*

The acquired *P. cf. balticum* sample did not amplify and the aligned sequence (AF042816) has its origin from Korea (Table 2.2). Accordingly, there is also a *P. balticum* in the oceans around Denmark (Thomsen 1992). However, this species has never been sequenced and is rarely found in survey samples (Gert Hansen, *pers. comm*.). In June 2017, a bloom of a *Prorocentrum* species was found in the Oslo Fjord that could be *P. balticum* (Wenche Eikrem, *pers. comm*.; Gert Hansen, *pers. comm*.). DNA was extracted from a Lugol fixated sample and included in the specificity assay. This sample amplified in 1/6 runs (data not shown). Further sequencing of isolated single algae may confirm the identity of the species in this bloom. The only other species to amplify was *P. triestinum* (Cq: 34.68, 35.68, 36.26, and 36.57). According to sequence alignment available from GenBank this was not expected (Figure 2.3), especially the geographical coverage of the sequences (Table 2.2).

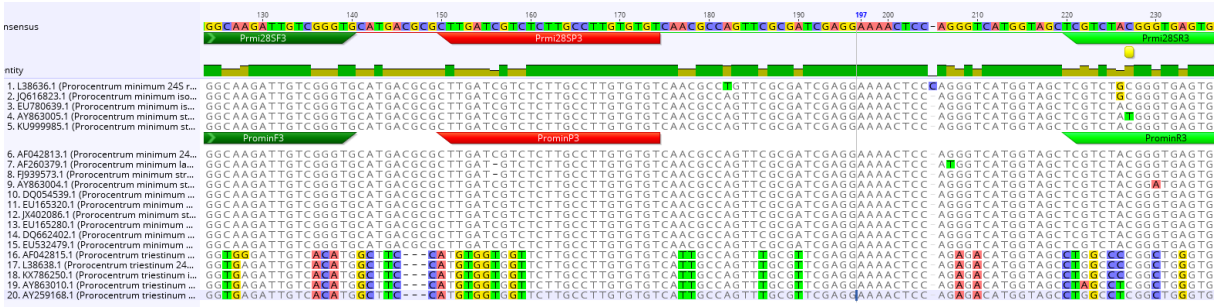

Figure 2.3 *Alignment of* Prorocentrum minimum *and* P. triestinum *species for the 28S gene region acquired from GenBank.*

Table 2.2 *GenBank sequences of* Prorocentrum triestinum *used for the detailed alignment with P. minimum and location of origin.*

Species	GenBank accession number	Sampling location
Prorocentrum triestinum	AF042815.1	Korea
Prorocentrum triestinum	L38638.1	Spain
Prorocentrum triestinum	KX786250.1	South Africa
Prorocentrum triestinum	AY863010.1	China
Prorocentrum triestinum	AY259168.1	Western Australia

Taxonomic inspection of strain K-1811 in NIVA's algae culture collection has identified the presence of an unidentified dinoflagellate contamination in the culture, however a decisive identification to determine if the microalgae is *P. triestinum* or not, despite originating from Ría de Vigo, Spain, has not yet been conducted. Despite this amplification, the designed assay is expected to only detect *Prorocentrum minimum* in Danish waters. Depending on amplification from environmental samples a maximum of 40 qPCR cycles could be recommended to avoid unspecific alignment of non-target species, as recommended for *Pseudochattonella* species (Eckford-Soper & Daugbjerg 2016).

3.3 Species no. 03: *Pseudochattonella farcimen*

Binomial nomenclature and author: *Pseudochattonella farcimen* W. Eikrem, B. Edvardsen & J. Throndsen 2009
English common name: -
Danish common name: -

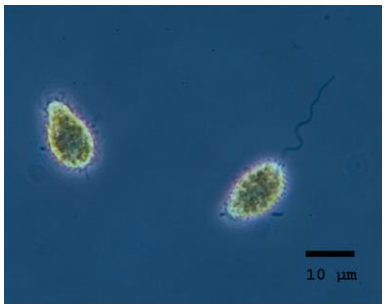

Figure 3.1. Pseudechattonelle farcimen. *Photo by Birger Skjelbred (NIVA, Olso, Norway)*

Species-specific assay development for *Pseudechattonelle farcimen* was not possible to develop. Assay development and testing was conducted together with *P. verruculosa*. Please see below.

3.4 Species no. 04: *Pseudochattonella verruculosa*

Binomial nomenclature and author: *Pseudochattonella verruculosa* Y. Hara & M. Chihara 2007
English common name: -
Danish common name: -

Figure 4.1 Pseudochattonella verruculosa. *Photo by Birger Skjelbred (NIVA, Olso, Norway).*

The genus *Pseudochattonella* only contains the two species *Pseudochattonella farcimen* and *Pseudochattonella verruculosa*. The two species are more or less impossible to tell apart morphologically, after fixation in lugol. Therefore, a molecular method has been developed and to date serves as the best solution to tell apart the two species (Dittami *et al.* 2013; Eckford-Soper & Daugbjerg 2016). However, the designed primers (best possible) only have two mismatches each (Dittami *et al*. 2013; Eckford-Soper & Daugbjerg 2016) and none in the probe (Eckford-Soper & Daugbjerg 2016). According to the published qPCR protocol by Dittami *et al.* (2013) it is relatively difficult even with the molecular method to determine if the amplification in the qPCR reaction is caused by the presence of the one or the other species. The assay published by Eckford-Soper & Daugbjerg (2016) was selected for species specific detection in this project (Table 4.1):

- PseverF: 5'-GGGAGAAGTCCTTTGGAACAAGG-3'.
- PseverR: 5'-GCAACTCGACTCCATTAGC-3'.
- PsefarF: 5'-GGGAGAAATTCTTTGGAACAAGG-3'.
- PsefarR: 5'-GCAACTCGACTCCACTAGG-3'.
- PseP: 5'-FAM-TCAGAGAGGGTGACAATCCCGTCT-BHQ1-3'.

Table 4.1 *Species-specific primer/probe assay for Pseudochattonella verruculosa (Psever) and* P. farcimen *(Psefar) with target gene, product size, melting temperature, primer/probe length, and GC ratio (%).*

Species	Gene	Size		Temp	Length	GC
Pseudochattonella sp.	28S	97 bp				
PseverF	GGGAGAAGTCCTTTGGAACAAGG			64.6	23	52
PseverR	GCAACTCGACTCCATTAGC			57.5	19	53
PsefarF	GGGAGAAATTCTTTGGAACAAGG			60.9	23	43
PsefarR	GCAACTCGACTCCACTAGG			59.5	19	58
PseP	TCAGAGAGGGTGACAATCCCGTCT			66.9	24	54
Related species	**Tested**	**Amplification**	**Source**	**Strain**	**Accession no.***	
Pseudochattonella verruculosa	Yes	Yes	NIVA CCA[1]	UIO-107	JF701986	
Pseudochattonella farcimen	Yes	Yes	NIVA CCA[1]	K-1804	JF030886.1	

** Additional sequences from each species and additional not listed species were used in the alignment for increased accuracy and diversity coverage within and among the two* Pseudochattonella *species. [1] Norwegian Institute for Water Research Culture Collection of Algae.*

Conditions of the specificity test

DNA from strains of *Pseudochattonella* were isolated using the DNeasy PowerPlant Pro Kit (Qiagen, Hilden, Germany) according to manufacturer's specifications. Primers and probe (BHQ1 and not BHQ2 as used by Eckford-Soper & Daugbjerg (2016) were run using concentrations specified by the authors (125 µM of each primer). Thermocycling was performed on a CFX96 Touch Real-Time PCR Detection Systems (BioRad, Hercules, California, Unites States) with an initial warming at 95°C for 10 minutes followed by 50 cycles of 95°C for 15 s and 60°C for 1 minute. The authors, however, recommend only running 40 cycles. The qPCR reaction was run in a 20 µL reaction volume of Applied Biosystems TaqMan Environmental Mastermix 2.0 (Thermo Fisher Scientific, Waltham, Massachusetts, United States) including 2.5 µL template DNA that was adjusted to a concentration around 1ng/µL. Target- and non-target species were run in duplicate reactions and four negative controls.

Assay specificity results

Despite the successful species-specific detection in the publication by Eckford-Soper & Daugbjerg (2016), we were not able to reproduce the results (Figure 4.2). In both assays the target species started amplifying about 10 Cq earlier than the non-target sister species. There may be a slight possibility of improving the assay designed by Eckford-Soper & Daugbjerg (2016) by adapting the forward

primer designed by Dittami *et al.* (2013) so the mismatches between the two species are positioned in the 3' end and not in the middle of the primer (Kwok *et al.* 1990; Bru *et al.* 2008).

Due to the toxic nature of both *Pseudochattonella* species (Edvardsen *et al.* 2007; Riisberg & Edvardsen 2008) a detection system that amplifies both species simultaneously, however at different intensities when Cq values are compared, may be good enough as a warning system.

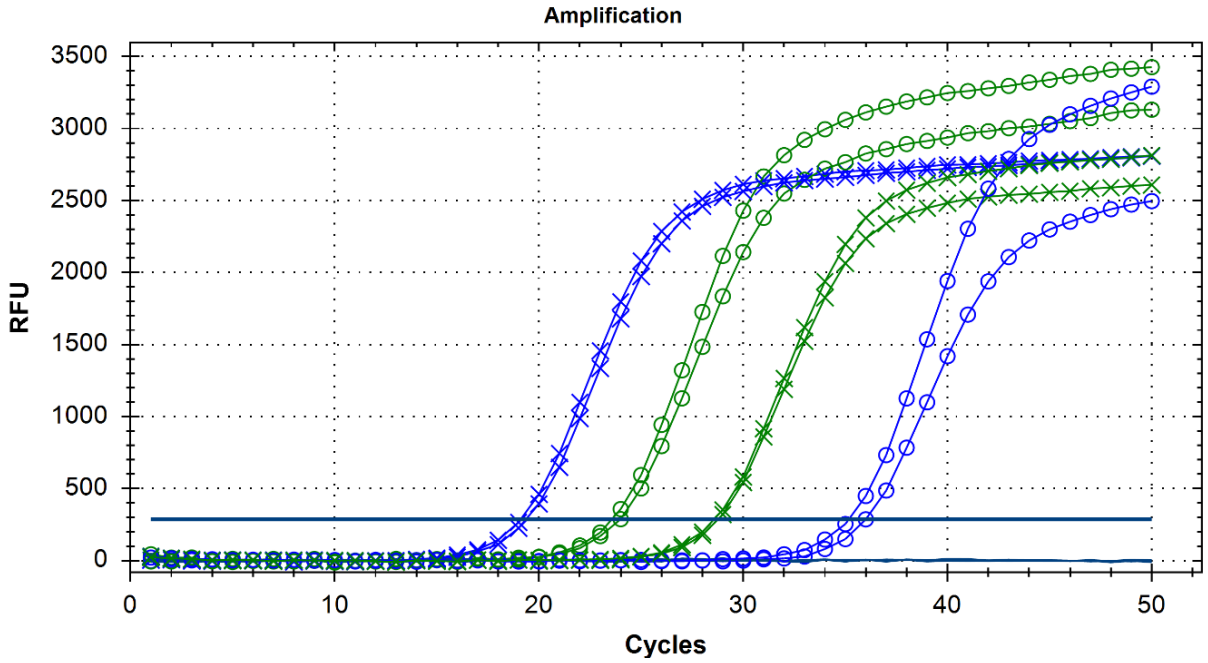

Figure 4.2 *qPCR amplification profile of the tested assay for species specific detection of either* Pseudochattonella farcimen *(green) or* P. verruculosa *(blue) in each their individual assays (*P. farcimen*, crosses; P.* verruculosa*, circles).*

3.5 Species no. 05: *Karenia mikimotoi*

Binomial nomenclature and author: *Karenia mikimotoi* (Miyake & Kominami ex Oda) (G. Hansen & Ø. Moestrup 2000).

English common name: -

Danish common name: -

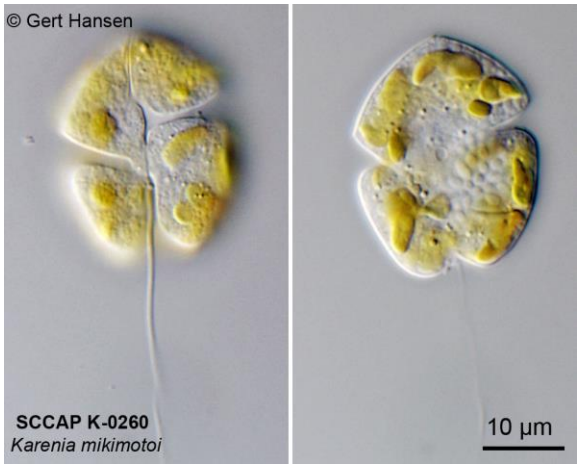

Figure 5.1 Karenia mikimotoi. *Photo by Gert Hansen (National History Museum, Copenhagen, Denmark).*

The genus *Karenia* comprises 9 species and due to the potential toxic fish-killing properties of these algae detection and separation assays have already been published (Smith *et al.* 2014, Yuan *et al.* 2012). Based on these published assay's, sequence alignment of several *Karenia*- and other closely related non-target species (Table 5.1), and specificity tests (see below), the following assay was found most species specific for *Karenia mikimotoi*:

- KarmikF3: 5'-CCGAGTGACTGAATGTCCTC-3'.
- KarmikR3: 5'-GATCGCAGGCAAGCACATGA-3'.
- KarmikP3: 5'-FAM-GCAGTGCTACCAGACACACAGAG-BHQ-1-3'.

Table 5.1 *Species specific primer/probe assay for* Karenia mikimotoi *with, target gene, product size, melting temperature, primer/probe length, GC ratio, tested target and non-target species with source and strain information, and GenBank accession number used for sequence alignment (not linked to strain).*

Species	**Gene**	**Size**		**Temp**	**Length**	**GC**
Karenia mikimotoi	18S	88 bp				
KarmikF3	CCGAGTGACTGAATGTCCTC			60.5	20	55
KarmikR3	TATTCACTCACCCATAGACGA			57.5	21	43
KarmikP3	GCAGTGCTACCAGACACACAGAG			66.6	23	57
Related species	**Tested**	**Amplification**	**Source**	**Strain**	**Accession no.***	
Karenia mikimotoi	Yes	Yes	NIVA CCA[1]	K-0260	KJ508364.1	
Karenia selliformis	Yes	No	NIVA CCA[1]	K-1319	KY580784.1	
Karenia brevis	Yes	No	NIVA CCA[1]	K-1274	EU165308.1	
Karlodinium armiger	Yes	No	NIVA CCA[1]	K-0668	KP790218.1	
Karlodinium decipiens	Yes	Yes	NIVA CCA[1]	K-1135	JF906079.1	
Karlodinium veneficum	Yes	No	NIVA CCA[1]	K-1634	JF906079.1	
Karenia bidigitata	Yes	No	CICCM[2]	CAWD81	AY947663.1	
Karenia brevisulcata	Yes	No	CICCM[2]	CAWD82	KJ508359.1	
Karenia papilionacea	Yes	No	CICCM[2]	CAWD91	LC055204.1	
Karenia umbella	Yes	No	CICCM[2]	CAWD131	KJ508368	
Gymnodinium aureolum	Yes	No	NIVA CCA[1]	K-1562	DQ779991.1	
Gymnodinium corollarium	Yes	No	NIVA CCA[1]	K-0983	FJ211386.1	
Gymnodinium nolleri	Yes	No	NIVA CCA[1]	K-0626	AF200673.1	

** Additional sequences from each species and additional not listed species were used in the alignment for increased accuracy and diversity coverage within and among* Karenia *species and closely related species. [1] Norwegian Institute for Water Research Culture Collection of Algae, [2] Cawthron Institute's Culture Collection of Micro-algae, New Zealand.*

Conditions of the specificity test

DNA from reference tissue samples were either supplied from external sources (Table 5.1) or extracted from tissue samples using the DNeasy PowerPlant Pro Kit (Qiagen, Hilden, Germany) according to manufacturer's specifications. Initial primer specificity, optimal annealing temperature and primer concentration was performed in a 25 µL reaction volume comprising forward and reverse primers (Eurofins Genomics, Ebersberg, Germany), SsoFast EvaGreen master Mix (BioRad, Hercules, California, Unites States) and 2.5 µL template DNA that was adjusted to a concentration around 1 ng/µL. QPCR reactions were run on a CFX96 Touch Real-Time PCR Detection Systems (BioRad, Hercules, California, Unites States). Conditions for the thermos cycling were run with an initial warming at 95°C for 10 minutes followed by 50 cycles of 95°C for 15 s and 60°C for 1 minute. Primer probe specificity test was run using 500 µM of each primer and 50 µM probe (TAG Copenhagen, Denmark) in a 20µL reaction volume of Applied Biosystems TaqMan Environmental Mastermix 2.0 (Thermo Fisher Scientific, Waltham, Massachusetts, United States) and 2.5 µL template DNA. Target- and non-target species were run in duplicate reactions and four negative controls.

Assay specificity results

The designed assay in the 28S gene showed high specificity towards *Karenia mikimotoi* (Cq: 21.58 and 21.13; Figure 5.2). In addition to *K. mikimotoi*, occasional amplification of *Karlodinium decipiens* was also observed (Cq: 37.60) in some of the replicates but not all. The amplification is rather suppressing due to the large sequence dissimilarity for *K. mikimotoi* versus the included available online sequences from *Karlodinium* species (data not shown). This closely related species however primarily occurs in the Oceans around Australia and New Zealand, but has also been detected in Spanish waters (Guiry & Guiry 2017). Due to the fish-killing mechanisms by *Karlodinium* blooms, potentially caused by karlotoxins, simultaneous detection of this *Karlodinium* species alongside *Karenia mikimotoi* may prove beneficial to prevent or forecast potential harmful algae blooms. Depending on amplification from environmental samples a maximum of 40 qPCR cycles could be recommended for microalgae to avoid unspecific alignment of non-target species, as recommended for *Pseudochattonella* species (Eckford-Soper & Daugbjerg 2016).

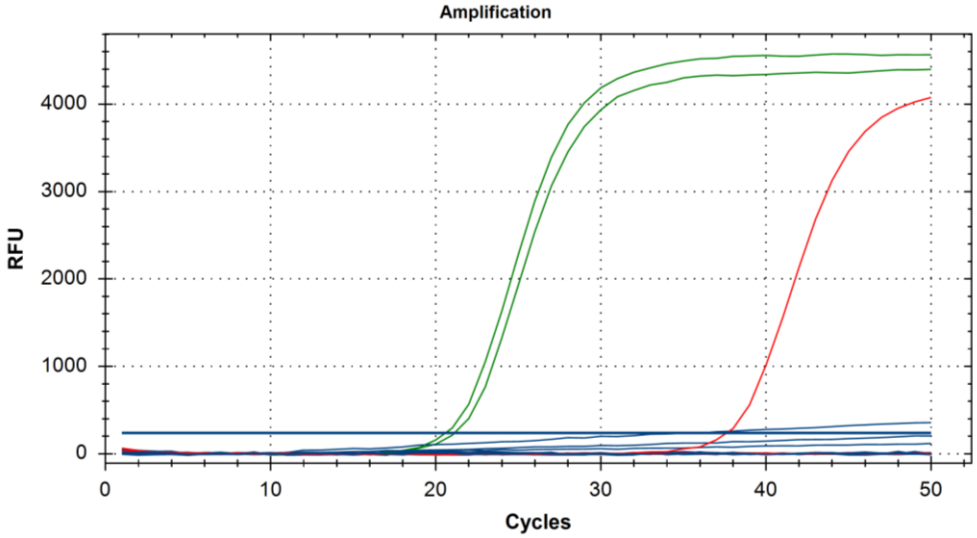

Figure 5.2 *Amplification of* Karenia *species using the F3R3P3 assay. Target species* K. mikimotoi *is shown in green and non-target sister species in blue.* Karlodinium decipiens *showed amplification with this assay and is marked in red.*

3.6 Species no. 06: *Carassius auratus auratus*

Binomial nomenclature and author:	*Carassius auratus auratus* Linnaeus, 1758.
English common name:	Goldfish.
Danish common name:	Sølvkarusse.

The genus *Carassius* belongs to the family Cyprinidae and includes five species (Fishbase 2015).

Comparisons of sequence data indicate relatively low sequence variation between *Carassius auratus auratus* and *Cyprinus gibelio* (Andersen *et al.* 2016) which confines the ability to distinguish these two species. This is potentially due to taxonomic confusion between *C. auratus auratus* and *C. gibelio* and their modes of reproduction (Froese & Pauly 2017) presumably resulting in substantial hybridization and introgression throughout their species distributions. Taxonomic relationships among Cyprinidae species are complex and in some cases unresolved (e.g. Carl 2012a), and it is e.g. questioned whether *C. gibleo* is in fact a separate species from *Carassius auratus auratus.* As *C. gibleo* is not reported in any Danish waters (Carl 2012a), it was therefore assumed that a specificity test of the

Carassius auratus auratus assay needed only include testing of well described non-target species (Table 6.1). An assay for *C. auratus auratus* based on the Cytochrome b sequence was published and validated by Nathan *et al.* (2014). Testing sequence variation for those primers revealed limited variation between *C. gibelio* and *C. auratus auratus* and attempts to increase specificity by using a probe showed no optimal binding sites and lack of intraspecific nucleotide polymorphisms. We instead tested the assay from Andersen *et al.* (2016), together with a second assay developed in the present study (Table 6.1). Both were based on the COI gene. As the second assay showed the best performance and was specific, we report results from that here.

Table 6.1 *Species-specific primer/probe assays for* Carassius auratus auratus *with target gene, product size, calculated melting temperature, primer/probe length and GC ratio (%) and target and relatives of target species and analysis results. Data from this study.*

Species	Gene	Product size	Temp	Length	GC
Carassius auratus	COI	97 bp			
Car_aur _COI_F01	5'- TTCTTCCCCCATCATTCCTGT -3'		58.6	21	47
Car_aur _COI_R01	5'- GTATACTGTCCATCCGGAGG -3'		59.7	20	55
Car_aur_COI_P02	5'FAM- TAGCTTCCTCTGGTGTTGAAGCCGGAG -BHQ3'		59.1	27	43
Related species	**Tested**	**Amplification**	**Source (Accession no.)**		
Carassius auratus	*Yes - Target*	*Yes*	Natural History Museum of Denmark (P264492)		
Carassius carassius	*Yes*	*No*	Natural History Museum of Denmark (P265763)		
Cyprinus carpio	*Yes*	*No*	Natural History Museum of Denmark (P265736)		

Initial evaluation of primer specificity, annealing temperature and optimal primer concentration was performed in a 10 µL reaction volume with 500 nM forward and reverse primers (Integrated DNA Technologies, U.S.A.), and 1µl template DNA at a concentration 0.1-1.0 ng/µL using a generic endpoint thermocycling programme: denaturing at 95°C for 1 minute, followed by 35 cycles of denaturing at 95°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 60 s, followed by final extension at 72°C for 5 minutes. Following PCR amplification, samples were electrophoresed on a 1% agarose gel stained with ethidium bromide and visualized under a UV light, and results documented by photography.

Primer-probe specificity test was performed in a 25 µL reaction volume containing 400 nM of each primer, 100 nM of BHQ probe and 10 µL of Applied Biosystems TaqMan Environmental Mastermix 2.0 (Thermo Fisher Scientific, Waltham, Massachusetts, United States) with 2 µL template DNA. Thermocycling qPCR reactions were run on a StepOne Plus Real-time PCR Instrument (Life Technologies, U.S.A.), using 10 minutes initial denaturation at 95°C, followed by 50 cycles of 95°C for 15 s and 60°C for 1 minute. Target- and non-target species were run in duplicate reactions and four negative controls were included.

Assay specificity results

The target species amplified successfully at 28 Cq (Figure 6.1) and no amplification was observed in any of the non-target species or the negative controls. Occurrences of hybridization between *C. auratus auratus* and other closely related species, including *Cyprinus carpio,* are reported. Since the mitochondrial genome of these hybrids are maternally inherited the assay will be unable to distinguish hybrids from normal individuals in cases where the mitochondrion genome originates from *C. auratus auratus* (Yan *et al.* 2005). However, both species are listed as potential invasive and the assay is therefore considered to be a good tool for identifying invasive cyprinid fishes in marine environments. In conclusion, it is concluded that the assay is specific for identifying the target in Danish marine waters.

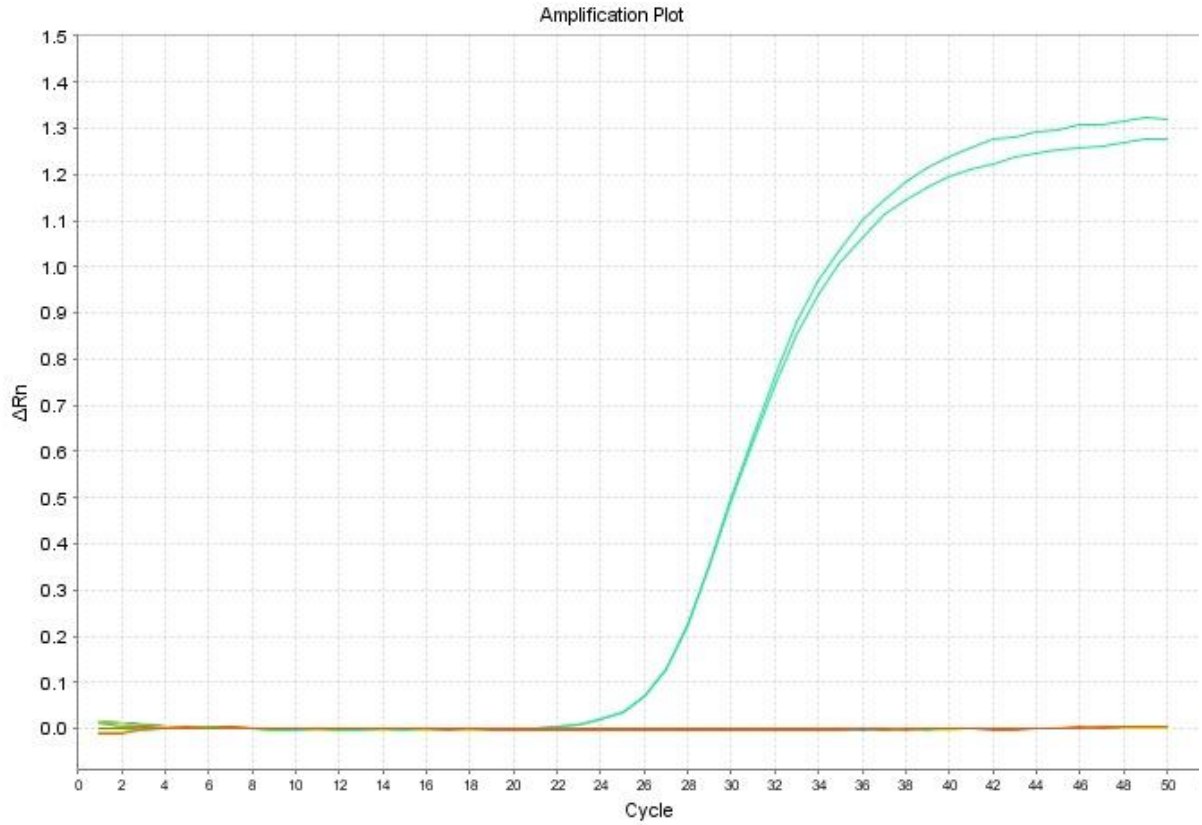

Figure 6.1 *Amplification of carp species using the Car_Aur_COI assay. Target species* Carassius auratus auratus *is shown in green and non-target species in red and yellow.*

3.7 Species no. 07: *Cyprinus carpio*

Binomial nomenclature and author: *Cyprinus carpio* Linnaeus, 1758.
English common name: Common carp.
Danish common name: Karpe.

Figure 7.1 Cyprinus carpio*. Photo by Søren Berg (DTU Aqua, Technical University of Denmark).*

The genus *Cyprinus* comprises two marine/brackish species and 24 species in total. The genus belongs to the family Cyprinidae containing 3032 species (Froese & Pauly 2017). Multiple qPCR assays have been published and are reported as specific to eDNA based detection of *C. carpio* (Takahara *et al.* 2012, Mahon *et al.* 2013, Eichmiller *et al.* 2014, Andersen *et al.* 2016). All of these systems were tested here, and one assay targeting the CytB region (from Eichmiller *et al.* 2012) showing specificity and reproducibility was selected to be reported here (Table 7.1). This assay was reported to be fully specific across all (obligate) freshwater fish species encountered in a small lake in the Upper Mississippi River Basin in the U.S.A (Eichmiller *et al.* 2012). Comparisons of sequence data indicate relatively low sequence variation among the congeners *C. acutidorsalis, C. rubrofuscus, C. pellegrini, C. multitaeniata, C. melanes, C. cuvieri, C. gibelio and Carassius auratus auratus* (Andersen *et al.* 2016). As the first six of these species are tropical and obligate freshwater species native to Southeast Asia they are unlikely to occur in Danish marine waters and were not included in specificity tests here. A comparison between published sequence for *C. auratus auratus* and *C. gibelio* (Prussian carp) showed limited sequence variation, which confines the ability to distinguish these two species. This is potentially due to taxonomic confusion between *C. auratus auratus* and *C. gibelio* and their complex modes of reproduction (Froese & Pauly 2017). Taxonomic relationships among Cyprinidae species are complex and in some cases unresolved (e.g. Carl 2012a), and it is questioned whether *C. gibleo* is in fact a separate species from *Carassius auratus auratus.* As *C. gibleo* is not reported in Danish waters, it was therefore concluded that a specificity test of the Common carp assay needed only include testing of the well described non-target species *Carassius auratus auratus* (Table 7.1).

Table 7.1 *Species-specific primer/probe assay for* Cyprinus carpio *with target gene, product size, calculated melting temperature, primer/probe length and GC ratio (%). Data from Takahara* et al. *(2012). Target and non-target species shown with qPCR analysis results***.**

Species	Gene	Product size	Temp	Length	GC
Cyprinus carpio	CytB	149 bp			
CCcytbF	5'- CTAGCACTATTCTCCCCTAACTTAC -3'		58.0	25	44.0
CCcytbR	5'- ACACCTCCGAGTTTGTTTGGA -3'		59.86	21	47.6
CCcytbP	5'-FAM- CCCTCTAGTTACACCACC -TAMRA-3'		53.2	18	55.6

Related species	Tested	Amplification	Source (Accession no.)
Cyprinus carpio	Yes – target	Yes	Natural History Museum of Denmark (P265736)
Carassius carassius	Yes	No	Natural History Museum of Denmark (P265763)
Carassius auratus auratus	Yes	No	Natural History Museum of Denmark (P264492)

Initial evaluation of primer specificity, annealing temperature and optimal primer concentration was performed in a 10 µL reaction volume with 500 nM forward and reverse primers (Integrated DNA Technologies, U.S.A.), and 1 µL template DNA at a concentration 0.1-1.0 ng/µL using a generic end-point thermocycling programme: denaturing at 95°C for 1 minutes, followed by 35 cycles of denaturing at 95°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 60 s, followed by final extension at 72°C for 5 minutes. Following PCR amplification, samples were electrophoresed on a 1% agarose gel stained with ethidium bromide and visualized under a UV light, and results documented by photography.

Primer-probe specificity test was performed in a 25 µL reaction volume containing 400 nM of each primer, 100 nM of BHQ probe and 10 µL of Applied Biosystems TaqMan Environmental Mastermix 2.0 (Thermo Fisher Scientific, Waltham, Massachusetts, United States) with 2 µL template DNA. Thermocycling qPCR reactions were run on a StepOne Plus Real-time PCR Instrument (Life Technologies, U.S.A.), using 10 minutes initial denaturation at 95°C, followed by 50 cycles of 95°C for 15 s and 60°C for 1 minute. Target- and non-target species were run in duplicate reactions and four negative controls were included.

Assay specificity results
The target species amplified successfully at 25 Cq (see Figure 7.2) and no amplification was observed in any of the non-target species or the negative controls. The mitochondrial genome of hybrids is maternally inherited, and the assay will hence not be able to distinguish hybrids from pure individuals in cases where the mitochondrion genome originates from *C. carpio* (Yan *et al.* 2005). However, both species are listed as potential invasive and the assay is therefore considered to be a good tool for identifying invasive cyprinid fishes in marine environments. In conclusion, as *C. carpio* is the only species representative of the genus *Cyprinus* occurring in Danish territorial waters it is therefore concluded that the assay is specific.

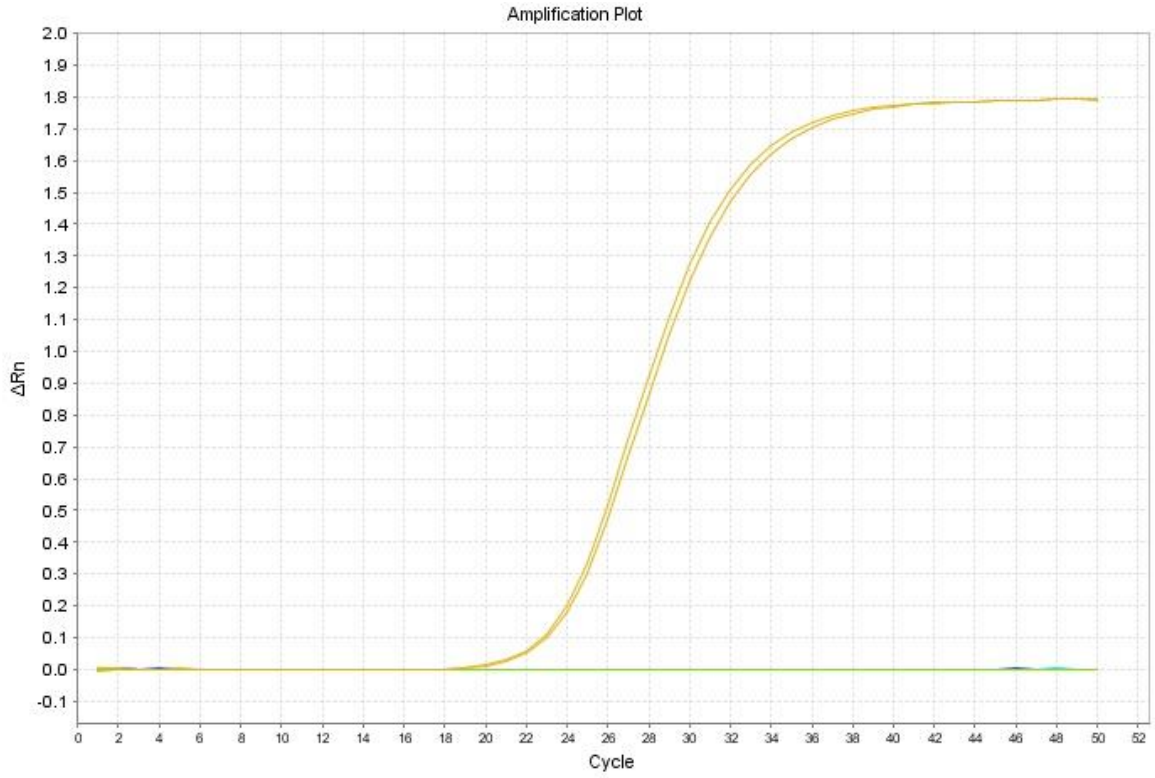

Figure 7.2 *Amplification of tested cyprinid species using the CCcytb assay. Target species* Cyprinus carpio *is shown in yellow (two replicates) and non-target species in greens and blues.*

3.8 Species no. 08: *Colpomenia peregrine*

Binomial nomenclature and author: *Colpomenia peregrine* (Sauvageau), Hamel 1937.
English common name: Oyster thief brown algae.
Danish common name: Østerstyv.

The genus *Colpomenia* contains 14 species, all native to the Pacific region and all with limited sequence information. Based on information in Andersen *et al.* (2016), sequence information was relatively ample for the COX3 gene, which was therefore targeted for assay development (Table 8.1).

Table 8.1 *Species-specific primer/probe assay for* Colpomenia peregrine *with target gene, produc size, calculated melting temperature, primer/probe length and GC ratio (%). Adapted from Andersen* et al. *(2016). Overview of primer and probe mismatches between target species and related species of* Colpomenia *(none occurring in Danish waters). In all cases there is low sequence similarity.*

Species	Gene	Product size	Temp	Length	GC
Colpomenia peregrine	COX3	116 bp			
Col_per_COX 3_F01	5'-GCAAGCTTTTGAATATGCTAATG-3'		57.6	23	35
Col_per_COX 3_R01	5'-CAGCTAAAAATATTGTACCGATTA-3'		56.6	24	29
Col_per_COX 3_P01	5'-FAM-TTCAGTTTTTTACATGGCTACAGGCTTC-TAMRA-3'		65.6	28	39

Related species	Forward	Reverse	Probe
Colpomenia bullosa	7	6	4
Colpomenia claytoniae	1	3	4
Colpomenia durvillei	7	3	4
Colpomenia ecuticulata	6	5	5
Colpomenia expansa	1	6	5
Colpomenia phaeodactyla	8	4	4
Colpomenia ramosa	7	5	7
Colpomenia sinuosa	4	6	6
Colpomenia tuberculata	7	5	7

DNA from reference tissue sample was extracted from archived dried tissue (collected at Tvillingholmen N, Grimstad, Aust-Agder, Norway, by NIVA, reference no. H-45) using the MoBio (now Qiagen) PowerPlant kit (Qiagen, Hilden, Germany) according to the manufacturer's specifications. Initial evaluation of primer specificity, annealing temperature and optimal primer concentration was performed in a 10 µL reaction volume with 500 nM forward and reverse primers (Integrated DNA Technologies, U.S.A.), and 1 µl template DNA at a concentration 0.1-1.0 ng/µL using a generic endpoint thermocycling programme: denaturing at 95°C for 1 minutes, followed by 35 cycles of denaturing at 95°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 60 s, followed by final extension at 72°C for 5 minutes. Following PCR amplification, samples were electrophoresed on a 1% agarose gel stained with ethidium bromide and visualized under a UV light, and results documented by photography.

Primer-probe specificity test was performed in a 25 µL reaction volume containing 400 nM of each primer, 100 nM of BHQ probe and 10 µL of Applied Biosystems TaqMan Environmental Mastermix 2.0 (Thermo Fisher Scientific, Waltham, Massachusetts, United States) with 2 µL template DNA. Reaction volumes of 10 µL were also tested and worked equally well. Thermocycling qPCR reactions were run on a StepOne Plus Real-time PCR Instrument (Life Technologies, U.S.A.), using 10 minutes initial denaturation at 95°C, followed by 50 cycles of 95°C for 15 s and 60°C for 1 minute. Target DNA was analysed in duplicate reactions and four negative controls were included.

Assay specificity results

The target species amplified successfully at 22 Cq (Figure 8.1) and no amplification was observed in any of the negative controls. No other related species are recorded from Danish waters and would be exotics if they were. The designed eDNA target assay for *Colpomenia peregrine* is therefore considered specific to invasive *Colpomenia* when tested on laboratory and environmental water samples

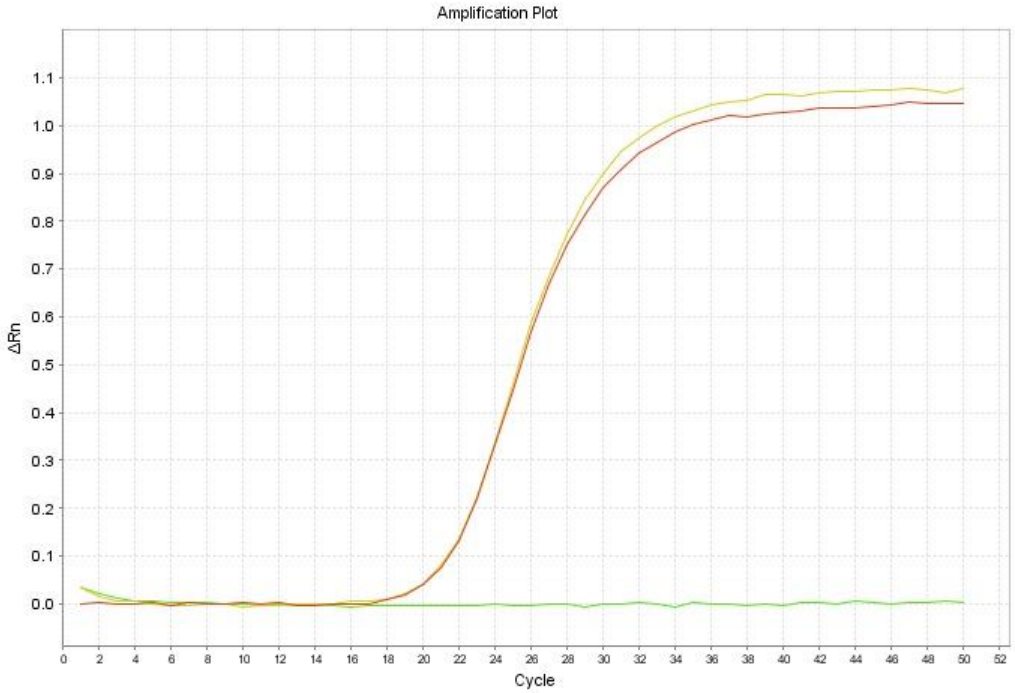

Figure 8.1 *Amplification of* Colpomenia peregrine *using the Col_per_COX 3 assay. Target species is shown in red and yellow (two replicates) and negative controls in green*.

3.9 Species no. 09: *Neogobius melanostomus*

Binomial nomenclature and author: *Neogobius melanostomus* Pallas, 1814.
English common name: Round goby.
Danish common name: Sortmundet kutling.

Figure 9.1 Neogobius melanostomus*. Photo by Mads Christoffersen (DTU Aqua, Technical University of Denmark)*

Neogobius comprises nine marine species and belongs to the family Gobiidae (Froese & Pauly 2017).

Apart from the assay developed in Andersen *et al.* (2016) another assay based on the COI region was validated for *Neogobius melanostomus* by Nathan et al. (2014; 2015). A third assay for *N. melanostomus* also based on the COI region was recently published and validated in an extensive number of

Goby species as well as other native European fish species (Adrian-Kalchhauser & Burkhardt-Holm 2016). Two assays were selected and tested for species specific detection here (Table 9.1). Both assays performed equally well in terms of specificity but we here report results for one of them (NeoMel_COI).

Table 9.1 *Species-specific primer/probe assays for* Neogobius melanostomus *with target gene, product size, calculated melting temperature, primer/probe length and GC ratio (%). #) adapted from Andesen* et al. *(2016). §) from Adrian-Kalchhauser & Burkhardt-Holm (2016). Target, relatives of target species and qPCR analysis results. The primer-probe assay by Adrian-Kalchhauser & Burkhardt-Holm (2016) was not used in the final analyses of water samples.*

Species	Gene	Product size	Temp	Length	GC
Neogobius melanostomus	COI	150 bp			
Neo_Mel_COI_F01#	5'-CTTCTRGCCTCCTCTGGWGTTG-3'		59.6 -62.8	22	54 -59
Neo_Mel_COI _R01#	5'-CCCWAGAATTGASGARATKCCGG-3'		58.9 -63.9	23	47-56
Neo_Mel_COI _P01#	5'-FAM-CAGGCAACTTRGCACATGCAG-BHQ-3'		60.1 - 62.9	21	52 -57
NeoMel_IK_F1§	5'- TATGTGATGATCGGACAGC-3'			19	53-56
NeoMel_IK_R1§	5'- GTTCTCTAGTCAGCTCGCT-3'			19	45-51
NeoMel_IK_Probe	5'-FAM-CATCTTTCTCGGCTTATTCCCCA-BHQ-3'			23	

Related species	Tested	Amplification
Neogobius melanostomus	Yes – target	Yes
Aphia minuta	Yes	No
Pomatoschistus minutus	Yes	No
Gobiusculus flavescens	Yes	No
Gobius niger	Yes	No
Pomatoschistus microps	Yes	No
Ponticola kessleri	No*	-
Neogobius rattan	No**	-
Neogobius rhodioni	No**	-

**) The assay was by Adrian-Kalchhauser & Burkhardt-Holm (2016) validated to not amplify for* P. kessleri *(i.e. was specific for the target* N. melanostomus*) but reference material not available for the current study.*
***) Reference material was not available for the current study but the species is exotic and is not expected to be invasive in Danish marine waters.*

DNA from target species was tested together with DNA from five related non-target species (Table 9.1).

Initial evaluation of primer specificity, annealing temperature and optimal primer concentration was performed in a 10 µL reaction volume with 500 nM forward and reverse primers (Integrated DNA Technologies, U.S.A.), and 1 µL template DNA at a concentration 0.1-1.0 ng/µL using a generic end-point thermocycling programme: denaturing at 95°C for 60 s, followed by 35 cycles of denaturing at 95°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 60 s, followed by final extension at 72°C for 5 minutes. Following PCR amplification, samples were electrophoresed on a 1% agarose gel stained with ethidium bromide and visualized under a UV light, and results documented by photography.

Primer-probe specificity test was performed in a 25 µL reaction volume containing 400 nM of each primer, 100 nM of BHQ probe and 10 µL of Applied Biosystems TaqMan Environmental Mastermix 2.0 (Thermo Fisher Scientific, Waltham, Massachusetts, United States) with 2 µL template DNA. Reaction volumes of 10 µL were also tested and worked equally well. Thermocycling qPCR reactions were run on a StepOne Plus Real-time PCR Instrument (Life Technologies, U.S.A.), using 10 minutes initial denaturation at 95°C, followed by 50 cycles of 95°C for 15 s and 60°C for 1 minute. Target- and non-target species were run in duplicate reactions and four negative controls were included.

Assay specificity results
The target species amplified successfully at 16 Cq (Figure 9.2) and no amplification was observed in any of the non-target species or the negative controls. It is therefore concluded that the assay is specific for identifying the target species in Danish marine waters.

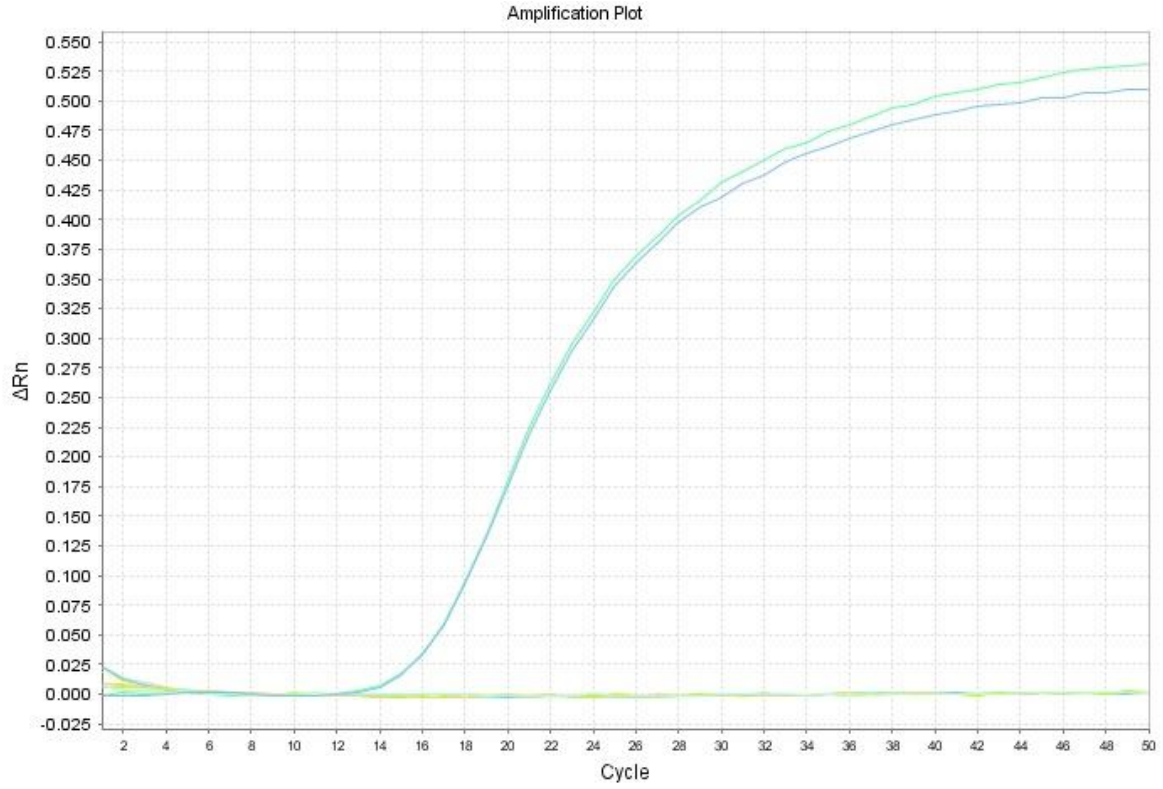

Figure 9.2 *Amplification of goby species using the NeoMel_COI assay.* Neogobius melanostomus *samples (two different individuals) are shown in blue and turquoise and non-target species and blanks in light greens.*

3.10 Species no. 10: *Oncorhynchus mykiss*

Binomial nomenclature and author: *Oncorhynchus mykiss* Walbaum, 1792.
English common name: Rainbow trout.
Danish common name: Regnbueørred.

The genus *Oncorhynchus* belongs to the family Salmonidae and comprises 15 species (Froese & Pauly 2017).

Andersen *et al.* (2016) developed a species-specific assay *in silico* and another qPCR assay was recently published and reported to be specific to eDNA from *Oncorhynchus mykiss* in its American homerange streams (Wilcox *et al.* 2015). However, the latter authors also noted that local sequence variation could pose a problem for specificity of assays. This was also evident for some of the assays tested here. We tested three systems (respectively from Wilcox *et al.* 2015, Andersen *et al.* 2016 and developed for this study), and report qPCR results for one of these (from Andersen *et al.* 2016), targeting the CytB region, which showed specificity and reproducibility (Table 10.1).

Table 10.1 *Species-specific primer/probe assay for* Oncorhynchus mykiss *with target gene, product size, calculated melting temperature, primer/probe length and GC ratio (%). Data from Andersen* et al. *(2016). Target and non-target species shown with qPCR analysis results.*

Species	Gene	Product size	Temp	Length	GC
Oncorhynchus mykiss	CytB	78 bp			
Onc_Myk_CytB_Forward_01	5'-ACCTCCAGCCATCTCTCAGT-3'		57.5	22	41
Onc_Myk_CytB_Reverse_01	5'-AGGACGGGGAGGGAAAGTAA-3'		56.9	26	39
Onc_Myk_CytB_Probe_01	5'-FAM-TGAGCCGTGCTAGTTACTGCTGTCCTT-BHQ-1-3'		60.2	28	32

Related species	Tested	Amplification	Source (Accession no.*)
Oncorhynchus mykiss	Yes – target	Yes	Natural History Museum of Denmark (P191985, P191984)
Salmo Trutta	Yes	No	Natural History Museum of Denmark (P191788, P192227)
Oncorhynchus gorbuscha	Yes	No	Natural History Museum of Denmark (P191563), DTU Aqua (PUK#01, PUK#02)
Thymallus thymallus	Yes	No	Natural History Museum of Denmark (P191624)
Salvelinus alpinus	Yes	No	Natural History Museum of Denmark (P191564, P191785)
Coregonus albula	Yes	No	Natural History Museum of Denmark (P191806, P191807)
Salvelinus fontinalis	Yes	No	Natural History Museum of Denmark (P191978, P191980)
Coregonus lavaretus	Yes	No	Natural History Museum of Denmark (P192242)

Initial evaluation of primer specificity, annealing temperature and optimal primer concentration was performed in a 10 µL reaction volume with 500 nM forward and reverse primers (Integrated DNA Technologies, U.S.A.), and 1 µL template DNA at a concentration 0.1-1.0 ng/µL using a generic end-point thermocycling programme: denaturing at 95°C for 1 minute, followed by 35 cycles of denaturing at 95°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 60 s, followed by final extension at 72°C for 5 minutes. Following PCR amplification, samples were electrophoresed on a 1% agarose gel stained with ethidium bromide and visualized under a UV light, and results documented by photography.

Primer-probe specificity test was performed in a 25 µL reaction volume containing 400 nM of each primer, 100 nM of BHQ probe and 10 µL of Applied Biosystems TaqMan Environmental Mastermix 2.0 (Thermo Fisher Scientific, Waltham, Massachusetts, United States) with 2µL template DNA. Thermocycling qPCR reactions were run on a StepOne Plus Real-time PCR Instrument (Life Technologies, U.S.A.), using 10 minutes initial denaturation at 95°C, followed by 50 cycles of 95°C for 15 seconds and 60°C for 1 minute. Target- and non-target species were run in duplicate reactions and four negative controls were included. DNA from target species was tested against DNA from seven related non-target species, all of which occur in Danish waters (Table 10.1).

Assay specificity results

The target species amplified successfully at 20-22 Cq (Figure 10.1) and no amplification was observed in any of the non-target species or in the negative controls. Occurrences of hybridization between *O. mykiss* and *Salmo salar* have been reported. Since the mitochondrial genome of these hybrids is maternally inherited the assay will be unable to distinguish hybrids from normal individuals in cases where the mitochondrion genome originates from *O. mykiss* (Wang et al. 2015). *Oncorhyncus mykiss* showed high similarity to *O. gilae* and *O. chrysogaster* both with regard to the COI and Cytochrome b sequences. However, as the latter two species are of Pacific Ocean origin and neither occurs in Danish territorial waters, they do not pose a risk of false positives. The assay is thus considered to be specific to the target species.

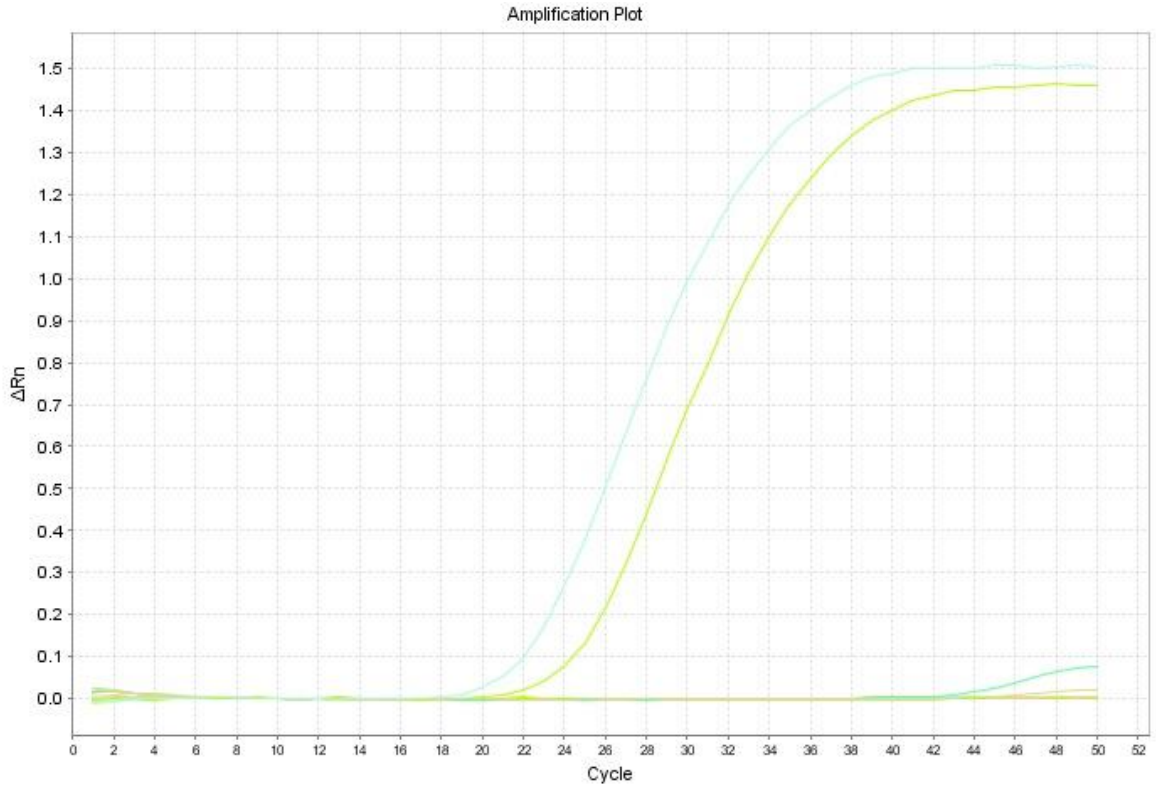

Figure 10.1 *Amplification of tested salmonid species using the Onc_Myk_CytB assay. Target species* Oncorhynchus mykiss *(two different individuals) is shown in light blue/light green and non-target species in other colours.*

3.11 Species no. 11: *Oncorhyncus gorbuscha*

Binomial nomenclature and author: *Oncorhyncus gorbuscha* (Walbaum, 1792).
English common name: Pink salmon.
Danish common name: Pukkellaks.

Figure 11.1 Oncorhyncus gorbuscha*. Photo by Henrik Carl (Natural History Museum of Denmark).*

The genus *Oncorhyncus* comprises 16 valid species (Eschmeyer & Fricke 2017): *Oncorhyncus aguabonita, O. apache, O. chrysogaster, O. clarkii, O. gilae, O. gorbuscha, O. iwame, O. kawamurae, O. keta, O. kisutch, O. masou formosanus, O. masou, O. mykiss, O. nerka, O. rhodurus*, and *O. tshawytscha.* Among these *O. gorbuscha* and *O. mykiss* have been found as introduced species in Northern Europe (Rasmussen 2012).

Among the sequences used for specific primer design were sequences representing the most commonly encountered salmonid species occurring in Danish waters (Carl 2012b, Carl & Møller 2012, Rasmussen 2012): *Coregonus albula*, *Oncorhynchus mykiss*, *Salmo trutta*, *Salvelinus alpelinus*, *Salvelinus fontinalis*, *Thymallus thymallus*. Sequences deposited in NCBI GenBank, and sequences obtained by *de novo* sequencing of vouchered samples held at the Natural History Museum of Denmark were compared for these salmonid species for the mitochondrial *cytochrome oxidase 1*(CO1) gene. This gene was selected as region for specific primer design, due to the level of variation among the native and non-native salmonid species (Table 11.1):

- Oncgor_CO1_F09: 5'-TCCTTCCTCCTCCTCCTTTC-3'.
- Oncgor_CO1_R06: 5'-TGGCCCCTAAAATTGATGAG-3'.
- Oncgor_CO1_P06: 5'-FAM-CAGGGGCATCCGTCGACTTAACTAT-BHQ-1-3.

The total length of the product was 163 bp.

Table 11.1 *Species specific primer/probe assay for* Oncorhynchus gorbuscha*, 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.*

Species	Gene	Size		Temp	Length	GC
Oncorhynchus gorbuscha	mtDNA-CO1	163 bp				
Oncgor_CO1_F09	TCCTTCCTCCTCCTCCTTTC			59.8	20	55
Oncgor_CO1_R06	TGGCCCCTAAAATTGATGAG			59.9	20	45
Oncgor_CO1_P06	CAGGGGCATCCGTCGACTTAACTAT			63.9	25	48
Related species*	**Tested**	**Amplification**	**Invasive**			
Coregonus albula	Yes	No	No			
Oncorhynchus mykiss	Yes	No	No			
Salmo trutta	Yes	No	No			
Salmo salar	Yes	No	No			
Oncorhynchus kisutch	Yes	No	Yes			
Salvelinus alpelinus	Yes	No	No			
Salvelinus fontinalis	Yes	No	No			
Thymallus thymallus	Yes	No	No			
Oncorhynchus gorbuscha	Yes	No	Yes			

** Not including all species in the genus* Oncorhynchus *but limited to those that are occurring in Northern Europe.*

The primers were designed by aligning sequences available from mitochondrial cytochrome oxidase 1 genes from salmonid species available from NCBI GenBank. The alignment was prepared using the following sequences from NCBI GenBank and sequences obtained by *de novo* sequencing of samples from the Natural History Museum of Denmark: *Coregonus lavaretus*: AB034824; *Oncorhynchus chrysogaster*: JX960908; *Oncorhynchus clarkii*: JX960909-JX960910; *Oncorhynchus gilae*: JX960907, JX960911; *Oncorhynchus gorbuscha*: AB712402-AB712411, EF455489, EU524202-EU524209, EU752126-EU752128, FJ998665-FJ998711, GU440431, HQ712698-HQ712701, JX960912-JX960913, KU867886, LC050854-LC050855, LC145744-LC145755; *Oncorhynchus keta*: JX960914-JX960915; *Oncorhynchus kisutch*: JX960916-JX960917; *Oncorhynchus masou*: DQ656543, JX960918-JX960919, JX960925; *Oncorhynchus mykiss*: JX960920-JX960922, KP085590, L29771, LC050735; *Oncorhynchus*

nerka: EF055889, JX960923-JX960924; *Oncorhynchus tshawytscha*: JX960926-JX960927; *Salmo salar*: AF133701, LC012541, U12143; *Salmo trutta*: AM910409, LC011387; *Salvelinus namaycush*: JX960965, KT630726, KU258418, KU867896-KU867898; *Thymallus thymallus*: FJ853655. Sequence alignment was performed using Geneious v. R7 (Kearse *et al.* 2012) and MAFFT 6.822 (Katoh & 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: *Oncorhynchus gorbuscha*, would be amplified by the F09_R06_P06systems 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 precise than the F09_R06_P06 systems: Oncgor_CO1_F01: 5'-TATCGCTATCCCCACAGGAG-3', Oncgor_CO1_F02: 5'-CGCTATCCCCACAGGAGTAA-3', Oncgor_CO1_F05: 5'-ACACTACACGGAGGCTCGAT-3', Oncgor_CO1_F06: 5'-CTGGTACCGGATGGACAGTT-3', Oncgor_CO1_F08: 5'-TGGTACCGGATGGACAGTTT-3', Oncgor_CO1_P01: 5'-FAM-ACGGAGGCTCGATCAAATGAGAGAC-BHQ-1-3', Oncgor_CO1_P03: 5'-FAM-TACACGGAGGCTCGATCAAATGAGA-BHQ-1-3', Oncgor_CO1_P05: 5'-FAM-TGAGAGACACCACTTCTCTGAGCCC-BHQ-1-3', Oncgor_CO1_P09: 5'-FAM-CGCTGGTACCGGATGGACAGTTTAT-BHQ-1-3', Oncgor_CO1_P10: 5'-FAM-AGGGGCATCCGTCGACTTAACTATCTT-BHQ-1-3', Oncgor_CO1_P11: 5'-FAM-AGCTGGAATCTCATCAATTTTAGGGGC-BHQ-1-3', Oncgor_CO1_R01: 5'-CCGCCCACTGTAAATAGGAA-3', Oncgor_CO1_R03: 5'-GGGCTCAGAGAAGTGGTGTC-3', Oncgor_CO1_R07: 5'-AGATTGCCGGTGGTTTTATG-3'.

Conditions of the specificity test

DNA from reference tissue samples were either supplied from external sources (Table 11.2) or extracted from tissue samples using the DNeasy Blood and tissue kit (Qiagen) according to manufacturer's specifications, and obtained through de novo sequencing performed at the NHMD. Initial primer specificity, test of annealing temperature and primer concentration was performed in a 25 µL reaction volume comprising forward and reverse primers (ordered through TAG Copenhagen A/S), using 1 µL forward and 1 µL reverse primer (with 10 µM initial concentrations per primer), and 0.1 µL 5U/µL AmpliTaq Gold Polymerase (Thermofisher, Applied Biosystems), 11.6 µL ddH_2O, 2 µL 25 mM $MgCl_2$ and 2 µL template DNA extracted from tissue samples and diluted 1:10 prior to usage. QPCR reactions were run on a Stratagene Mx3005P qPCR Machine (Agilent, Santa Clara, California, United States). Primer probe specificity test was run using 1 µL forward and 1 µL reverse primer (with 10 µM initial concentrations per primer) and 1 µL probe (with 2.5 µM initial concentration) in a 25 µL reaction volume, including 10 µL Applied Biosystems TaqMan Environmental Mastermix 2.0 (Thermo Fisher Scientific, Waltham, Massachusetts, United States), 10 µL ddH_2O and 2 µL 1:10 diluted template DNA from tissue extractions. Target- and non-target species were run in duplicate reactions and two negative controls.

Table 11.2 *List of tested species with information regarding collector and origin.*

Species*	Tissue sample at Zoological Museum of Copenhagen	Collector	Sampling location
Coregonus albula	Coralb	NHMD, P.R. Møller and H. Carl	Denmark
Oncorhynchus mykiss	Oncmyk	NHMD, P.R. Møller and H. Carl	Denmark
Salmo trutta	Saltru	NHMD, P.R. Møller and H. Carl	Denmark
Salvelinus alpelinus	Salalp	NHMD, P.R. Møller and H. Carl	Denmark
Salvelinus fontinalis	Salfon	NHMD, P.R. Møller and H. Carl	Denmark
Oncorhynchus kisutch	Onckis	NHMD, P.R. Møller and H. Carl	Denmark
Salmo salar	Salsal	NHMD, P.R. Møller and H. Carl	Denmark
Thymallus thymallus	Thythy	NHMD, P.R. Møller and H. Carl	Denmark
Oncorhynchus gorbuscha	Oncgor	NHMD, P.R. Møller and H. Carl	Denmark

** Species closely related to* Oncorhynchus gorbuscha *co-occruing with* O. gorbuscha *in Danish waters.*

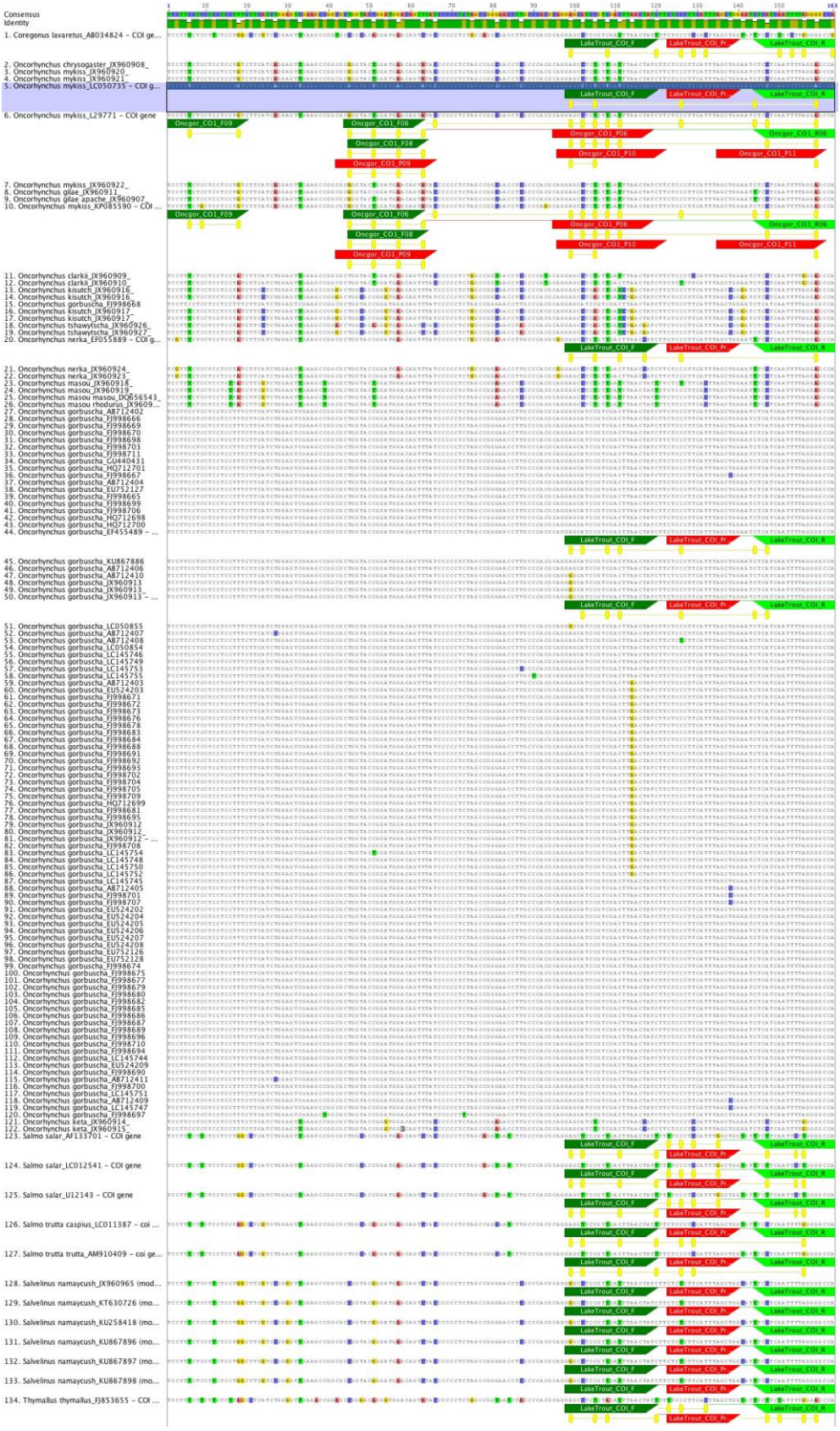

Figure 11.2 *Alignment of sequences from salmonid species and occurring in North European seas for the mtDNA-cytochrome oxidase 1 gene. Sequences were acquired from NCBI GenBank, and from de novo sequencing of vouchered samples held at NHMD.*

Assay specificity results
The two replicates of *Oncorhynchus gorbuscha* amplified at a Cq of 18.93 and 19.00, the replicates of (Figure 11.3). None of the native species amplified with this primer-probe assay.

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

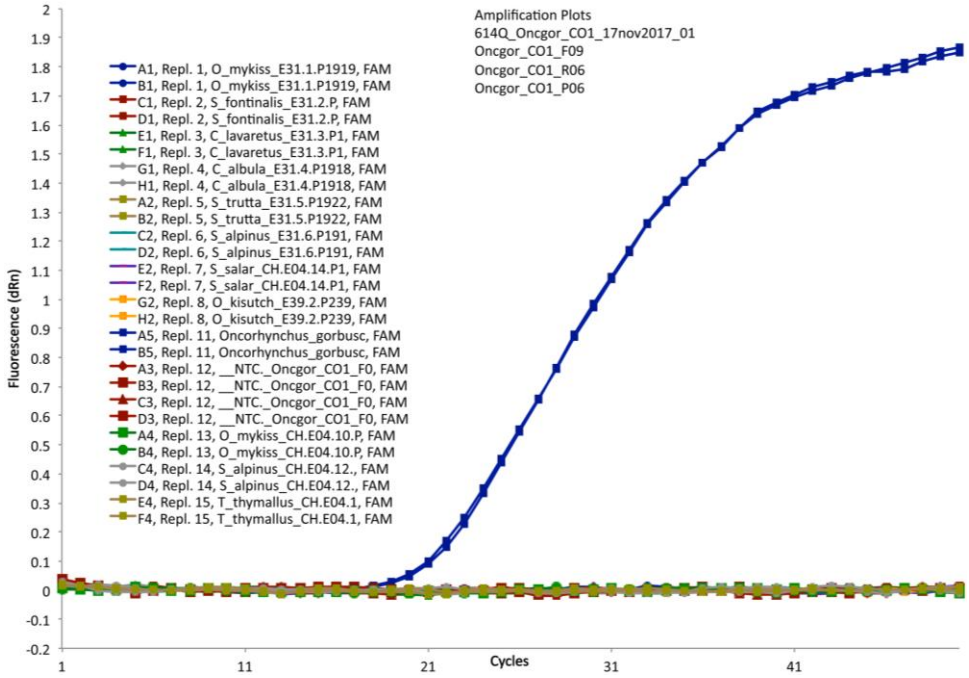

Figure 11.3 *Amplification of* Oncorhynchus gorbuscha *using the following assays: F09_R06_P09 assay targeting mtDNA-cytochrome oxidase 1. Target species* Oncorhynchus gorbuscha*, dark blue. Other non-target species:* Coregonus albula, Oncorhynchus mykiss, Oncorhynchus kisutch, Salmo trutta, Salmo salar, Salvelinus alpelinus, Salvelinus fontinalis *and* Thymallus thymallus*, in: red, green, gray, yellow-green, cyan, purple, orange, green and gray, respectively. Non-Target Control samples red.*

3.12 Species no. 12: *Crassostrea gigas* / *Magallana gigas*

Binomial nomenclature and author:	*Crassostrea gigas* (Thunber, 1793) or *Magallana gigas* (Thunberg 1793).
English common name:	Pacific oyster.
Danish common name:	Stillehavsøsters.

Figure 12.1 Magallana gigas*. Photo by Peter Rask Møller (Natural History Museum of Denmark).*

The genus *Crassostrea* comprises 11 extant valid species: *Crassostrea aequatorialis*, *C. angulata*, *C. brasiliana*, *C. chilensis*, *C. columbiensis*, *C. corteziensis*, *C. cuttackensis*, *C. dianbaiensis*, *C. rhizophorae*, *C. tulipa*, *C. virginica* in the family Ostreidae. Recently, the genus *Crassostrea* has been found to be paraphyletic, and *Crassostrea gigas* have been moved to the genus *Magallana*. The genus *Magallana* comprises nine species: *Magallana ariakensis*, *M. belcheri*, *M. bilineata*, *M. dactylena*, *M. gigas*, *M. hongkongensis*, *M. nippona*, *M. rivularis*, *M. sikamea*. All species of both *Crassostrea* and *Magallana* are considered non-native In North-European seas. The family Ostreidae comprise 16 genera: *Alectryonella*, *Anomiostrea*, *Booneostrea*, *Crassostrea*, *Magallana*, *Cryptostrea*, *Dendostrea*, *Lopha*, *Nanostrea*, *Ostrea*, *Planostrea*, *Pretostrea*, *Pustulostrea*, *Saccostrea*, *Striostrea*, and *Teskeyostrea*, where *Ostrea* is native in north European seas. A selection of species representing Bivalvia occurring in Danish seas, 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 (Table 12.1). The subsequent tests showed that primers and probes designed for mitochondrial DNA cytochrome oxidase 1 sequences were optimal for distinguishing between *C. gigas* and *Ostrea edulis* (English common name: European flat oyster; Danish common name: Limfjordsøsters):

- Cragig_CO1_F07: 5'-TTGAGTTTTGCCAGGGTCTC-3'.
- Cragig_CO1_R09: 5'-ACCAGCAAGGTGAAGGCTTA-3'.
- Cragig_CO1_P06: 5'-FAM-AACATTGTAGAAAACGGAGTTGGGGC-BHQ-1-3'.

The total length of the product was 154 bp.

Table 12.1 *Species-specific primer/probe assay for* Crasssostrea gigas *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.*

Species	**Gene**	**Size**	**Temp**	**Length**	**GC**
Crasssostrea gigas	*mtDNA-CO1* 154 bp				
Cragig_CO1_F07:	5'-TTGAGTTTTGCCAGGGTCTC-3'		60.2	20	50
Cragig_CO1_R09:	5'-ACCAGCAAGGTGAAGGCTTA-3'		59.9	20	50
Cragig_CO1_P06:	5'- AACATTGTAGAAAACGGAGTTGGGGC- 3'		66.1	26	42
Related species*	**Tested**	**Amplification**			
Aequipecten opercularis	Yes	No			
Arctica islandica	Yes	No			
Ensis sp.	Yes	No			
Modilus modilus	Yes	No			
Mya arenaria	Yes	No			
Mytilus edulis	Yes	No			
Crassostrea gigas	Yes	Yes			

** Not necessarily closely related to the genus* Crassostrea*, but these species are all species representing Bivalvia, and co-occuring with species from the family Ostreidae, and all are commonly encountered in North European seas.*

The primers were designed by aligning sequences available from mitochondrial cytochrome oxidase 1 from North European species of Bivalvia available from NCBI GenBank. This included: *Crassostrea angulata*: AB904879-AB904883, AB904885-AB904888, AB904890, AY397685, AY397686, AY455664, DQ659372-DQ659374, HQ661008, HQ661009, JQ027306-JQ027308; *Crassostrea ariakensis*: AF300617, AY160752-AY160754, FJ743512-FJ743527, HQ661020, HQ661021, KF272857-KF272859; *Crassostrea belcheri*: AY038077, AY160755, GU591436, GU591437-GU591441, GU591448-GU591467, JF915473-JF915497, JF915505, JF915509-JF915511; *Crassostrea brasiliana*: FJ717640-FJ717651; *Crassostrea columbiensis*: KP455017, KP455051, KP455052, KP455054, KP455055; *Crassostrea cuttackensis*: FJ262983; *Crassostrea gasar*: FJ717611, HM003499-HM003524; *Crassostrea gigas*:

AB636166-AB636263, AB641329-AB641334, AB904884, AB904889, AF280608, AJ553907-AJ553911, DQ417690-DQ659371, FJ717608, FJ743528, HM626169, HM626170, HQ661002-HQ661007, JF700177, KF643519, KF643604, KF643857, KF644048, KJ801546-KJ801559, KJ818236, KJ818240, KP099007-KP099052, KR084465, KR084582, KR084717, KR084750, KR084770, KR084952; *Crassostrea gryphoides*: FJ262985; *Crassostrea hongkongensis*: EU266073, HM627878; *Crassostrea iredalei*: AY038078, FJ948053-FJ948056, FJ948058-FJ948067, FJ948072-FJ948077, GU591412-GU591426, GU591429, GU591430, HQ661022, HQ661023, JF802601-JF802608, JF915438-JF915455, JF915458-JF915462, JF915467-JF915470, JF915472, JF915503, JQ991023-JQ991025, KF479204, KM373585-KM373588; *Crassostrea madrasensis*: FJ428750-FJ428752, FJ948057, FJ948068-FJ948071, GU591427, GU591428, GU591431-GU591435, JF915456, JF915457, JF915463-JF915466, JF915471, JF915504, JF915506, JF915507; *Crassostrea nippona*: AF300616, FJ743531; *Crassostrea rhizophorae*: FJ717612-FJ717639, HM003475-HM003498, KP455016, KP455025, KP455026, KP455028, KP455050; *Crassostrea sikamea*: AB641324-AB641328, AB675947-AB675955, AB904872-AB904878, HQ661010-HQ661019, JQ027288, JQ027289, KC683503; *Crassostrea* sp.: AJ553912, HQ661024; *Crassostrea virginica*: AY905542, FJ717609-FJ973668, FJ973670, JX468896-JX468944, KC429098, KF245599-KF245601, KF643392, KF643482, KF643557, KF643598, KF644075, KF644145, KF644147, KF644230, KF644323, KP455015, KP455023, KP455024, KP455029; *Ostrea angas*: AF540598; *Ostrea conchaphila*: DQ464125; *Ostrea edulis*: AF120651, AF540599, JF274008, KJ818233-KJ818235, KT988326, KU714734, KX713488, NC_016180; *Ostrea stentina*: AY376617, AY376623, AY376624, DQ226516, DQ226517, DQ313181-DQ313183; *Saccostrea cucullata*: AB721961, AY038076; *Saccostrea* sp.: JF915508. 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: *Crassostrea gigas*, would be amplified by the co1-F06_R07_P09 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 precise than the co1- F06_R07_P09 system: Cragig_CO1_F05: 5'-AAGCCTTCACCTTGCTGGTA-3', Cragig_CO1_R05: 5'-CTAGTAAATGGCCCCCAACA-3', Cragig_CO1_P05: 5'-GCTCTATTTTCAGGTCAATTAATTTC-3', Cragig_CO1_F06: 5'-TGAGTTTTGCCAGGGTCTCT-3', Cragig_CO1_R06: 5'-CCAGCAAGGTGAAGGCTTAG-3', Cragig_CO1_F08: 5'-TAGGCATGCGTTGGTTATGA-3', Cragig_CO1_R08: 5'-AGAGACCCTGGCAAAACTCA-3', Cragig_CO1_P08: 5'-FAM-GGGGTTTGGTAACTGGCTTATCCCTT-BHQ-1-3', Cragig_CO1_F10: 5'-TTTGAGTTTTGCCAGGGTCT-3'.

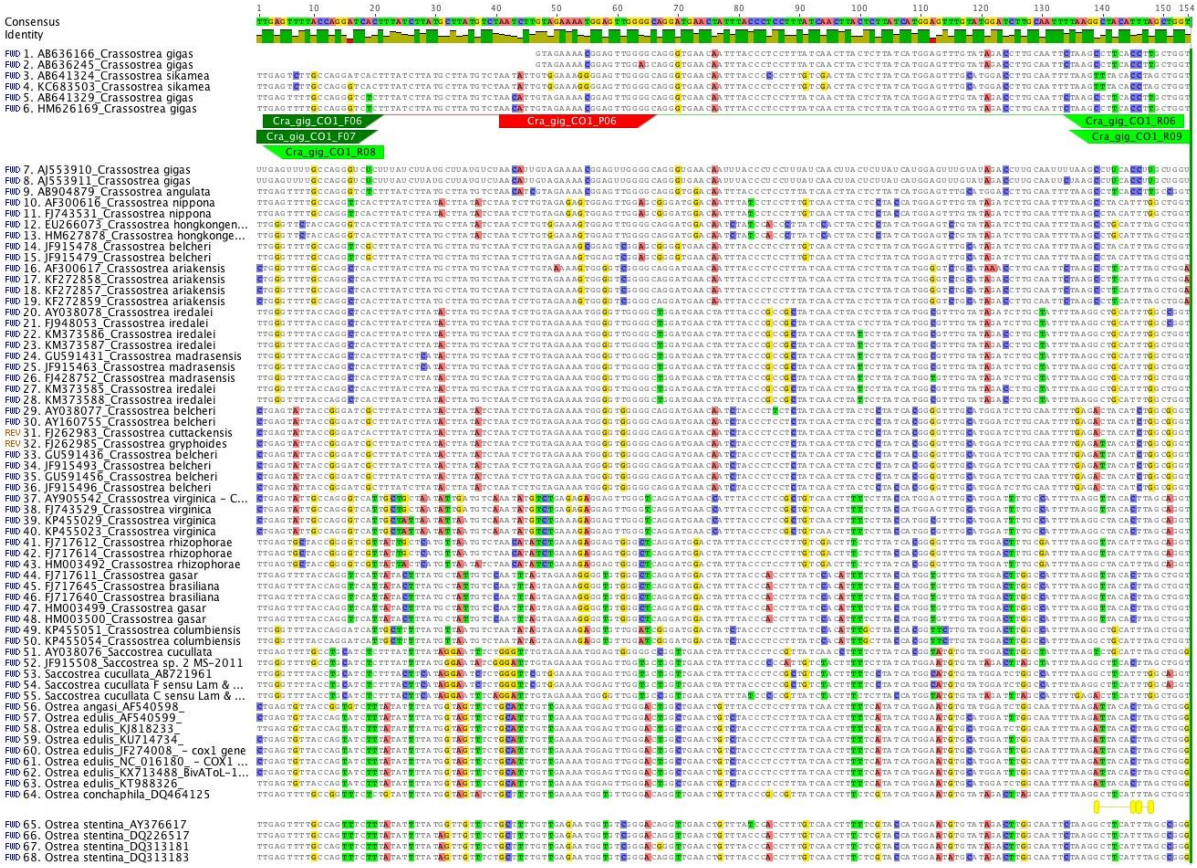

Figure 12.2 *Alignment of* Paralithodes camtschaticus *and other species of the order Decapoda for the mtDNA-cytochrome oxidase 1 gene. All sequences were acquired from NCBI GenBank.*

Conditions of the specificity test

DNA from reference tissue samples were either supplied from external sources (Table 12.2) or extracted from tissue samples using the DNeasy Blood and tissue kit (Qiagen) according to manufacturer's specifications. Initial primer specificity, test of annealing temperature and primer concentration was performed in a 25 µL reaction volume comprising forward and reverse primers (ordered through TAG Copenhagen A/S), using 1 µL forward and 1 µL reverse primer (with 10 µM initial concentrations per primer), and 0.1 µL 5U/µL AmpliTaq Gold Polymerase (Thermofisher, Applied Biosystems), 11.6 µL ddH_2O, 2 µL 25 mM $MgCl_2$ and 2 µL template DNA extracted from tissue samples and diluted 1:10 prior to usage. QPCR reactions were run on a Stratagene Mx3005P qPCR Machine (Agilent, Santa Clara, California, United States). Primer probe specificity test was run using 1 µL forward and 1 µL reverse primer (with 10 µM initial concentrations per primer) and 1 µL probe (with 2.5 µM initial concentration) in a 25 µL reaction volume, including 10 µL Applied Biosystems TaqMan Environmental Mastermix 2.0 (Thermo Fisher Scientific, Waltham, Massachusetts, United States), 10 µL ddH_2O and 2 µL 1:10 diluted template DNA from tissue extractions. Target- and non-target species were run in duplicate reactions and two negative controls.

Table 12.2 *List of tested species with information regarding collector and origin.*

Species*	Tissue samples at Zoological Museum of Co-penhagen	Collector	Origin, collection locality
Aequipecten opercularis	Aeqope	Øresundsakvariet, S.W. Knudsen	The Sound outside Helsingør
Arctica islandica	Arcisl	Øresundsakvariet, S.W. Knudsen	The Sound outside Helsingør
Ensis sp	Enssp	Øresundsakvariet, S.W. Knudsen	The Sound outside Helsingør
Modilus modilus	Modmod	Øresundsakvariet, S.W. Knudsen	The Sound outside Helsingør
Mya arenaria	Myaare	M. Krag, NHMD	The Sound outside Helsingør
Mytilus edulis	Mytedu	M. Krag, NHMD	The Sound outside Helsingør
Crassostrea gigas	Cragig	M. Krag, NHMD	The Sound outside Helsingør

** Not necessarily closely related to* Crassostrea gigas*, but these species are all common and species of Bivalvia, and all are commonly encountered in North European seas.*

Assay specificity results

The two replicated of *Crassostrea gigas* amplified at a Cq of 23.78 and 24.72, respectively (Figure 12.3). None of the non-target species amplified with the co1-F02_R05_P02 assay.

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

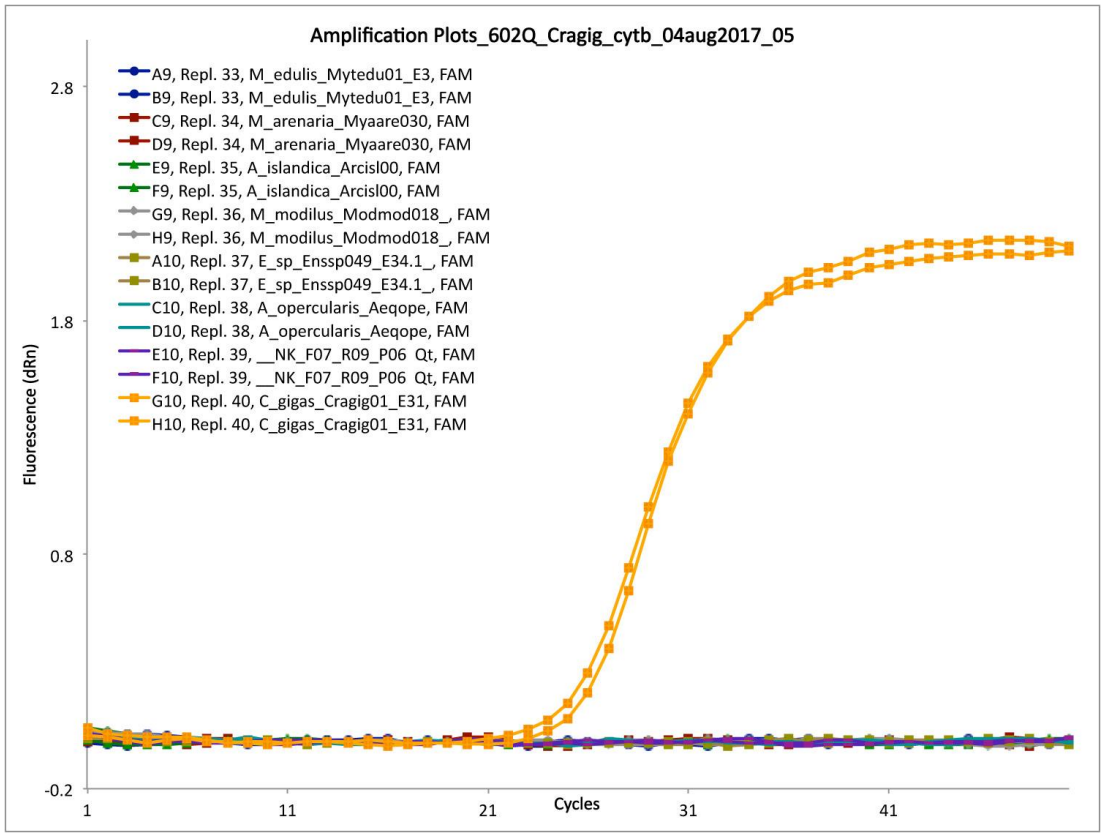

Figure 12.3 *Amplification of* Crassostrea gigas *using the co1-F02_R05_P02 assay. Target species Crassostrea gigas is shown in orange. Non-target species:* Aequipecten opercularis, Arctica islandica, Ensis *sp,* Modilus modilus, Mya arenaria, *and* Mytilus edulis *in: blue, red, green, grey, light brown, respectively. Non-Target Control samples are purple.*

Remarks
The developed co1-F02_R05_P02 assay has (October-2017) not yet been tested *in vitro* on DNA extracted from *Ostrea edulis*, that is commonly found in Northen Europe. However, the *in silico* test shows that with 18 base pair differences in the primers and probes targeting *C. gigas*, will make it impossible for this co1-F02_R05_P02 assay in a qPCR at these settings.

3.13 Species no. 13: *Mya arenaria*

Binomial nomenclature and author: *Mya arenaria* Linneaus, 1758.
English common name: Soft-shell clam.
Danish common name: Almindelig sandmusling.

The genus *Mya* comprise seven extant valid species: *Mya arenaria, M. baxteri, M. eideri, M. neoovata, M. neouddevallensis, M. pseudoarenaria* and *M. truncata*, in the family Myidae. In North-European seas only *Mya arenaria* and *Mya truncata* are found. A representation of species of bivalvia commonly found in Danish seas, were selected for mitochondrial DNA cytochrome b sequences and mitochondrial DNA cytochrome oxidase 1 deposited on NCBI GenBank. Mitochondrial DNA cytochrome oxidase 1 (CO1) showed potential for assay design (Table 13.1). The subsequent tests showed that primers and probes designed for mitochondrial DNA cytochrome oxidase 1 sequences were optimal for distinguishing between *Mya arenaria* and *Mya truncata*:

- Mya_are_CO1_F01: 5-CCCTCCGTTGTCGAGAAATA-3'.
- Mya_are_CO1_R02: 5-ACGCATGTTACCCCAAGTTC-3'.
- Mya_are_CO1_P06: 5-FAM-TATCCCTTCATATTGGAGGGGCTTCAT-BHQ1-3'.

The total length of the product was 133 bp.

Table 13.1 *Species-specific primer/probe assay for* Mya arenaria *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.*

Species	Gene	Size	Temp	Length	GC
Mya arenaria	CO1	133 bp			
Mya_are_CO1_F01	CCCTCCGTTGTCGAGAAATA		60.07	20	50.00
Mya_are_CO1_R02	ACGCATGTTACCCCAAGTTC		59.86	20	50.00
Mya_are_CO1_P06	TATCCCTTCATATTGGAGGGGCTTCAT		68.11	27	44.44
Related species*	**Tested**	**Amplification**			
Mytilus edulis	Yes	No			
Crassostrea gigas	Yes	No			
Arctica islandica	Yes	No			
Modilus modilus	Yes	No			
Ensis sp.	Yes	No			
Aequipecten opercularis	Yes	No			
Mya arenaria	Yes	Yes			

** Not necessarily closely related to the genus* Mya*, but these species are all common species of bivalvia, and all are commonly encountered in North European seas.*

The primers were designed by aligning sequences available from mitochondrial cytochrome oxidase 1 from North European species of *Mya* available from NCBI GenBank. This included: *Mya arenaria*: KX576728, KX576732-KX576733, KX576735-KX576736; *Mya truncata*: KF643403, KF643675, KF643769, KF644116, KF644129, KF644154. 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: *Mya arenaria*, would be amplified by the Mya_are_CO1_F01_R02_P06 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 precise than the Mya_are_CO1_F01_R02_P06: Mya_are_CO1_F03: 5-CATGGCCTTCCCTCGTATAA-3', Mya_are_CO1_F04: 5-CTTTACCCTCCGTTGTCGAG-3', Mya_are_CO1_F09: 5-TGGGGGATGAACACTTTACC-3', Mya_are_CO1_F10: 5-GGTGGGGGATGAACACTTTA-3', Mya_are_CO1_P01: 5-FAM-TGCGGCGGGAGACTTTTTAATTTTATC-BHQ1-3', Mya_are_CO1_P03: 5-FAM-ATGAACACTTTACCCTCCGTTGTCGAG-BHQ1-3', Mya_are_CO1_P04: 5-FAM-AATATAATAGGACACTCTGGTGCGGCG-BHQ1-3', Mya_are_CO1_P05: 5-FAM-TATTTTGTTCAATGTTTAGAGAGGGGG-BHQ1-3', Mya_are_CO1_R01: 5-CGCATGTTACCCCAAGTTCT-3', Mya_are_CO1_R03: 5-CCGCACCAGAGTGTCCTATT-3', Mya_are_CO1_R05: 5-CTCGACAACGGAGGGTAAAG-3'.

Figure 13.1 *Alignment of* Mya arenaria *and other common species of Bivalvia in Danish Seas for the mtDNA-cytochrome oxidase 1 gene. All sequences were acquired from NCBI GenBank.*

Conditions of the specificity test

DNA from reference tissue samples were either supplied from external sources (Table 13.2) or extracted from tissue samples using the DNeasy Blood and tissue kit (Qiagen) according to manufacturer's specifications. Initial primer specificity, test of annealing temperature and primer concentration was performed in a 25 µL reaction volume comprising forward and reverse primers (ordered through TAG Copenhagen A/S), using 1 µL forward and 1 µL reverse primer (with 10 µM initial concentrations per primer), and 0.1 µL 5U/µL AmpliTaq Gold Polymerase (Thermofisher, Applied Biosystems), 11.6 µL ddH_2O, 2 µL 25 mM $MgCl_2$ and 2 µL template DNA extracted from tissue samples and diluted 1:10 prior to usage. QPCR reactions were run on a Stratagene Mx3005P qPCR Machine (Agilent, Santa Clara, California, United States). Primer probe specificity test was run using 1 µL forward and 1 µL reverse primer (with 10 µM initial concentrations per primer) and 1 µL probe (with 2.5 µM initial concentration) in a 25 µL reaction volume, including 10 µL Applied Biosystems TaqMan Environmental Mastermix 2.0 (Thermo Fisher Scientific, Waltham, Massachusetts, United States), 10 µL ddH_2O and 2 µL 1:10 diluted template DNA from tissue extractions. Target- and non-target species were run in duplicate reactions and two negative controls.

Table 13.2 *List of tested species with information regarding collector and origin*.

Species*	Tissue samples at the Zoological Museum of Copenhagen	Collector	Sampling locality
Mytilus edulis	Mytedu	M. Krag, NHMD	The Sound
Crassostrea gigas	Cragig	M. Krag, NHMD	The Sound
Arctica islandica	Arcisl	S.W. Knudsen	The Sound outside Helsingør
Modilus modilus	Modmod	S.W. Knudsen	Sjælland, Denmark
Ensis sp.	Enssp	M. Krag, NHMD	The Sound
Aequipecten opercularis	Aeqope	Øresundsakvariet, S.W. Knudsen	The Sound outside Helsingør
Mya arenaria	Myaare	Øresundsakvariet, S.W. Knudsen	The Sound outside Helsingør

** Not necessarily closely related to the genus* Mya*, but these species are all common species of bivalvia, and all are commonly encountered in North European seas.*

Assay specificity results

The two replicated of *Mya arenaria* amplified at a Cq of 32.45 and 32.57, respectively (Figure 13.2 and 13.3). None of the non-target species amplified with the co1-F01_R02_P06 assay.

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

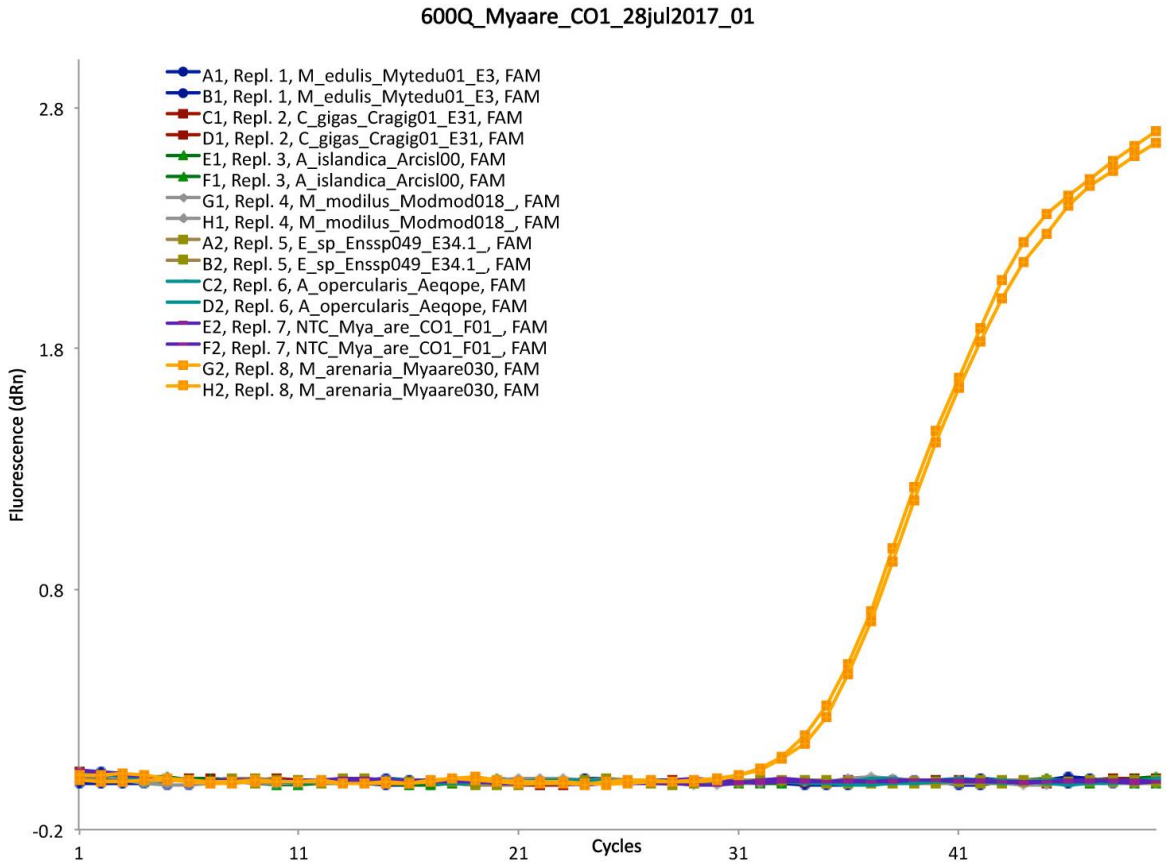

Figure 13.2 *Amplification of* Mya arenaria *using the Mya_are_CO1_F01_R02_P06 assay. Target species* Mya arenaria *is shown in orange. Non-target species:* Mytilus edulis, Crassostrea gigas, Arctica islandica, Modilus modilus, Ensis *sp. and* Aequipecten opercularis*, in: blue, red, green, grey, light brown, turquoise respectively. Non-Target Control samples are purple.*

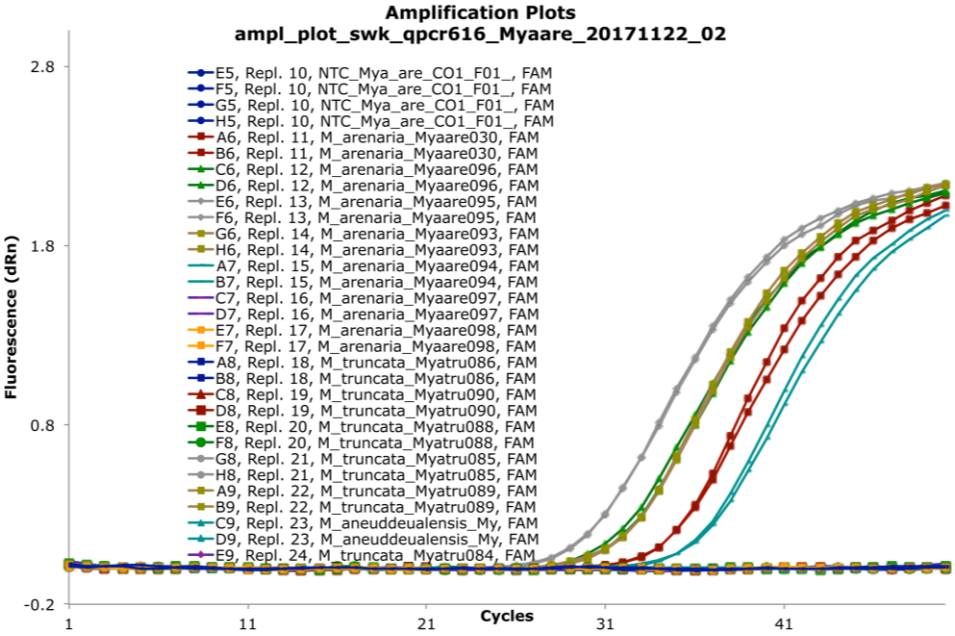

Figure 13.3 *Amplification of* Mya arenaria *using the Mya_are_CO1_F01_R02_P06 assay. With mulitple individuals of* M. arenaria *included and* M. truncata *included. Target species Mya arenaria is shown in grey, green, yellow-green, red and turquise. Non-target species:* Mya arenaris*, in: blue, red, green, grey, light brown, turquoise respectively. Non-Target Control samples are blue.*

3.14 Species no. 14: *Rhithropanopeus harrisii*

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 14.1 Rhithropanopeus harrisii. *Photo by Henrik Carl (Natural History Museum of Denmark*)

The genus *Rhithropanopeus* comprises one valid species, *Rhithropanopeus harrisii*, in the family Panopeidae. *Rhithropanopeus* is non-native in European seas, and no other genera in the family Panopeidae occur natively in North European seas. 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 gene showed potential for assay design (Table 14.1):

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

The total length of the product was 164 bp.

Table 14.1 *Species specific primer/probe assay for* Rhithropanopeus harrisii *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.*

Species	Gene	Size	Temp	Length	GC
Rhithropanopeus harrisii	*mtDNA-CO1*	164 bp			
Rhihar_cytb_F03	GTCAACCTGGTACTCTCATTGGT		63	23	48
Rhihar_cytb_R03	ACGAGGAAATGCTATATCAGGGG		63	23	48
Rhihar_cytb_P03	TGTTGTAGTAACAGCTCACGCCTTTGT		67	27	44
Related species*	**Tested**	**Amplification**			
Cancer pagurus	Yes	No			
Carcinus maenus	Yes	No			
Paralithodes camtschaticus	Yes	No			
Hyas araneus	Yes	No			
Pagurus bernhardus	Yes	No			
Hyas coarctatus	Yes	No			
Rhithropanopeus harrisii	Yes	Yes			

** Not necessarily closely related to the genus* Rhithropanopeus*, but these species are all representatives of the order Decapoda, and all are occurring in Northern Europe.*

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* was 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 (NHMD): *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 precise 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 Forsström & Vasemägi (2016) were also tested in an initial pilot study, but the primers recommended by Forsström & Vasemägi (2016) were found to be unable to distinguish between *Rhithropanopeus* and *Hyas*. Since the genus *Hyas* is widely distributed and common in the Skagerak Sea, Kattegat Sea and North Sea the primers recommended by Forsström & Vasemägi (2016) were found unsuitable for species-specific detection of eDNA from *R. harrisii*.

Conditions of the specificity test

DNA from reference tissue samples were either supplied from external sources (Table 14.2) or extracted from tissue samples using the DNeasy Blood and tissue kit (Qiagen) according to manufacturer's specifications, and obtained through previous work (Aagaard, 2015). Initial primer specificity, test of annealing temperature and primer concentration was performed in a 25 µL reaction volume comprising forward and reverse primers (ordered through TAG Copenhagen A/S), using 1 µL forward and 1 µL reverse primer (with 10 µM initial concentrations per primer), and 0.1 µL 5U/µL AmpliTaq Gold Polymerase (Thermofisher, Applied Biosystems), 11.6 µL ddH_2O, 2 µL 25 mM $MgCl_2$ and 2 µL template DNA extracted from tissue samples and diluted 1:10 prior to usage. QPCR reactions were run on a Stratagene Mx3005P qPCR Machine (Agilent, Santa Clara, California, United States). Primer probe specificity test was run using 1 µL forward and 1 µL reverse primer (with 10 µM initial concentrations per primer) and 1 µL probe (with 2.5 µM initial concentration) in a 25 µL reaction volume, including 10 uL 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. Target- and non-target species were run in duplicate reactions and two negative controls.

Table 14.2 *List of tested species with information regarding collector and origin*

Species*	Tissue sample at zoological museum of Copenhagen	Collector	Sampling location
Cancer pagurus	Canpag	Øresundsakvariet, S.W. Knudsen	The Sound outside Helsingør
Carcinus maenus	Carmae	Øresundsakvariet, S.W. Knudsen	The Sound outside Helsingør
Paralithodes camtschaticus	Parcam	Hjelset, A.M., Havforskningsinstitutet, Tromsø, Norway	North Atlantic Sea, off the coast of Norway
Hyas araneus	Hyaara	Øresundsakvariet, S.W. Knudsen	The Sound outside Helsingør
Pagurus bernhardus	Pagber	Øresundsakvariet, S.W. Knudsen	The Sound outside Helsingør
Hyas coarctatus	Hyacoa	Øresundsakvariet, S.W. Knudsen	The Sound outside Helsingør
Rhithropanopeus harrisii	Rhihar	A.B Aagaard, S.W. Knudsen, The Natural History Museum of Denmark	Køge Bugt, Denmark

** Not necessarily closely related to* Rhithropanopeus harrisii*, but these species are all common species of the order Decapoda, and all are commonly encountered in North European seas. The invasive species Paralithodes camtschaticus was also included as reference.*

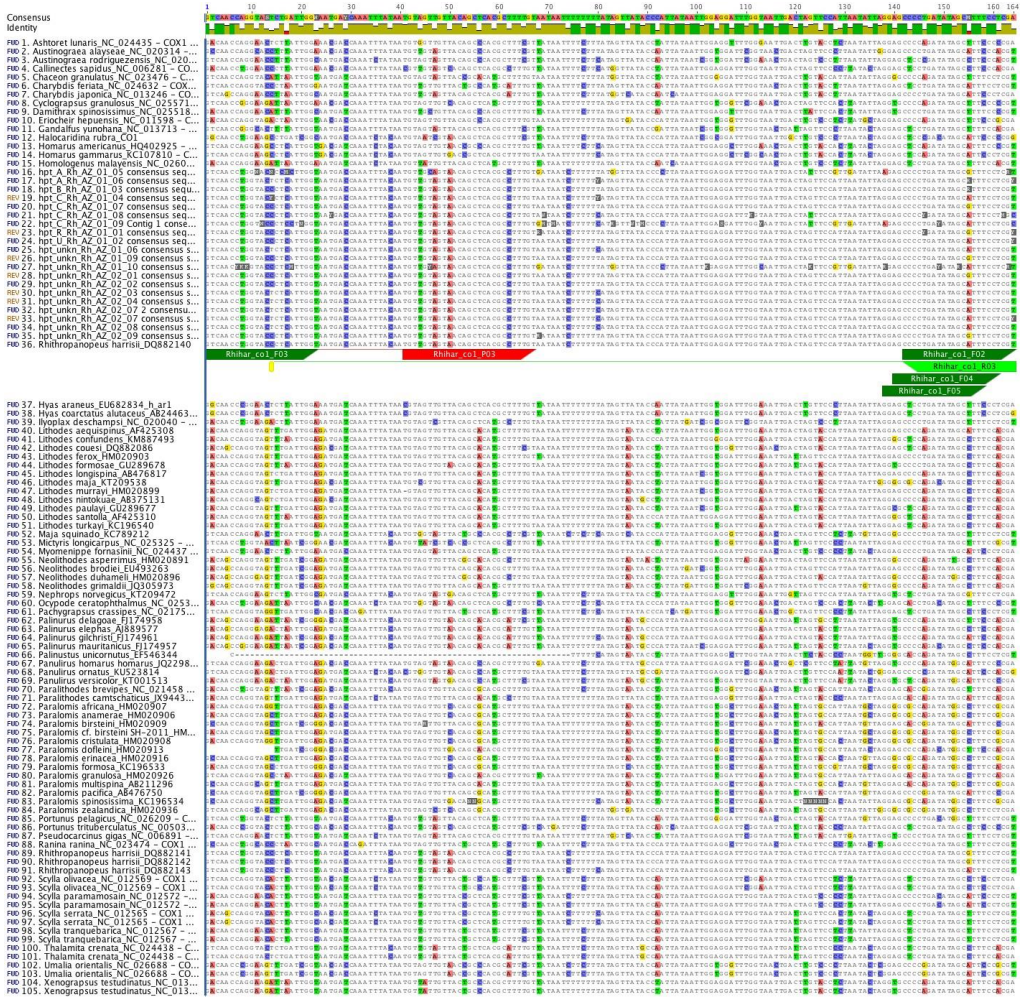

Figure 14.2 *Alignment of* Rhithropanopeus harrisii *and other species of the order Decapoda occurring in North European seas for the mtDNA-cytochrome oxidase 1 gene. Sequences were acquired from NCBI GenBank, and from a bachelor project (Aagaard, 2015) focusing on mitochondrial population genetic variation among* R. harrisii *in Øresund in Denmark.*

Assay specificity results
The two replicated of *Rhithropanopeus harrisii* amplified at a Cq of 26.80 and 27.42, respectively (Figure 14.3). None of the non-target species amplified with the F03R03P03 assay.

The designed eDNA target assay for *Rhithropanopeus harrisii* is expected to only amplify the target species when tested on laboratory or environmental water samples.

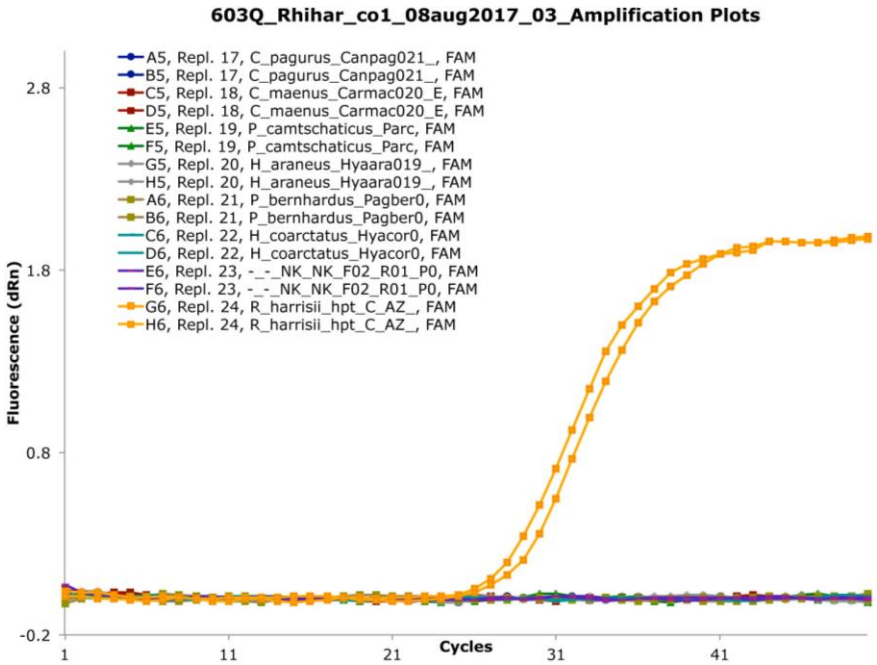

Figure 14.3 *Amplification of* Rhithropanopeus harrisii *using the F03R03P03 assay targeting mtDNA-cytochrome oxidase 1. Target species* Rhithropanopeus harrisii *is shown in orange and non-target species:* Cancer pagurus, Carcinus maenus, Paralithodes camtschaticus, Hyas araneus, Pagurus bernhardus, Hyas coarctatus*, in: blue, red, green, grey, light brown, and turquoise, respectively. Non-Target Control samples are purple.*

3.15 Species no. 15: *Paralithodes camtschaticus*

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

The genus *Paralithodes* comprises five extant valid species, *Paralithodes brevipes; P. californiensis; P. camtschaticus; P. platypus and P. rathbuni* in the family Lithodidae. 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 seas 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 (Table 15.1). The subsequent tests showed that primers and probes designed for mitochondrial DNA cytochrome oxidase 1 sequences were optimal for distinguishing between *Homarus americanus* and *H. gammarus* and *Nephrops norvegicus* (Norway lobster):

- Parcam_co1_F02: 5'-GGGCTTGAGCTGGAATAGTG-3'.
- Parcam_co1_R05: 5'-CAATTTCCAAACCCTCCAAT-3'.
- Parcam_co1_P02: 5'-FAM-ATTCGAGCTGAACTAGGACAACCAGGT-BHQ-1-3'.

The total length of the product was 175 bp.

Table 15.1 *Species specific primer/probe assay for* Paralithodes camtschaticus *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.*

Species	**Gene**	**Size**	**Temp**	**Length**	**GC**
Paralithodes camtschaticus	*mtDNA-CO1*	175 bp			
Parcam_co1_F02	GGGCTTGAGCTGGAATAGTG		59.8	20	55
Parcam_co1_R05	CAATTTCCAAACCCTCCAAT		58.7	20	40
Parcam_co1_P02	ATTCGAGCTGAACTAGGACAACCAGGT		67.2	27	48
Related species*	**Tested**	**Amplification**			
Nephrops norvegicus	Yes	No			
Paralomis sp	Yes	No			
Cancer pagurus	Yes	No			
Carcinus maenus	Yes	No			
Paralomis spectabilis	Yes	No			
Lithodes maja	Yes	No			
Paralithodes camtschaticus	Yes	Yes			

** Not necessarily closely related to the genus* 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.*

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 precise than the co1- F02_R05_P02 system: Parcam_co1_F07: 5'-AGGAGCATCAGTGGATTTAGGT-3', Parcam_co1_F12: 5'-CGTCCACAAGGAATAACCTTAGAC-3', Parcam_co1_P12: 5'-FAM-TTTGTGTGATCCGTATTTATTACTGCAA-BHQ-1-3', Parcam_co1_R02: 5'-GTCAATTTCCAAACCCTCCA-3', Parcam_co1_R03: 5'-TCAATTTCCAAACCCTCCAA-3', Parcam_co1_R06: 5'-TCAATTTCCAAACCCTCCAAT-3', Parcam_co1_R07: 5'-CGGTCTAAGGTTATTCCTTGTGG-3', Parcam_co1_R09: 5'-AAGGTTATTCCTTGTGGACGTA-3', Parcam_co1_R10: 5'-ACGGTCTAAGGTTATTCCTTGTG-3', Parcam_co1_R11: 5'-GGTCTAAGGTTATTCCTTGTGGA-3', Parcam_co1_R12: 5'-AACTGGGTCTCCTCCTCCTG-3', Parcam_co1_R14: 5'-AAAACTGGGTCTCCTCCTCCT-3', Parcam_co1_R16: 5'-CTCCTCCTGCAGGGTCAA-3'.

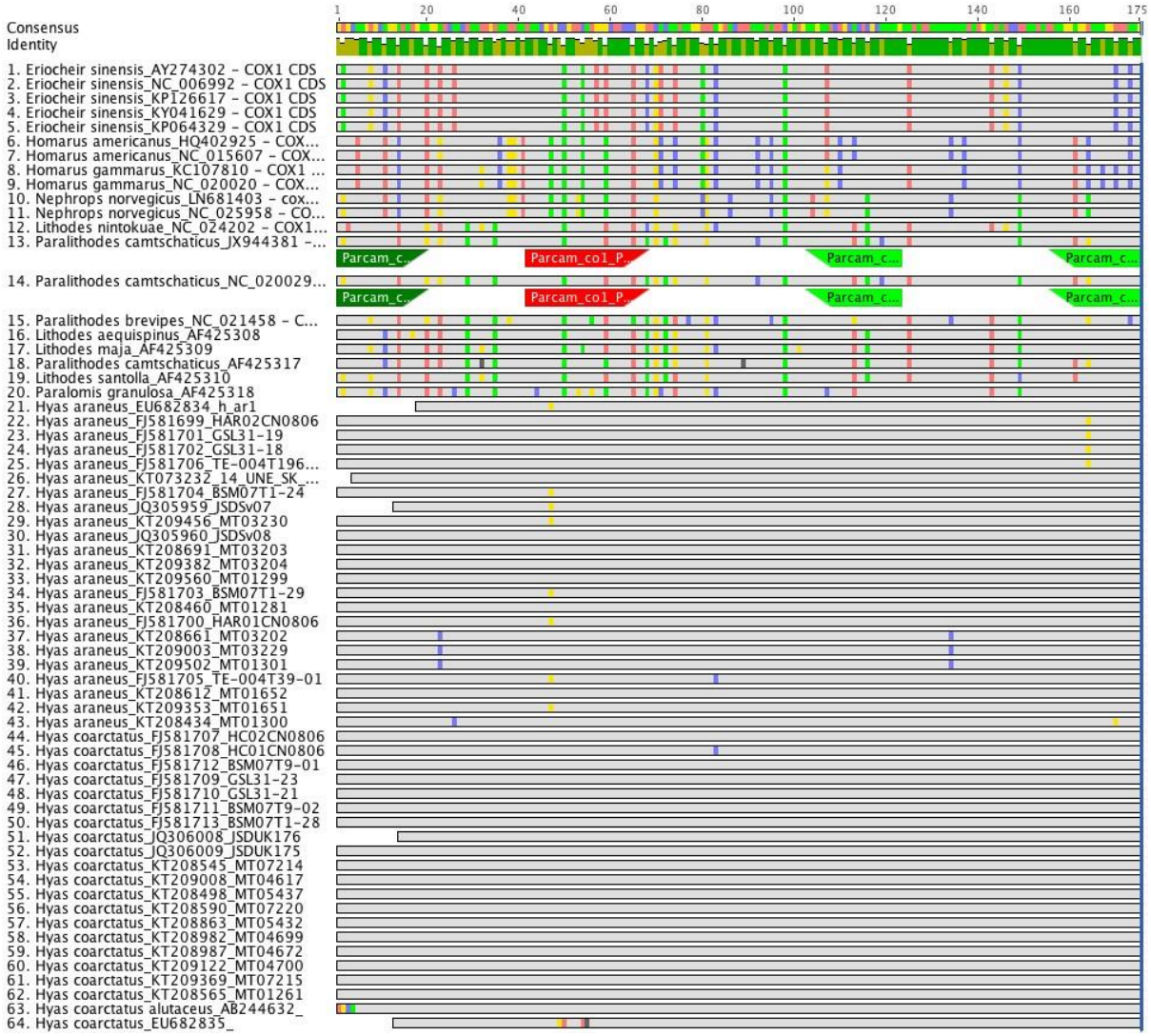

Figure 15.1 *Alignment of* Paralithodes camtschaticus *and other species of the order Decapoda for the mtDNA-cytochrome oxidase 1 gene. All sequences were acquired from NCBI GenBank.*

Conditions of the specificity test

DNA from reference tissue samples were either supplied from external sources (Table 15.2) or extracted from tissue samples using the DNeasy Blood and tissue kit (Qiagen) according to manufacturer's specifications. Initial primer specificity, test of annealing temperature and primer concentration was performed in a 25 µL reaction volume comprising forward and reverse primers (ordered through TAG Copenhagen A/S), using 1 µL forward and 1 µL reverse primer (with 10 µM initial concentrations per primer), and 0.1 µL 5U/µL AmpliTaq Gold Polymerase (Thermofisher, Applied Biosystems), 11.6 µL ddH_2O, 2 µL 25 mM $MgCl_2$ and 2 µL template DNA extracted from tissue samples and diluted 1:10 prior to usage. QPCR reactions were run on a Stratagene Mx3005P qPCR Machine (Agilent, Santa Clara, California, United States). Primer probe specificity test was run using 1 µL forward and 1 µL reverse primer (with 10 µM initial concentrations per primer) and 1 µL probe (with 2.5 µM initial concentration) in a 25 µL reaction volume, including 10 µL Applied Biosystems TaqMan Environmental Mastermix 2.0 (Thermo Fisher Scientific, Waltham, Massachusetts, United States), 10 µL ddH_2O and 2 µL 1:10 diluted template DNA from tissue extractions. Target- and non-target species were run in duplicate reactions and two negative controls.

Table 15.2 *List of tested species with information regarding collector and origin*.

Species*	Tissue sample at Zoological mueum of Copenhagen	Collector	Sampling locality
Nephrops norvegicus	Nepnor	S.W. Knudsen	North Sea, NHMD
Paralomis sp	Parsp	Øresundsakvariet, S.W. Knudsen	The Sound outside Helsingør
Cancer pagurus	Canpag	Øresundsakvariet, S.W. Knudsen	The Sound outside Helsingør
Carcinus maenus	Carmae	Øresundsakvariet, S.W. Knudsen	The Sound outside Helsingør
Paralomis spectabilis	Parspe	S.W. Knudsen	North Sea, NHMD
Lithodes maja	Litmaj	NHMD	NHMD
Paralithodes camtschaticus	Parcam	Hjelset, A.M., Havforskningsinstitutet, Tromsø, Norway	North Atlantic Sea, off the coast of Norway

** Not necessarily closely related to* Paralithodes camtschaticus*, but these species are all common and species of the order Decapoda, and all are commonly encountered in North European seas.*

Assay specificity results

The two replicated of *Paralithodes camtschaticus* amplified at a Cq of 21.96 and 21.97, respectively (Figure 15.2). None of the non-target species amplified with the co1-F02_R05_P02 assay.

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

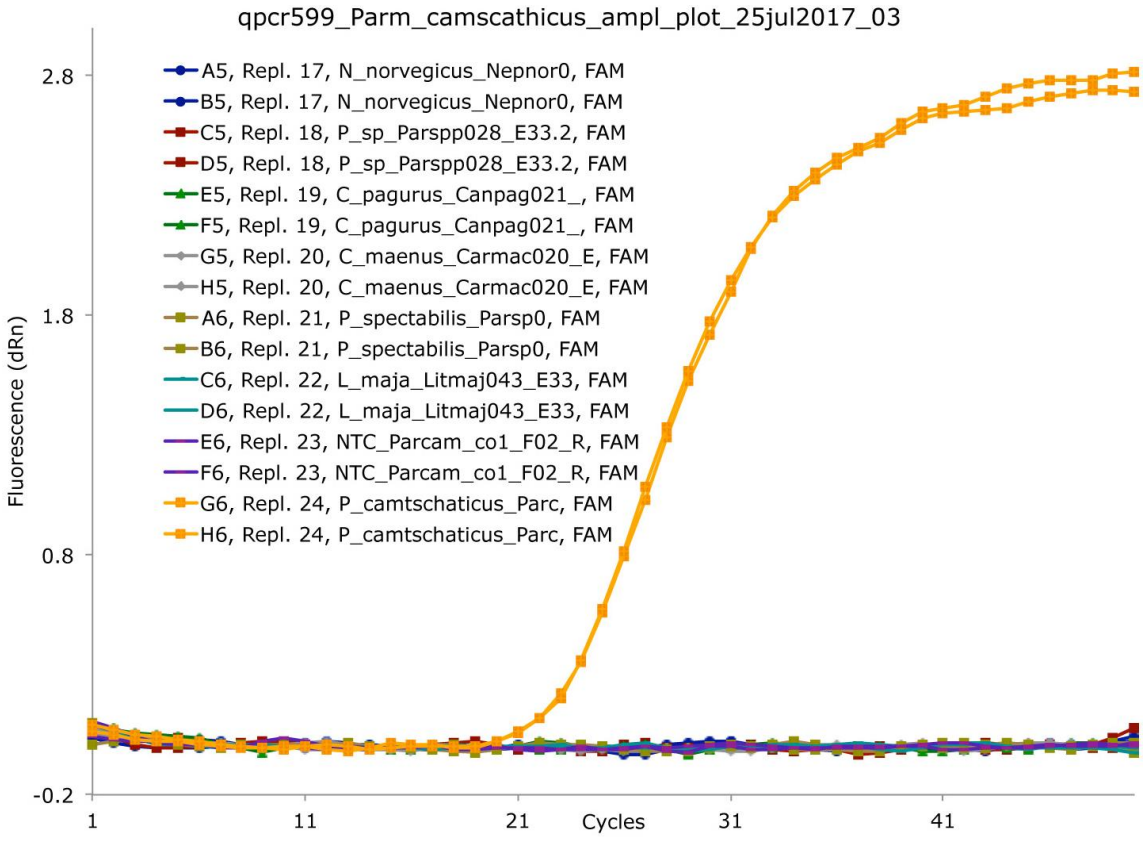

Figure 15.2 *Amplification of* Paralithodes camtschaticus *using the co1-F02_R05_P02 assay. Target species* Paralithodes camtschaticus *is shown in orange. Non-target species:* Nephrops norvegicus, Paralomis *sp.,* Cancer pagurus, Carcinus maenus, Paralomis spectabilis*, and* Lithodes maja*, in: blue, red, green, grey, light brown, respectively. Non-Target Control samples are purple.*

3.16 Species no. 16: *Eriocheir sinensis*

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

Figure 16.1 Eriocheir sinensis*. Photo by Henrik Carl (Natural History Museum of Denmark*)

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. Among the sequences for *E. sinensis* deposited in NCBI GenBank the mtDNA-cytochrome b gene showed potential for assay design (Table 16.1):

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

The total length of the product was 114 bp.

Table 16.1 *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*

Species	**Gene**	**Size**	**Temp**	**Length**	**GC**
Eriocheir sinensis	*mtDNA-Cytb*	114 bp			
Erisin_cytb_F02	ACCCCTCCTCATATCCAACCA		62.7	21	52
Erisin_cytb_R02	AAGAATGGCCACTGAAGCGG		64.7	20	55
Erisin_cytb_P02	TTTGCTTACGCTATTTTACGATCAATTCCT		66.3	30	33
Related species*	**Tested**	**Amplification**			
Cancer pagurus	Yes	No			
Carcinus maenus	Yes	No			
Hyas araneus	Yes	No			
Hyas coarctatus	Yes	No			
Pagurus bernhardus	Yes	No			
Rhithropanopeus harrisii	Yes	No			
Eriocheir sinensis	Yes	Yes			

** Not necessarily closely related to the genus* Eriocheir*, 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*.*

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 precise 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'.

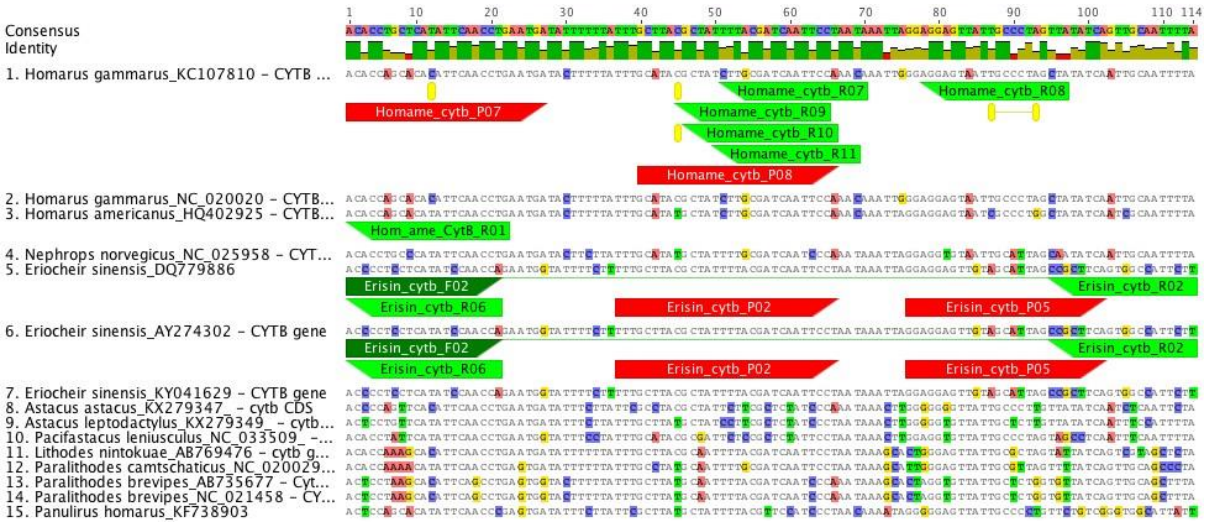

Figure 16.2 *Alignment of* Eriocheir sinensis *and other native species of the order Decapoda occurring in North European seas for the mtDNA-cytochrome b gene. All sequences were acquired from NCBI GenBank.*

Conditions of the specificity test

DNA from reference tissue samples were either supplied from external sources (Table 16.2) or extracted from tissue samples using the DNeasy Blood and tissue kit (Qiagen) according to manufacturer's specifications. Initial primer specificity, test of annealing temperature and primer concentration was performed in a 25 µL reaction volume comprising forward and reverse primers (ordered through TAG Copenhagen A/S), using 1 µL forward and 1 µL reverse primer (with 10 µM initial concentrations per primer), and 0.1 µL 5U/µL AmpliTaq Gold Polymerase (Thermofisher, Applied Biosystems), 11.6 µL ddH_2O, 2 µL 25 mM $MgCl_2$ and 2 µL template DNA extracted from tissue samples and

diluted 1:10 prior to usage. QPCR reactions were run on a Stratagene Mx3005P qPCR Machine (Agilent, Santa Clara, California, United States). Primer probe specificity test was run using 1 µL forward and 1 µL reverse primer (with 10 µM initial concentrations per primer) and 1 uL 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. Target- and non-target species were run in duplicate reactions and two negative controls.

Table 16.2. *List of tested species with information regarding collector and origin*.

Species*	Tissue sample at Zoological Museum of Copenhagen	Collector	Sampling locality
Cancer pagurus	Canpag	Øresundsakvariet, S.W. Knudsen	The Sound outside Helsingør
Carcinus maenus	Carmae	Øresundsakvariet, S.W. Knudsen	The Sound outside Helsingør
Hyas araneus	Hyaara	Øresundsakvariet, S.W. Knudsen	The Sound outside Helsingør
Hyas coarctatus	Hyacoa	Øresundsakvariet, S.W. Knudsen	The Sound outside Helsingør
Pagurus bernhardus	Pagber	Øresundsakvariet, S.W. Knudsen	The Sound outside Helsingør
Rhithropanopeus harrisii	Rhihar	A.B Aagaard, S.W. Knudsen, Natural History Museum of Denmark	Køge Bugt, Denmark
Eriocheir sinensis	Erisin	Natural History Museum of Denmark	Denmark

** Not necessarily closely related to* Eriocheir sinensis*, but these species are all common and species of the order Decapoda, and all are commonly encountered in North European seas. The invasive species* Rhithropanopeus harrisii *was also included as reference.*

Assay specificity results

The two replicated of *E. sinensis* amplified at a Cq of 24.46 and 24.76, respectively (Figure 16.3). None of the non-target species amplified with the F02_R02_P02 assay.

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

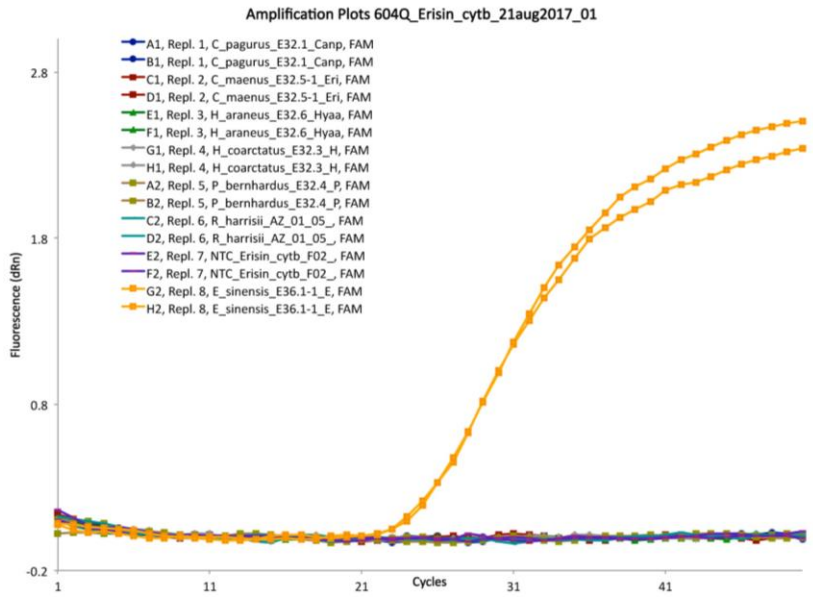

Figure 16.3. *Amplification of* Eriocheir sinensis *using the F02_R02_P02 assay. Target species* Eriocheir sinensis *is shown in orange and non-target species:* Cancer pagurus, Carcinus maenus, Hyas araneus, Hyas coarctatus, Pagurus bernhardus, Rhithropanopeus harrisii*, in: blue, red, green, grey, light brown, and turquoise, respectively. Non-Target Control samples are purple.*

3.17 Species no. 17: *Homarus americanus*

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

The genus *Homarus* comprise 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 Northeast Atlantic is only inhabited by *Homarus*, *Nephrops*, *Nephropsis* and *Thymopides*, where the latter two are considered deep-sea species >500 m depth, and rare. In the order Decapoda a broad representation of species occurring in Danish seas were selected for mitochondrial DNA cytochrome b sequences and mitochondrial DNA cytochrome oxidase 1 deposited on NCBI GenBank, showed potential for assay design (Table 17.1). The subsequent tests showed that primers and probes designed for mitochondrial DNA cytochrome oxidase 1 sequences were optimal for distinguishing between *Homarus americanus* and *H. gammarus* and *Nephrops norvegicus*:

- Homame_co1_F06: 5'-TTACAGCAGTTCTTTTACTACTCTCG-3'.
- Homame_co1_R08: 5'-ACTGGGTCTCCACCTCCAG-3'.
- Homame_co1_P08: 5'-FAM-TCGAAATTTAAATACTTCATTCTTCGATCCA-BHQ-1-3'.

The total length of the product was 118 bp.

Table 17.1 *Species specific primer/probe assay for* Homarus americanus *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.*

Species	Gene	Size	Temp	Length	GC
Homarus americanus	*mtDNA-CO1*	118 bp			
Homame_co1_F06	TTACAGCAGTTCTTTTACTACTCTCG		57.8	26	38
Homame_co1_R08	ACTGGGTCTCCACCTCCAG		60.1	19	63
Homame_co1_P08	TCGAAATTTAAATACTTCATTCTTCGATCCA		66.2	31	29
Related species*	**Tested**	**Amplification**			
Homarus gammarus	Yes	No			
Nephrops norvegicus	Yes	No			
Paralomis sp.	Yes	No			
Astacus leptodactylus	Yes	No			
Pacifastacus leniusculus	Yes	No			
Homarus americanus	Yes	Yes			

** Not necessarily closely related to the genus* Homarus*, but these species are all species of the order Decapoda, and evolutionary closely related to the family Nephropidae, and all are commonly encountered in North European seas.*

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; *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. 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 precise 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'.

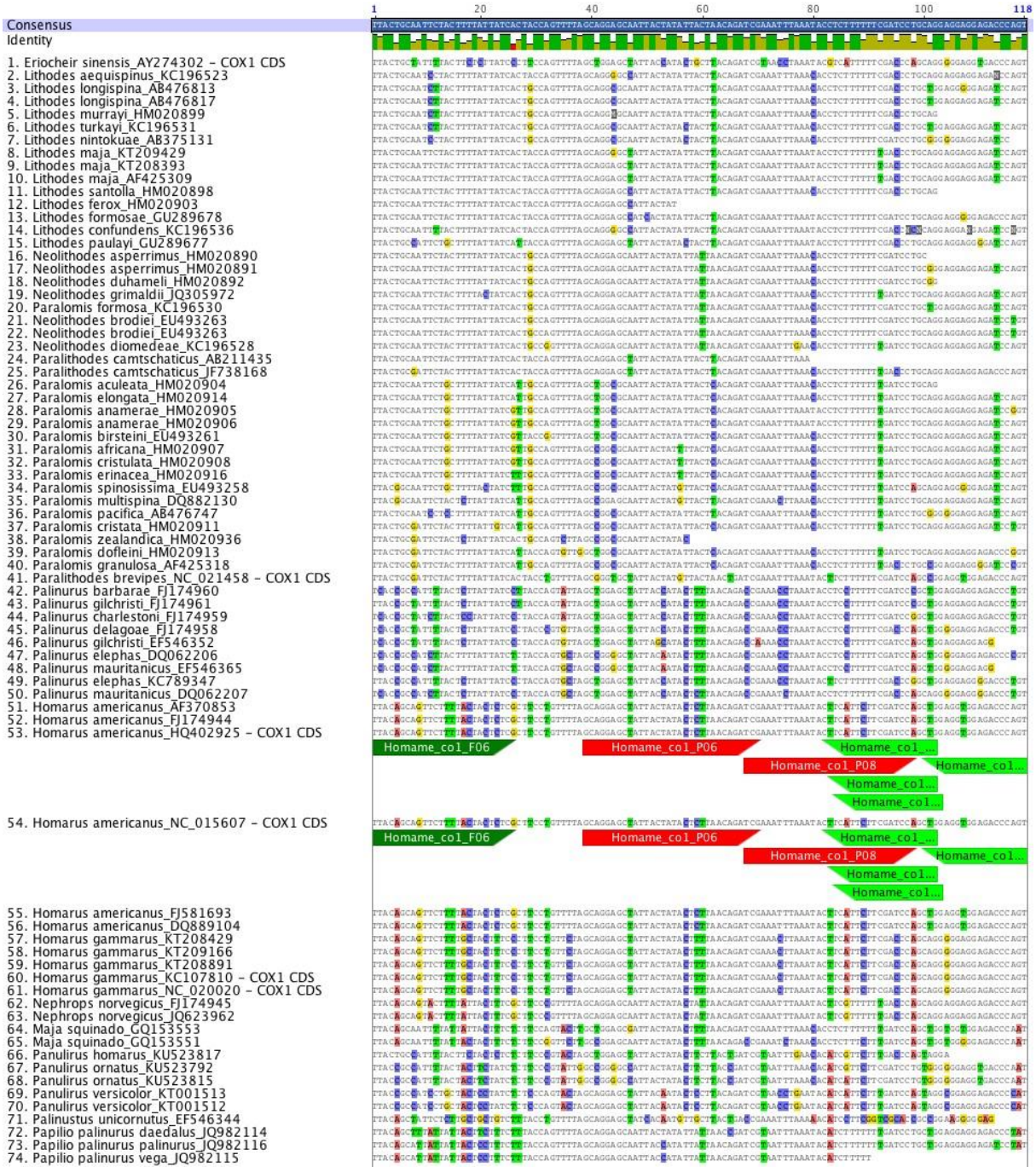

Figure 17.1 *Alignment of* Homarus americanus *and other species of the order Decapoda for the mtDNA-cytochrome oxidase 1 gene. All sequences were acquired from NCBI GenBank.*

Conditions of the specificity test

DNA from reference tissue samples were either supplied from external sources (Table 17.2) or extracted from tissue samples using the DNeasy Blood and tissue kit (Qiagen) according to manufacturer's specifications. Initial primer specificity, test of annealing temperature and primer concentration was performed in a 25 µL reaction volume comprising forward and reverse primers (ordered through TAG Copenhagen A/S), using 1 µL forward and 1 µL reverse primer (with 10 µM initial concentrations per primer), and 0.1 µL 5U/µL AmpliTaq Gold Polymerase (Thermofisher, Applied Biosystems), 11.6 µL ddH_2O, 2 µL 25 mM $MgCl_2$ and 2 µL template DNA extracted from tissue samples and

diluted 1:10 prior to usage. QPCR reactions were run on a Stratagene Mx3005P qPCR Machine (Agilent, Santa Clara, California, United States). Primer probe specificity test was run using 1 µL forward and 1 µL reverse primer (with 10 µM initial concentrations per primer) and 1 µL probe (with 2.5 µM initial concentration) in a 25 µL reaction volume, including 10 µL Applied Biosystems TaqMan Environmental Mastermix 2.0 (Thermo Fisher Scientific, Waltham, Massachusetts, United States), 10 µL ddH_2O and 2 µL 1:10 diluted template DNA from tissue extractions. Target- and non-target species were run in duplicate reactions and two negative controls.

Table 17.2 *List of tested species with information regarding collector and origin.*

Species*	Tissue sample at Zoological Museum of Copenhagen	Collector	Sampling locality
Homarus gammarus	Homgam	Øresundsakvariet, S.W. Knudsen	The Sound outside Helsingør
Nephrops norvegicus	Nepnor	S.W. Knudsen	North Sea, NHMD
Paralomis sp	Parsp	Øresundsakvariet, S.W. Knudsen	The Sound outside Helsingør
Astacus leptodactylus	Astlep	S. Agersnap; W.B. Larsen, NHMD	Sjælland, Denmark
Pacifastacus leniusculus	Paclen	S. Agersnap; W.B. Larsen, NHMD	Sjælland, Denmark
Homarus americanus	Homame	"Danmarks akvarium, Den Blå Planet", S.W. Knudsen	"Danmarks akvarium, Den Blå Planet"

** Not necessarily closely related to* Homarus americanus*, but these species are all common and species of the order Decapoda, and all are commonly encountered in North European seas.*

Assay specificity results

The two replicates of the two samples from *Homarus americanus* amplified at a Cq of 21.90, 22.00, 23.07 and 23.25, respectively (Figure 17.2). None of the non-target species amplified with the co1-F06_R08_P08 assay.

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

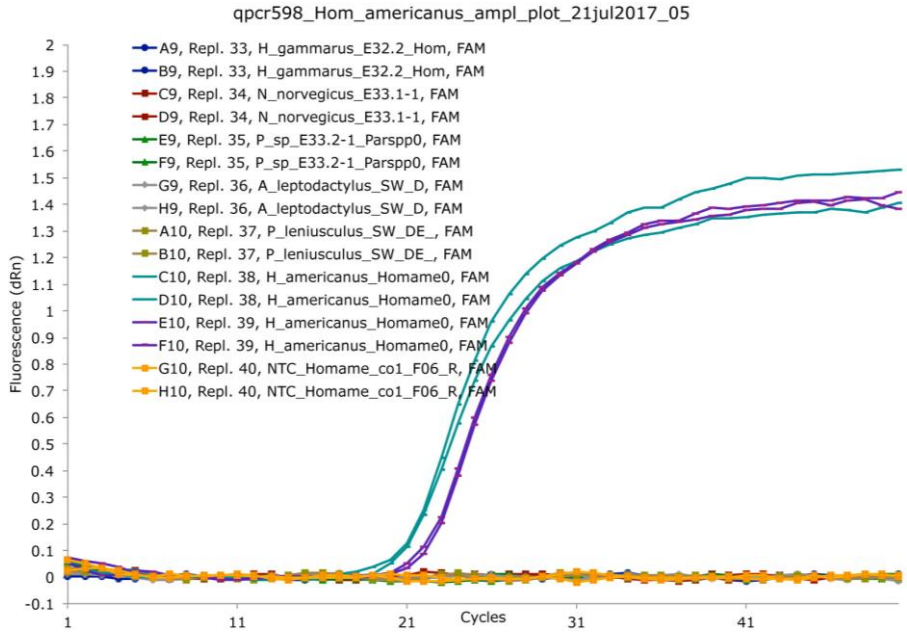

Figure 17.2 *Amplification of* Homarus americanus *using the co1-F06_R08_P08 assay. Target species* Homarus americanus *is shown in turquoise, and purple. Non-target species:* Homarus gammarus, Nephrops norvegicus, Paralomis *sp*., Astacus leptodactylus, Pacifastacus leniusculus*, in: blue, red, green, grey, light brown, respectively. Non-Target Control samples are orange.*

3.18 Species no. 18: *Cordylophora caspia*

Binomial nomenclature and author: *Cordylophora caspia* Pallas, 1771.
English common name: Freshwater hydroid.
Danish common name: Brakvands-køllepolyp.

Figure 18.1 Cordylophora caspia. *Photo by Magnus W. Jacobsen (DTU Aqua, Technical University of Denmark).*

The genus *Cordylophora* comprises three valid species of colonial hydroids: *Cordylophora caspia* (Pallas, 1771), *Cordylophora japonica* Itô, 1951 and *Cordylophora solangiae* Redier, 1967 (WoRMS Editorial Board 2015). Two of the species are strictly marine (*Cordylophora japonica* and *C. solangiae*) and native to the Pacific, while *C. caspia* is native to brackish and freshwater habitats in the Black Sea and the Caspian Sea. *Cordylophora* belongs to the family Cordylophoridae, which only contains this one genus. Two assays were originally developed for the present study using available sequence data on NCBI GenBank. Here only nucleotide sequence data for *Cordylophora caspia* is available while no data is published for the two sister-species of *C. japonica* and *C. solangia*e. Hence, the primer and probes were chosen based on comparisons between sequences from other representatives of the family Oceanidae. Without sequence information or DNA samples available from *C. japonica* and *C. solangia*e it has not been possible to determine whether the recommended primer-probe system for detection of eDNA from *Cordylophora caspia* will be able to differentiate between *C. japonica* and *C. solangia*e and *Cordylophora caspia*.

Table 18.1 *Species-specific primer/probe assays for* Cordylophora caspia *with target gene, product size, calculated melting temperature, primer/probe length and GC percentage.*

Primer/Probe	Gene	PS (bp)	Sequence	Temp (°C)	Length (bp)	GC%
Cor_cas_COI_F01	COI	76	5'- TCATCTGTACAAGCACATTCTGG -3'	60.9	23	43.5
Cor_cas_COI_R01	COI	76	5'- TTGAAGAAGCTCCTGCACAGT -3'	59.5	21	47.6
Cor_cas_COI_P01	COI	76	5'-FAM-CCTTCTGTAGACATGGCTATATTTAGTC-BHQ-1-3'	65.6	28	39.3
Cor_cas_28S_F02	28S	70	5'- ACTGGACAGAGGAGGAGTCG -3'	62.5	20	60.0
Cor_cas_28S_R02	28S	70	5'- CGACGACCAACAGTGACAAG -3'	60.5	20	55.0
Cor_cas_28S_P02	28S	70	5'-FAM-ACATGCTCTTTTGGGCTGGCCTCT-BHQ-1-3'	66.9	24	54.0

The primers and probe (Table 18.1) were designed by aligning sequences available from the mitochondrial cytochrome oxidase 1 (COI) gene and from nuclear 28S ribosomal RNA (rRNA28S) gene from *cordylophora* and distantly related *Turritopsis* species available from NCBI GenBank. The COI alignment was prepared using the following sequences from NCBI GenBank: *Cordylophora caspia*: KC489509; *Cordylophora* sp.; EF540792-EF54793; *Turritopsis lata*: JX965908; *Turritopsis nutricula*:

JQ716084; *Turritopsis rubra*: EF540792. The rRNA28S alignment was prepared using the following sequences from NCBI GenBank: *Cordylophora caspia*: EU272556; *Turritopsis lata*: KF962372; *Turritopsis rubra*: EF547133 and *Turritopsis dohrnii*: EU272596. Sequence alignment was performed using Geneious v. R7 (Kearse et al. 2012) and MAFFT 6.822 (Katoh & Toh, 2010) and primers matched against target-species sequence with Primer3 v2.3.4 (Untergasser et al., 2012), as implemented in Geneious. The sequence alignment indicated that only the target-species: *Cordylophora caspia*, would be amplified by the primer systems in a qPCR setup (Figure 19.2, Table 19.2 and 19.3).

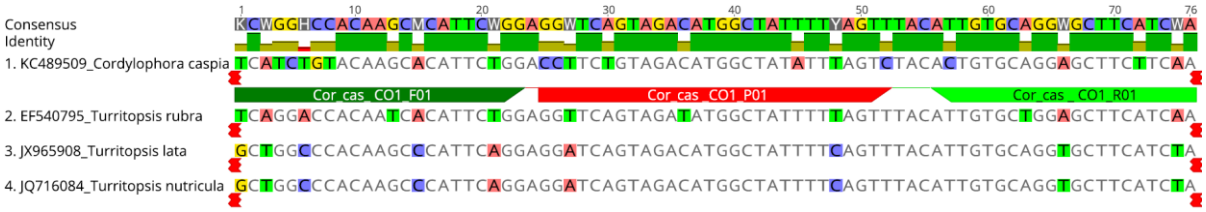

Figure 18.2 *Alignment of COI gene sequences with the developed primers and probe. Original sequences were acquired from NCBI GenBank (see text).*

Table 18.2 *Overview of primer and probe mismatches for COI gene between target species and distantly related species of* Turritopsis*. In all cases there is low sequence similarity.*

Potential non-target species	Forward	Reverse	Probe
Cordylophora caspia	0	0	0
Turritopsis lata	9	4	7
Turritopsis nutricula	9	4	7
Turritopsis rubra	6	3	6

Table 18.3 *Overview of primer and probe mismatches for 28S gene between target species and distantly related species of* Turritopsis*. In all cases there is low sequence similarity.*

Potential non-target species	Forward	Reverse	Probe
Cordylophora caspia	0	0	0
Turritopsis dohrnii	9	9	5
Turritopsis lata	9	9	5
Turritopsis rubra	9	9	4

DNA from a reference tissue sample was extracted from archived tissue preserved in 96% alcohol (collected at Lake Havasu, AZ, USA by biologist Sherri Pucherelli, Bureau of Reclamation, Technical Service Center, Hydraulic Investigations and Lab Services (86-68560)) using the DNEasy Blood & Tissue Extraction Kit (Qiagen Inc., Valencia, CA USA) according to the manufacturer's recommendations. Initially the performance of the two different primer sets was tested (Table 19.1). Evaluation of primers was performed in a 10 µL reaction volume with 500 nM forward and reverse primers (Integrated DNA Technologies, U.S.A.) and 1 µl template DNA at a concentration of 16.9 ng/µL. A generic endpoint thermocycling programme was used: denaturing at 95°C for 2 minutes, followed by 35 cycles of denaturing at 95°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 30 s, followed by final extension at 72°C for 5 minutes. Following PCR amplification, amplicons were visualized following electrophoresis on a 2% agarose gel stained with ethidium bromide and visualized under a UV light, and results documented by photography. Both primer sets generated the correct size PCR product, which supported specific amplification. However, the mitochondrial COI primer set generated more amplicons, which likely reflects the higher number of copies of mitochondrial versus nuclear DNA molecules. In order to maximize detection potential, the COI assay was therefore used for further downstream qPCR analysis.

A primer-probe specificity test was performed in a 10 µL reaction volume containing 400 nM of each primer, 200 nM of BHQ probe and 4 µL of Applied Biosystems TaqMan Environmental Mastermix 2.0

(Thermo Fisher Scientific, Waltham, Massachusetts, United States). A total of 1 µL template DNA of a concentration of 1 ng/µL was used. Thermocycling qPCR reactions were run on a StepOne Plus Real-time PCR Instrument (Life Technologies, U.S.A.), using 10 minutes initial denaturation at 95°C, followed by 50 cycles of 95°C for 15 s and 60°C for 1 minute. Target DNA was analysed in duplicate reactions and four negative controls were included. As DNA or tissue could not be obtained for any closely related species, the specificity of the assay was tested only on a distantly related hydroid species of *Tubularia* sp. found in Denmark, which potential could be mistaken for *C. caspia*.

Assay specificity results

The target species amplified successfully at 22 Cq (Figure 19.3) and no amplification was observed in any of the four negative controls or in reactions containing DNA from the distantly related species of *Tubularia* sp. The specificity of the assay against sister-species of *Cordylophora* was not tested. However, no closely related species are recorded from Danish waters and would be exotics if they were. The designed eDNA target assay for *Cordylophora caspia* is therefore considered specific to invasive *Cordylophora* when tested on laboratory and environmental water samples.

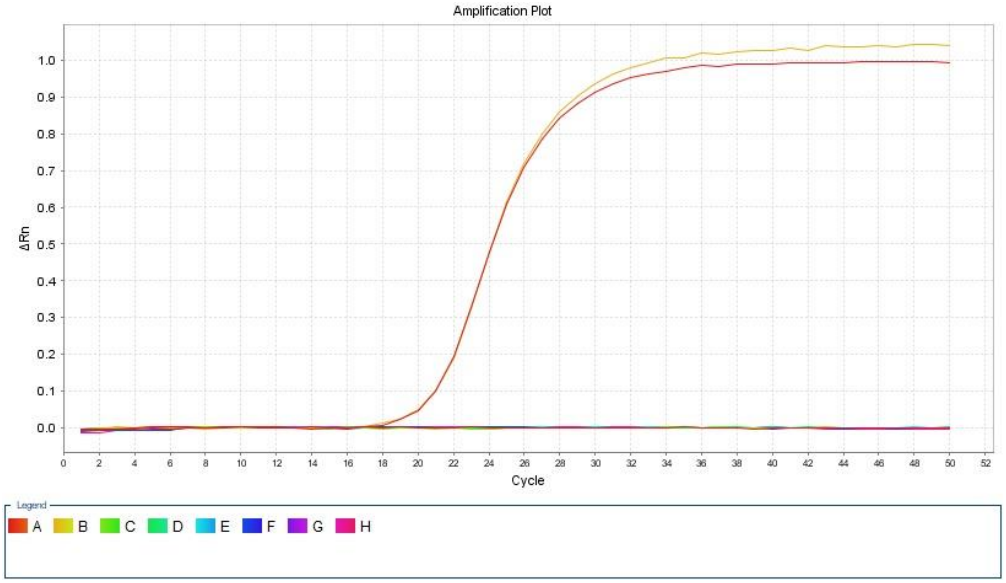

Figure 18.3 *Amplification of* Cordylophora caspia *using the Col_cas_COI assay. Target species is shown in red and yellow (two replicates),* Tubularia *sp. is shown in green (two replicates) and negative controls in blue and purple.*

3.19 Species no. 19: *Mnemiopsis leidyi*

Binomial nomenclature and author: *Mnemiopsis leidyi* A. Agassiz, 1865.
English common name: Warty comb jelly or sea walnut.
Danish common name: Amerikansk ribbegople.

Figure 19.1 *Mnemiopsis leidyi.* Photo by Peter Rask Møller (Natural History Museum of Denmark)

The ctenophore family Bolinopsidae comprise three genera: *Bolinopsis*, *Lesueuria* and *Mnemiopsis*, and together comprise the species: *Bolinopsis chuni, B. elegans, B. indosinensis, B. infundibulum, B. microptera, B. mikado, B. ovalis, B. rubripunctata, B. vitrea, Lesueuria hyboptera, L. pinnata, L. tiedemanni, L. vitrea* and *Mnemiopsis leidyi*. Where *Bolinopsis alala*, *B. microptera*, *B. septentrionalis vitrea* and *B. hydatina*; all are considered junior synonyms of *B. infundibulum* (van der Land, 2001). *Bolinopsis infundibulum*, *Leucothea multicornis* and *Mnemiopsis leidyi* inhabits the Atlantic Ocean (van der Land, 2001). *Mnemiopsis leidyi* is native to the Western Atlantic Ocean and is considered an invasive species in European Seas, in the North Sea, the Black Sea and the Caspian Sea (van der Land, 2001).

Evolutionary phylogenetic studies on ctenophores have focused on the nuclear DNA 18s rRNA and internal transcribed spacer (ITS) 1 and ITS2 gene regions (Podar et al., 2001). As a consequence of this focus on this specific gene region, the nuclear DNA18s rRNA and flanking gene regions comprise the broadest representation of ctenophore species available from NCBI GenBank. Hence, the primers and probes have been designed with the intend of amplifying only eDNA from *Mnemiopsis leidyi* and are based on alignment of sequences from the nuclear DNA18s rRNA and flanking gene regions. The primers are designed within the gene regions for ITS 1 and ITS2, as these sequences appeared best for distinguishing between *Mnemiopsis leidyi* and other species of Bolinopsidae. Based on comparison of sequences, and test of primers in PCR setups as well as quantitative PCR setup, the following combination of primers and probes was selected as optimal for detecting eDNA specifically from *Mnemiopsis leidyi:*

- Mnelei_its2_F04: 5'-ACGGTCCCTTGAAGTAGAGC-3'.
- Mnelei_its2_R06: 5'-TCTGAGAAGGCTTCGGACAT-3'.
- Mnelei_its2_P06: 5'-FAM-GTGCCTCTCGGTGTGGTAGCAATATCT-BHQ1-3'.

The total length of the product was 150 bp.

Table 19.1 *Species specific primer/probe assay for* Mnemiopsis leidyi *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.*

Species	**Gene**	**Size**	**Temp**	**Length**	**GC**
Mnemiopsis leidyi	*nDNA-its2*	150 bp			
Mnelei_its2_F04	ACGGTCCCTTGAAGTAGAGC		57.5	19	53
Mnelei_its2_R06	TCTGAGAAGGCTTCGGACAT		58.4	20	50
Mnelei_its2_P06	GTGCCTCTCGGTGTGGTAGCAATATCT		69.9	27	52
Related species*	**Tested**	**Amplification**			
Aurealia aurita	Yes	No			
Bolinopsis sp.	Yes	No			
Mnemiopsis leidyi	Yes	Yes			

** Not closely related to* Mnemiopsis leidyi*, but commonly encountered in North European seas.*

The primers were designed by aligning sequences available from nuclear 18s rRNA and internal transcribed spacer (ITS) 1 and ITS2 available from NCBI GenBank. This included: *Bolinopsis* sp.: U65480; *Leucothea pulchra*: AF293688; *Lobata* sp.: AF293686; *Mnemiopsis leidyi*: AF293700, HM007193-HM007195, HM147259-HM147261, HM147266-HM147269, JQ071530; *Ocyropsis crystallina*: AF293690-AF293691; *Ocyropsis maculata*: AF293689. Sequence alignment was performed using Geneious v. R7 (Kearse et al. 2012) and MAFFT 6.822 (Katoh & 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: *Mnemiopsis leidyi*, would be amplified by the

Mnelei_its2_F06_R06_P06 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 precise than the Mnelei_its2_F06_R06_P06: Mnelei_its1_F01: 5'-ACCCACGAGTTCAAACCTCA-3', Mnelei_its1_F02: 5'-GCGGTTTCACTTGATGCTGT-3', Mnelei_its1_F03: 5'-ACCGTTAAATCGTCAGTGGGT-3', Mnelei_its1_F04: 5'-CCTTAGCGGTTTCACTTGATGC-3', Mnelei_its1_F05: 5'-ACGAATCCAATCTTACCTGCCT-3', Mnelei_its1_F06: 5'-CGGGTTGCGCCTTCAC-3', Mnelei_its1_F07: 5'-CGGGTTGCGCCTTCAC-3', Mnelei_its1_F08: 5'-CGGGTTGCGCCTTCAC-3', Mnelei_its1_F09: 5'-CGGGTTGCGCCTTCAC-3', Mnelei_its1_F10: 5'-CGGGTTGCGCCTTCAC-3', Mnelei_its1_P01: 5'-FAM-CTTAACGTATCGATTCCTCGGTCGGGC-BHQ1-3', Mnelei_its1_P02: 5'-FAM-GAGCTCGACCGTTAAATCGTCAGTGGG-BHQ1-3', Mnelei_its1_P03: 5'-FAM-ACCCACGAGTTCAAACCTCAATGCCTT-BHQ1-3', Mnelei_its1_P04: 5'-FAM-CGGAGCTCGACCGTTAAATCGTCA-BHQ1-3', Mnelei_its1_P06: 5'-FAM-CGAGTTCAAACCTCAATGCCTTAACGT-BHQ1-3', Mnelei_its1_P07: 5'-FAM-CTCGACCGTTAAATCGTCAGTGGGTAC-BHQ1-3', Mnelei_its1_P08: 5'-FAM-CGAGTTCAAACCTCAATGCCTTAACGT-BHQ1-3', Mnelei_its1_P09: 5'-FAM-CTCGACCGTTAAATCGTCAGTGGGTAC-BHQ1-3', Mnelei_its1_P10: 5'-FAM-CTCGACCGTTAAATCGTCAGTGGGTAC-BHQ1-3', Mnelei_its1_R01: 5'-GGCAACTCTCGTTCGGGAC-3', Mnelei_its1_R02: 5'-CCGCCTAGCAGACCGAAG-3', Mnelei_its1_R03: 5'-GAGCCCGACCGAGGAATC-3', Mnelei_its1_R04: 5'-GAAGTCCGCCGTACCCAC-3', Mnelei_its1_R05: 5'-GAGGTTTGAACTCGTGGGTA-3', Mnelei_its1_R06: 5'-CGCTTTTAGGCAACTCTCGT-3', Mnelei_its1_R07: 5'-GGCATTGAGGTTTGAACTCG-3', Mnelei_its1_R08: 5'-TCGCTTTTAGGCAACTCTCG-3', Mnelei_its1_R09: 5'-GCCTAGCAGACCGAAGTCC-3', Mnelei_its1_R10: 5'-CGTTAAGGCATTGAGGTTTGA-3', Mnelei_its2_F01: 5'-GCGAACGGTCCCTTGAAGTA-3', Mnelei_its2_F02: 5'-AAAGCGAACGGTCCCTTGAA-3', Mnelei_its2_F03: 5'-GTTAAAGCGAACGGTCCCTT-3', Mnelei_its2_F04: 5'-ACGGTCCCTTGAAGTAGAGC-3', Mnelei_its2_F05: 5'-CCCTTGAAGTAGAGCGATCCC-3', Mnelei_its2_P01: 5'-FAM-GTGTGGTAGCAATATCTCACCGAGCGG-BHQ1-3', Mnelei_its2_P02: 5'-FAM-GTGTGGTAGCAATATCTCACCGAGCGG-BHQ1-3', Mnelei_its2_P03: 5'-FAM-GAAGTAGAGCGATCCCGAGTTGCG-BHQ1-3', Mnelei_its2_P04: 5'-FAM-GTGTGGTAGCAATATCTCACCGAGCGG-BHQ1-3', Mnelei_its2_P05: 5'-FAM-GTGTGGTAGCAATATCTCACCGAGCGG-BHQ1-3', Mnelei_its2_R01: 5'-TTCGGACATCCTGCAAAGCT-3', Mnelei_its2_R02: 5'-GGCTTCGGACATCCTGCAAA-3', Mnelei_its2_R03: 5'-CCGCTCGGTGAGATATTGCT-3', Mnelei_its2_R04: 5'-AAGGCTTCGGACATCCTGC-3', Mnelei_its2_R05: 5'-GGACATCCTGCAAAGCTCGG-3'.

Figure 18.2 *Alignment of* Mnemiopsis leidyi *and other species of the order* Lobata *for the nDNA-ITS2 gene. The selection of species was limited by the availability of sequences on NCBI GenBank. All sequences were acquired from NCBI GenBank.*

Conditions of the specificity test

DNA from reference tissue samples were either supplied from external sources (Table 18.2) or extracted from tissue samples using the DNeasy Blood and tissue kit (Qiagen) according to manufacturer's specifications. Initial primer specificity, test of annealing temperature and primer concentration was performed in a 25 µL reaction volume comprising forward and reverse primers (ordered through TAG Copenhagen A/S), using 1 µL forward and 1 µL reverse primer (with 10 µM initial concentrations per primer), and 0.1 µL 5U/µL AmpliTaq Gold Polymerase (Thermofisher, Applied Biosystems), 11.6 µL ddH_2O, 2 µL 25 mM $MgCl_2$ and 2 µL template DNA extracted from tissue samples and diluted 1:10 prior to usage. QPCR reactions were run on a Stratagene Mx3005P qPCR Machine (Agilent, Santa Clara, California, United States). Primer probe specificity test was run using 1 µL forward and 1 µL reverse primer (with 10 µM initial concentrations per primer) and 1 µL probe (with 2.5 µM initial concentration) in a 25 µL reaction volume, including 10 µL Applied Biosystems TaqMan Environmental Mastermix 2.0 (Thermo Fisher Scientific, Waltham, Massachusetts, United States), 10 µL ddH_2O and 2 µL 1:10 diluted template DNA from tissue extractions. Target- and non-target species were run in duplicate reactions and two negative controls.

Table 19.2 *List of tested species with information regarding collector and origin.*

Species*	Tissue sample at Zoological Museum of Copenhagen	Collector	Sampling Location
Aurelia aurita	Auraur	S. W. Knudsen, NHMD	Gedser Harbour, Denmark
Mnemiopsis leidyi	Mnelei	Syddansk Universitet, Florian Lüskow	outside Kerteminde
Bolinopsis sp.	Bolsp	Syddansk Universitet, Florian Lüskow	outside Kerteminde

** Not necessarily closely related to the genus* Mnemiopsis *but commonly encountered in Danish waters.*

Assay specificity results

The two replicated of *Mnemiopsis leidyi* amplified at a Cq of 24.53 and 23.41, respectively (Figure 18.3). None of the non-target species amplified with the Mnelei_its2_F06_R06_P06 assay.

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

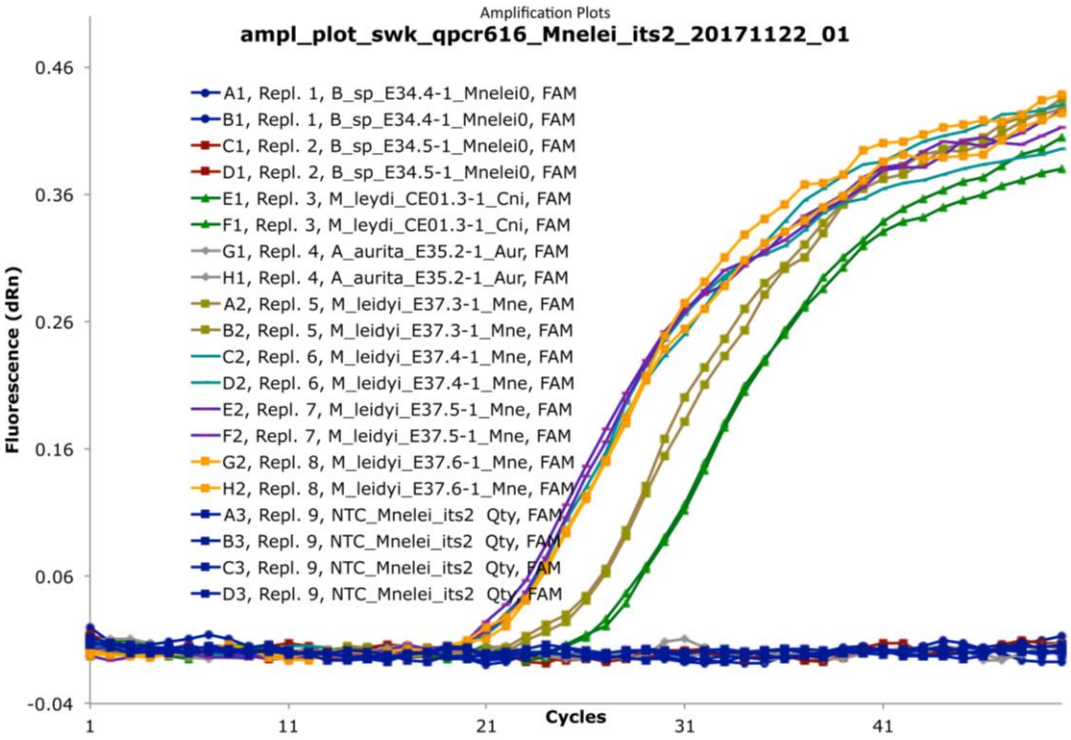

Figure 18.3 *Amplification of* Mnemiopsis leidyi *using the Mnelei_its2_F06_R06_P06 assay. Target species* Mnemiopsis leidyi *is amplified in well: E1, F1, A2, B2, C2, D2, E2, F2, G2 and H2. Non-target species:* Aurelia aurita *(A7, B7) and* Bolinopsis *sp. (well A1, B1, C1, D1) are not amplified. Non-Target Control samples are dark blue.*

3.20 Species no. 20: *Acipenser baerii*

Binomial nomenclature and author: *Acipenser baerii,* Brandt 1896.
English common name: Siberian sturgeon.
Danish common name: Sibirisk stør.

In Danish Seas the following species of sturgeons occurs: *Acipenser baerii*, *A. gueldenstaedtii*, *A. oxyrhynchus*, *A. ruthenus*, *A. stellatus*, *A. sturio*, *Huso huso*. Among these *A. oxyrhynchus* and *A. sturio* are considered indigenous in Denmark (Carl, 2012b; Møller, 2012). Furthermore, *Acipenser baerii*, *A. gueldenstaedtii*, *A. ruthenus* and *A. stellatus* are known to hybridize (Carl, 2012b; Dudu et al., 2011; Ludwig et al., 2009), and each of these four non-indigenous species can thus carry a mitochondrial genome (mt-genomes) from either one of these four species.

Individuals of *A. baerii* and can thus carry an mt-genome from *A. gueldenstaedtii* and vice versa.
An alternative primer and probe assay was therefore developed to distinguish *A. bearii* and *A. gueldenstaedtii* from the other two non-indigenous species of *Acipenser* (i.e. *Acipenser ruthenus* and *A. stellatus*) occurring in Danish waters.
Based on comparison of sequences, and test of primers in PCR setups as well as quantitative PCR setup, the following combination of primers and probes was selected as optimal for detecting eDNA specifically from *Acipenser baerii/Acipenser gueldenstaedtii:*

- Acibae_CR_F02 5'- CAGTTGTATCCCCATAATCAGCC -3'.
- Acibae_CR_R03 5'- TTATTCATTATCTCTGAGCAGTCGTGA-3'.
- Acibae_CR_P01 5'- FAM-ATGCCGAGAACCCCATCAACATTTGGT-BHQ1 -3'.

The total length of the product was 214 bp.

Table 20.1 *Species specific primer/probe assay for* Acipenser baerii /Acipenser gueldenstaedtii *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.*

Species	Gene	Size		Temp	Length	GC
Acipenser baerii	*mtDNA-Control Region*	214 bp				
Acibae_CR_F02	CAGTTGTATCCCCATAATCAGCC			62.9	23	48
Acibae_CR_R03	TTATTCATTATCTCTGAGCAGTCGTGA			63.7	27	37
Acibae_CR_P01	ATGCCGAGAACCCCATCAACATTTGGT			68.2	27	48
Related species*	**Tested**	**Amplification**	**Sample No.**			
Acipenser stellatus	Yes	No	ZMUC_P10283			
Huso huso	Yes	No	ZMUC_P10276			
Acipenser sturio	Yes	No	ZMUC_P10302			
Acipenser oxyrhynchus	Yes	No	ZMUC_P191563			
Acipenser gueldenstaedtii	Yes	Yes**	ZMUC_P10248			
Acipenser ruthenus	Yes	No	ZMUC_P10272			
Acipenser baerii	Yes	No**	ZMUC_P10277			

** Not all species of* Acipenser *were included in this test, only the species commonly encountered in Danish seas*
*** The Acibae_CR_F02-R03-P01 assay resulted in positive amplification from a sample of* A. gueldenstaedtii*, but not from the sample from* A. baerii*. Because the sample from ZMUC_P10277 (a specimen of* A. gueldenstaedtii*) contains a mitochondrial genome from* A. baerii *– verified by de novo sequencing.*

The primers were designed by aligning sequences available from mitochondrial control region available from NCBI GenBank for *Acipenser baerii*, *A. gueldenstaedtii*, *A. oxyrhynchus*, *A. ruthenus*, *A. stellatus*, *A. sturio* and *Huso huso*. Sequence alignment was performed using Geneious v. R7 (Kearse et al. 2012) and MAFFT 6.822 (Katoh & 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: *Acipenser baerii /Acipenser gueldenstaedtii*, would be amplified by the Acibae_CR_F02-R03-P01 system in a qPCR setup.

Conditions of the specificity test

DNA from reference tissue samples were either supplied from external sources (Table 20.2) or extracted from tissue samples using the DNeasy Blood and tissue kit (Qiagen) according to manufacturer's specifications. Initial primer specificity, test of annealing temperature and primer concentration was performed in a 25 µL reaction volume comprising forward and reverse primers (ordered through TAG Copenhagen A/S), using 1 µL forward and 1 µL reverse primer (with 10 µM initial concentrations per primer), and 0.1 µL 5U/µL AmpliTaq Gold Polymerase (Thermofisher, Applied Biosystems), 11.6 µL ddH_2O, 2 µL 25 mM $MgCl_2$ and 2 µL template DNA extracted from tissue samples and diluted 1:10 prior to usage. QPCR reactions were run on a Stratagene Mx3005P qPCR Machine (Agilent, Santa Clara, California, United States). Primer probe specificity test was run using 1 µL forward and 1 µL reverse primer (with 10 µM initial concentrations per primer) and 1 µL probe (with 2.5 µM initial concentration) in a 25 µL reaction volume, including 10 µL Applied Biosystems TaqMan Environmental Mastermix 2.0 (Thermo Fisher Scientific, Waltham, Massachusetts, United States), 10 µL ddH_2O and 2 µL 1:10 diluted template DNA from tissue extractions. Target- and non-target species were run in duplicate reactions and two negative controls.

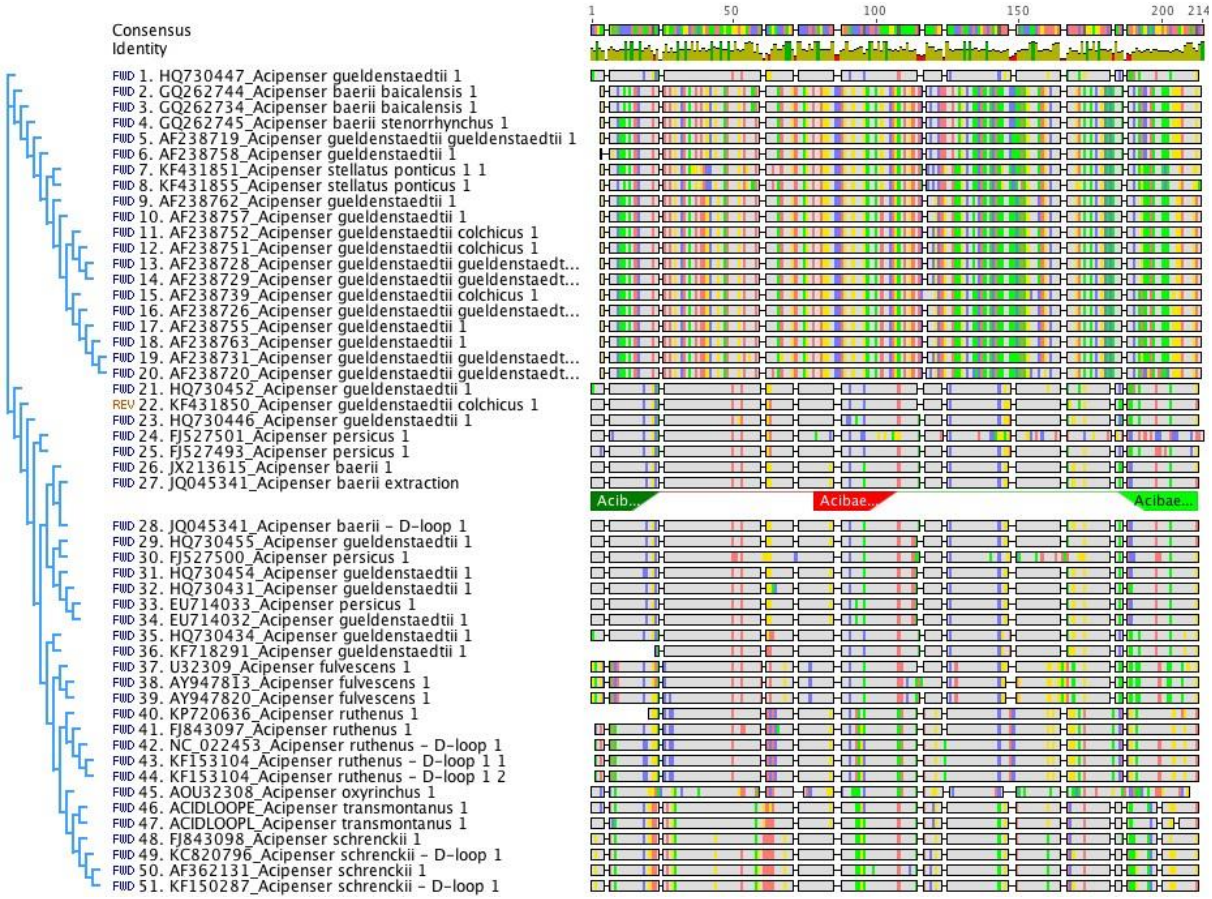

Figure 20.1 *Alignment of* Acipenser *for the mtDNA-Control region gene. The selection of species was limited by the availability of sequences on NCBI GenBank. All sequences were acquired from NCBI GenBank.*

Table 20.2 *List of tested species with information regarding collector and origin*

Species*	Tissue sample at Zoological Museum of Copenhagen	Collector	Sampling locality and sample
Acipenser baerii	Acibae	NHMD, P.R. Møller and H. Carl	Denmark; ZMUC_P10277
Acipenser gueldenstaedtii	Acigue	NHMD, P.R. Møller and H. Carl	Denmark; ZMUC_P10248
Acipenser ruthenus	Acirut	NHMD, P.R. Møller and H. Carl	Denmark; ZMUC_P10272
Acipenser stellatus	Aciste	NHMD, P.R. Møller and H. Carl	Denmark; ZMUC_P10283
Acipenser sturio	Acistu	NHMD, P.R. Møller and H. Carl	Denmark; ZMUC_P10302
Acipenser oxyrinchus	Acioxy	NHMD, P.R. Møller and H. Carl	Denmark; ZMUC_P10260
Huso huso	Hushus	NHMD, P.R. Møller and H. Carl	Denmark; ZMUC_P10276

** Species closely related to* Acipenser ruthenus *and* A. gueldenstaedtii*.*

Assay specificity results

The two replicated extractions from specimen ZMUC_P10248 (i.e. a specimen of *Acipenser gueldenstaedtii* with an *Acipenser baerii* mt-genome) amplified at a Cq of 20.10 and 20.20, respectively (Figure 20.2).

The designed eDNA target assay Acibae_CR_F02-F03-P01-assay is expected to only amplify DNA from the *A. gueldenstaedtii* and *A. baerii* when tested on laboratory or environmental water samples.

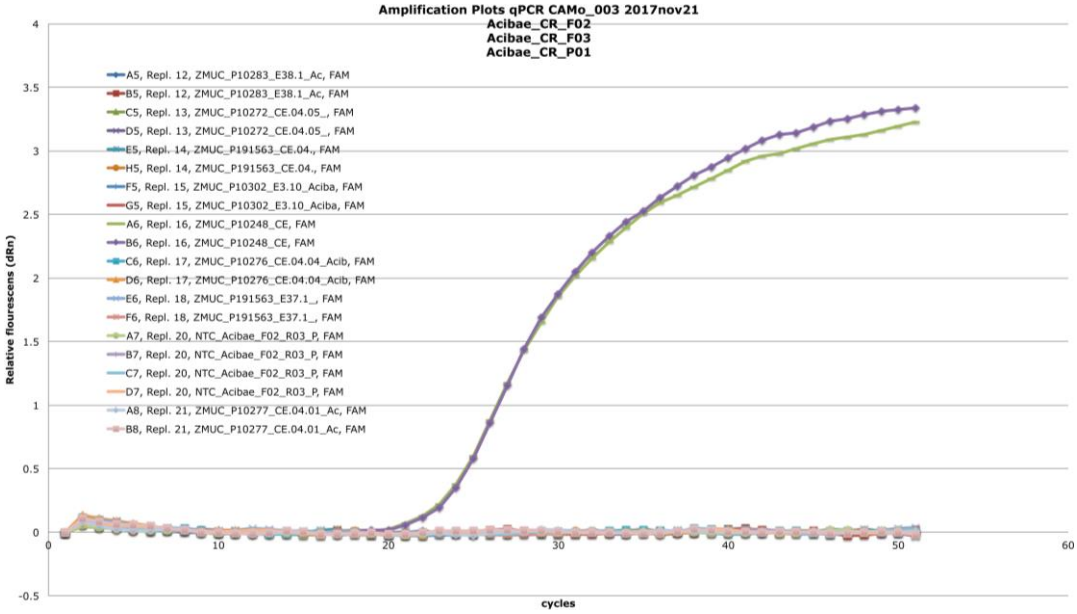

Figure 20.2 *Amplification of an* Acipenser baerii *mt-genome control region using the Acibae_CR_F02-F03-P01-assay. Sample ZMUC_P10248 represents an mt-genome from* A. baerii*. Non-target samples are from ZMUC_P191563, ZMUC_P10272, ZMUC_P10283, ZMUC_P10302, ZMUC_P10276, and are not amplified. Sample ZMUC_P10277 (i.e. a specimen of* A. baerii *with an mt-genome from* A. gueldenstaedtii*, confirmed by de novo sequencing) did not give rise to amplification. Non-Target Control samples in wells number (repl. 20) did not amplify.*

3.21 Species no. 21: *Acipenser gueldenstaedtii*

Binomial nomenclature and author: *Acipenser gueldenstaedtii* von Brandt & Ratzeburg 1833.
English common names: Russian sturgeon.
Danish common name: Diamant stør.

Figure 21.1 Acipenser gueldenstaedtii. *Photo by Henrik Carl (Natural History Museum of Denmark).*

3.22 Species no. 22: *Acipenser ruthenus*

Binomial nomenclature and author: *Acipenser ruthenus* Linnaeus, 1758.
English common names: Sterlet.
Danish common name: Sterlet.

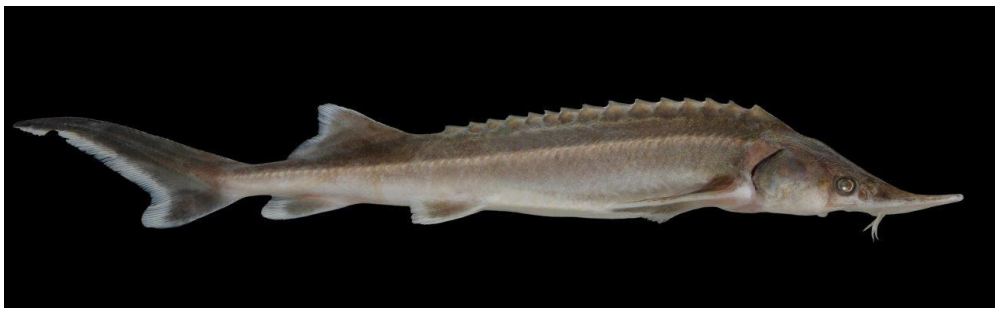

Figure 22.1 Acipenser ruthenus*. Photo by Henrik Carl (Natural History Museum of Denmark).*

The genus *Acipenser* comprise the species: *Acipenser baerii, A. brevirostrum, A. dabryanus, A. fulvescens, A. gueldenstaedtii, A. medirostris, A. mikadoi, A. naccarii, A. nudiventris, A. oxyrinchus, A. persicus, A. ruthenus, A. schrenckii, A. sinensis, A. stellatus, A. sturio*, and *A. transmontanus.* Among these *A. sturio* and *A. oxyrinchus* are native in Northern European seas. Four species of *Acipenser: A. baerii, A. gueldenstaedtii, A. ruthenus*, and *A. stellatus*, are considered introduced in Northern Europe (Carl, 2012b; Møller, 2012). These four non-native species are able to hybridize, and the offspring can thus carry mitochondrial inheritance from any of these four species. However, the four species of non-native *Acipenser* do not hybridize with the inherent species: *A. sturio* and *A. oxyrinchus* (Carl, 2012b; Dudu et al., 2011; Ludwig et al., 2009). The design of primers and probes for targeting eDNA from non-native species of *Acipenser* in Northern Europe was therefore set primarily to distinguish between the four non-native and the two inherent species (i.e. between *Acipenser baerii, A. gueldenstaedtii, A. ruthenus*, *A. stellatus* and *A. sturio*, *A. oxyrinchus)*. Among the sequences used for specific primer design wereseqeunces from: *Acipenser baerii, A. gueldenstaedtii, A. ruthenus*, *A. stellatus*, *A. sturio*, and *A. oxyrinchus* deposited in NCBI GenBank, and the sequences obtained in by de novo sequencing of vouchered samples held at the Natural History Museum of Denmark. The mitochondrial *cytochrome b* gene was selected as region for specific primer design, due to the level of variation among the native and non-native species of *Acipenser* (Table 22.1). Five different combinations of primers and probes were tested out:

- Acibae_*cytb*_F11: 5'-TTCCACCCGTACTTCTCATAC-3'.
- Acibae_*cytb*_P11: 5'-FAM-CCTAATGCTAGTCGGACTCACCTCCGT-BHQ-1-3'.
- Acibae_*cytb*_R16: 5'-GGCGTAGGCGAAGAGAAAGTA-3'.
- Acibae_*cytb*_R17: 5'-TATTTGGGATGGATCGGAGA-3'.
- Acibae_*cytb*_R18: 5'-CCACCTAGTTTATTTGGGATGG-3'.
- Acibae_*cytb*_R19: 5'-GCGTAGGCGAAGAGAAAGTATC-3'.
- Acibae_*cytb*_R20: 5'-TTATTTGGGATGGATCGGAGA-3'.

The total length of the product was 180-216 bp.

Table 22.1 *Species specific primer/probe assay for:* Acipenser baerii*,* A. gueldenstaedtii*,* A. ruthenus*, and* A. stellatus*, 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.*

Species	Gene	Size		Temp	Length	GC
Acipenser gueldenstaedtii	*mtDNA-cytb*	180-216 bp				
Acipenser ruthenus	*mtDNA- cytb*	180-216 bp				
Acibae_cytb_F11	TTCCACCCGTACTTCTCATAC			56.7	21	47
Acibae_cytb_P11	CCTAATGCTAGTCGGACTCACCTCCGT			69.2	27	55
Acibae_cytb_R16	GGCGTAGGCGAAGAGAAAGTA			60.9	21	52
Acibae_cytb_R17	TATTTGGGATGGATCGGAGA			60.2	20	45
Acibae_cytb_R18	CCACCTAGTTTATTTGGGATGG			59.6	22	45
Acibae_cytb_R19	GCGTAGGCGAAGAGAAAGTATC			59.6	22	50
Acibae_cytb_R20	TTATTTGGGATGGATCGGAGA			61.5	21	42
Related species*	**Tested**	**Amplification**	**Invasive**			
Acipenser baerii	Yes	Yes	Yes			
Acipenser gueldenstaedtii	Yes	Yes	Yes			
Acipenser ruthenus	Yes	Yes	Yes			
Acipenser stellatus	Yes	Yes	Yes			
Acipenser sturio	Yes	No	No			
Acipenser oxyrinchus	Yes	No	No			
Huso huso	Yes	No	Yes			

** Not including all species in the genus* Acipenser *but limited to those that are occurring in Northern Europe.*

The primers were designed by aligning sequences available from mitochondrial cytochrome b genes from species of the family Acipenseridae available from NCBI GenBank. The alignment was prepared using the following sequences from NCBI GenBank and sequences obtained by *de novo* sequencing of samples from the Natural History Museum of Denmark: *Acipenser baerii*: ZMUC_P10277, *Acipenser ruthenus*: ZMUC_P10272, *Acipenser ruthenus*: GU647225, *Acipenser gueldenstaedtii*: ZMUC_P10248, *Acipenser gueldenstaedtii*: FJ974040, *Acipenser gueldenstaedtii*: AJ277596, *Acipenser baerii*: JQ045341, *Acipenser baerii*: NC_017603, *Acipenser baerii*: NC_017603, *Acipenser gueldenstaedtii*: AJ277595, *Acipenser gueldenstaedtii*: AJ277594, *Acipenser gueldenstaedtii*: GU647227, *Acipenser gueldenstaedtii*: KC130091, *Acipenser gueldenstaedtii*: KC130092, *Acipenser gueldenstaedtii*: NC_012576, *Acipenser gueldenstaedtii*: KC130090, *Acipenser stellatus*: ZMUC_P10283, *Acipenser stellatus*: GU647226, *Acipenser stellatus*: NC_005795, *Acipenser gueldenstaedtii*: EU919434, *Acipenser gueldenstaedtii*: EU910274, *Acipenser sturio*: FJ974043, *Acipenser sturio*: KP997216, *Acipenser oxyrinchus*: KP013103, *Acipenser oxyrinchus*: KP997217, *Acipenser oxyrinchus*: KP997218. 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: *Acipenser baerii, A. gueldenstaedtii, A. ruthenus*, and *A. stellatus*, would be amplified by the F11_R16-R20_P11 systems 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 precise than the F11_R16-R20_P11 systems: Acibae_cytb_F16: 5'-CTATTTTCCCCCAACCTCCTG-3', Acibae_cytb_F21: 5'-ATCCCAAATAAACTAGGTGGAGTT-3', Acibae_cytb_F26: 5'-TCTGCCTTGTCACACAAATCC-3', Acibae_cytb_P01: 5'-FAM-ATGAATCTGAGGCGGCTTTTCAGTAGA-BHQ-1-3', Acibae_cytb_P02: 5'-FAM-GAGGACAGATATCATTTTGAGGGGCAA-BHQ-1-3', Acibae_cytb_P09: 5'-FAM-TTCCGTACATCGGCGACACACTAGTAC-BHQ-1-3', Acibae_cytb_P12: 5'-FAM-ACTCACCTCCGTAGCACTATTTTCCCC-BHQ-1-3', Acibae_cytb_P16: 5'-FAM-CCTTGTCACTCCCCCACACATCAAG-BHQ-1-3', Acibae_cytb_P17: 5'-FAM-TCAAGCCCGAATGATACTTTCTCTTCG-BHQ-1-3', Acibae_cytb_P21: 5'-FAM-CGTTCCGACCCCTTTCTCAAATTCTAT-BHQ-1-3', Acibae_cytb_P22: 5'-FAM-

TCCTAATATTAGTGCCAGTACTCCACACC-BHQ-1-3', Acibae_cytb_P24: 5'-FAM-TCCACACCTCTAAACAACGAGGAAACA-BHQ-1-3', Acibae_cytb_P26: 5'-FAM-CACTACACAGCTGACATTTCAACAGCC-BHQ-1-3', Acibae_cytb_P28: 5'-FAM-ATTTCTTGCAATACACTACACAGCTGA-BHQ-1-3', Acibae_cytb_P29: 5'-FAM-ACACTACACAGCTGACATTTCAACAGC-BHQ-1-3', Acibae_cytb_R01: 5'-GAAAGTGGAAGGCGAAAAATC-3', Acibae_cytb_R02: 5'-GGTGGCGTTGTCTACTGAAAA-3', Acibae_cytb_R03: 5'-TAAGGGTGGCGTTGTCTACTG-3', Acibae_cytb_R04: 5'-TGTACTAGTGTGTCGCCGATG-3', Acibae_cytb_R05: 5'-AAGAAAGTGGAAGGCGAAAAA-3', Acibae_cytb_R07: 5'-TCCAGCGATTACGAATGGTAG-3', Acibae_cytb_R08: 5'-CCAGCGATTACGAATGGTAGA-3', Acibae_cytb_R11: 5'-AGGAGGTTGGGGGAAAATAGT-3', Acibae_cytb_R12: 5'-TATCATTCGGGCTTGATGTGT-3', Acibae_cytb_R13: 5'-GTATCATTCGGGCTTGATGTG-3', Acibae_cytb_R14: 5'-GAAAGTATCATTCGGGCTTGA-3', Acibae_cytb_R15: 5'-AAGTATCATTCGGGCTTGATG-3', Acibae_cytb_R21: 5'-GGCCTCCAATTCATGTGAGTA-3', Acibae_cytb_R22: 5'-GAACGTGTTTCCTCGTTGTTT-3', Acibae_cytb_R23: 5'-GGAACGTGTTTCCTCGTTGT-3', Acibae_cytb_R24: 5'-CCAGGGCTCAGAATAGAATTTG-3', Acibae_cytb_R25: 5'-CCACCAGGGCTCAGAATAGA-3', Acibae_cytb_R26: 5'-CAGATGTGGGCAATAGAGGAG-3', Acibae_cytb_R27: 5'-GATGTGGGCAATAGAGGAGAA-3', Acibae_cytb_R28: 5'-GAGGAGAAGGCTGTTGAAATG-3', Acibae_cytb_R29: 5'-GATGTGGGCAATAGAGGAGAAG-3', Acibae_cytb_R30: 5'-AGAGGAGAAGGCTGTTGAAATG-3', Acibae_cytb_F01: 5'-GCCTTCGTGGGATATGTACTG-3', Acibae_cytb_F06: 5'-GGGCAACCGTCATCACC-3'.

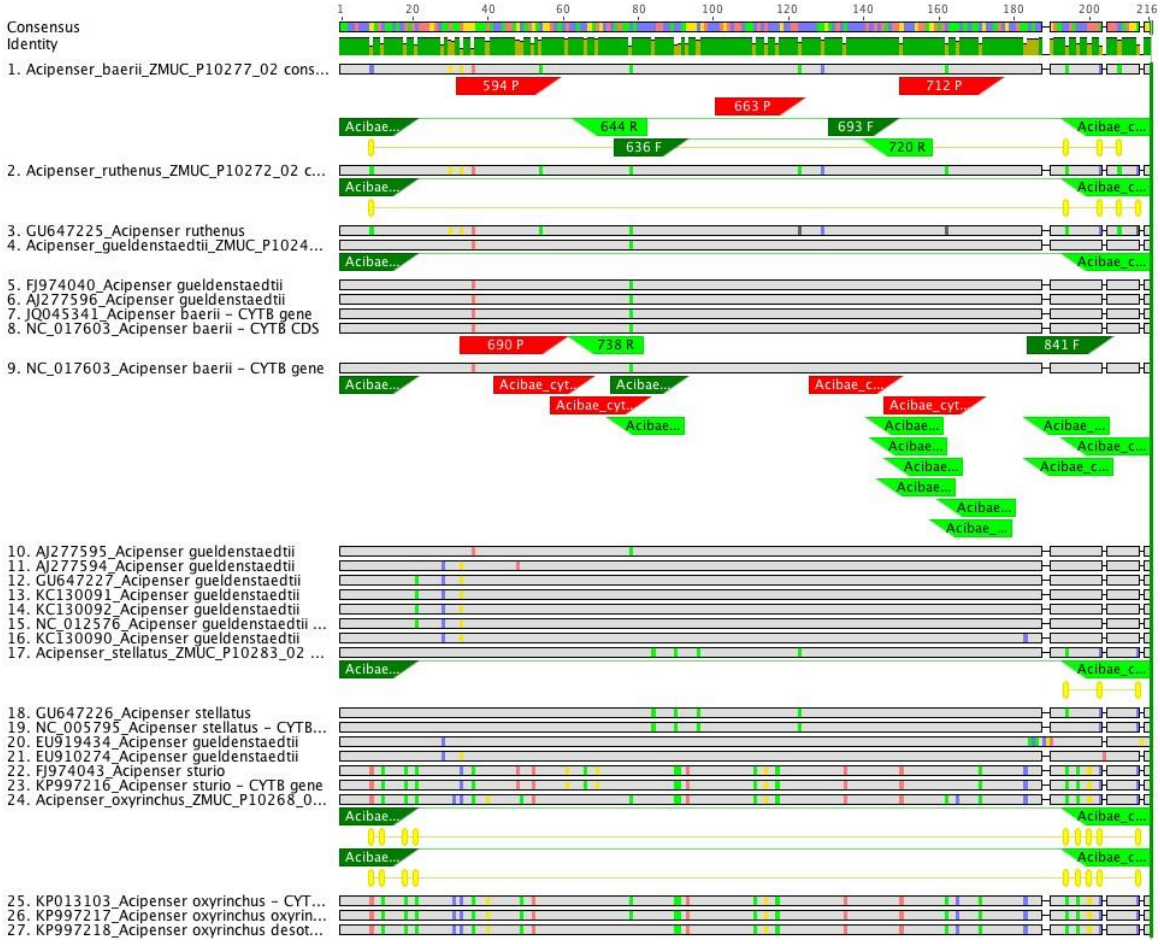

Figure 22.2 *Alignment of sequences from* Acipenser *and occurring in North European seas for the mtDNA-cytochrome b gene. Sequences were acquired from NCBI GenBank, and from de novo sequencing of vouchered samples held at NHMD.*

Conditions of the specificity test

DNA from reference tissue samples were either supplied from external sources (Table 21.2) or extracted from tissue samples using the DNeasy Blood and tissue kit (Qiagen) according to manufacturer's specifications and obtained through de novo sequencing performed at the NHMD. Initial primer specificity, test of annealing temperature and primer concentration was performed in a 25 µL reaction volume comprising forward and reverse primers (ordered through TAG Copenhagen A/S), using 1 µL forward and 1 µL reverse primer (with 10 µM initial concentrations per primer), and 0.1 µL 5U/µL AmpliTaq Gold Polymerase (Thermofisher, Applied Biosystems), 11.6 µL ddH_2O, 2 µL 25 mM $MgCl_2$ and 2 µL template DNA extracted from tissue samples and diluted 1:10 prior to usage. QPCR reactions were run on a Stratagene Mx3005P qPCR Machine (Agilent, Santa Clara, California, United States). Primer probe specificity test was run using 1 µL forward and 1 µL reverse primer (with 10 µM initial concentrations per primer) and 1 µL probe (with 2.5 µM initial concentration) in a 25 µL reaction volume, including 10 µL Applied Biosystems TaqMan Environmental Mastermix 2.0 (Thermo Fisher Scientific, Waltham, Massachusetts, United States), 10 µL ddH_2O and 2 µL 1:10 diluted template DNA from tissue extractions. Target- and non-target species were run in duplicate reactions and two negative controls.

Table 22.2 *List of tested species with information regarding collector and origin.*

Species*	Tissue sample at Zoological Museum of Copenhagen	Collector	Sampling locality and sample
Acipenser baerii	Acibae	NHMD, P.R. Møller and H. Carl	Denmark; ZMUC_P10277
Acipenser gueldenstaedtii	Acigue	NHMD, P.R. Møller and H. Carl	Denmark; ZMUC_P10248
Acipenser ruthenus	Acirut	NHMD, P.R. Møller and H. Carl	Denmark; ZMUC_P10272
Acipenser stellatus	Aciste	NHMD, P.R. Møller and H. Carl	Denmark; ZMUC_P10283
Acipenser sturio	Acistu	NHMD, P.R. Møller and H. Carl	Denmark; ZMUC_P10302
Acipenser oxyrinchus	Acioxy	NHMD, P.R. Møller and H. Carl	Denmark; ZMUC_P10260
Huso huso	Hushus	NHMD, P.R. Møller and H. Carl	Denmark; ZMUC_P10276

** Species closely related to* Acipenser ruthenus *and* A. gueldenstaedtii*.*

Assay specificity results

The two replicates of *Acipenser gueldenstaedtii* and *Acipenser ruthenus* amplified at a Cq of 31.34, 31.35, 31.90, 31.79, with assay F11_R16_P11, at a Cq of 29.96, 30.49, 31.02, 28.70, with assay F11_R17_P11, 36.12, 37.53, 28.93, 28.80, with assay F11_R18_P11, at a Cq of 32.07, 28.53, 29.76, 29.61, with assay F11_R19_P11, and at a Cq of 33.21, 33.12, 30.31, 30.35, with assay F11_R20_P11 (Figure 22.3). None of the native species (*Acipenser sturio*, and *Acipenser oxyrinchus,*) amplified with any of the assays.

The designed eDNA target assay for *Acipenser gueldenstaedtii*, and *Acipenser ruthenus* is expected to only amplify DNA from the invasive species: *A. baerii, A. gueldenstaedtii, A. ruthenus*, and *A. stellatus* when tested on laboratory or environmental water samples.

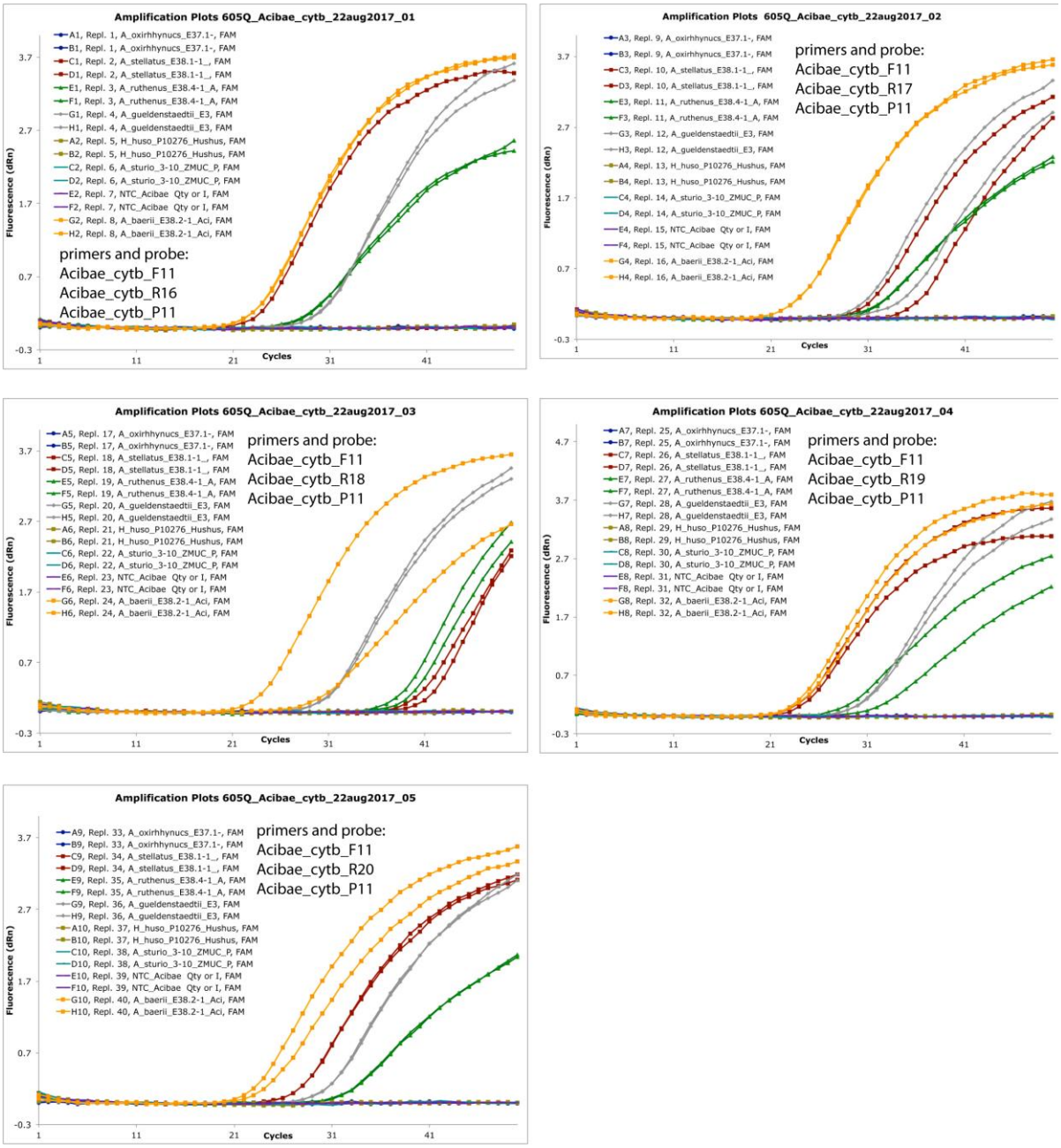

Figure 22.3 *Amplification of* Acipenser *using the following assays: F11_R16_P11, F11_R17_P11, F11_R18_P11, F11_R19_P11 and F11_R20_P11 assay targeting mtDNA-cytochrome b. Target species* Acipenser gueldenstaedtii, Acipenser ruthenus *is shown in grey and green, respectively, the other invasive target species:* Acipenser baerii, Acipenser stellatus *in orange and red, respectively, and non-target species:* Acipenser sturio, Acipenser oxyrinchus, Huso huso*, in: blue, light brown, and turquoise, respectively. Non-Target Control samples are purple.*

4 Discussion and conclusions

Based on the analyses and results of this study (see Chapter 3), we take stock of progress by answering the questions (1) where are we now? and (2) where are we going? And based on the answers to these two questions we draw up conclusions and perspectives of this study.

4.1 Where are we now?

The species-specific primer-probe assays developed and tested in this report are now ready for a large-scale implementation in a eDNA monitoring scheme in the Danish seas. The collection of samples and survey can be set up in such a way that it resembles the study by Knudsen *et al.* (2018b), where commercial species of marine fishes were monitored in the Baltic Sea. For optimal collection of eDNA the usage of Sterivex-filters is recommended as described by Spens *et al.* (2016) and Knudsen *et al.* (2018a).

The specificity of each of the species-specific primer-probe systems is shortly summarized here:

Species no. 01: *Bonnemaisonia hamifera:* The developed test system is described and documented in section 3.1. The species-specific assay targeting eDNA from *Bonnemaisonia hamifera* was tested *in vivo* on water samples collected in 16 Danish ports in 2017 during the MONIS 4 project. The *in vivo* test of this assay confirmed the previous records of this species in Danish seas found by conventional monitoring.

Species no. 02: *Prorocentrum minimum:* The developed test system is described and documented in section 3.2. The species-specific assay targeting eDNA from *Prorocentrum minimum* was tested *in vivo* on water samples collected in 16 Danish ports in 2017 during the MONIS 4 project. The *in vivo* test of this assay confirmed the previous records of this species in Danish seas found by conventional monitoring.

Species no. 03: *Pseudochattonella farcimen:* The developed test system is described and documented in section 3.3. The species-specific assay targeting eDNA from *Pseudochattonella farcimen* was tested *in vivo* on water samples collected in 16 Danish ports in 2017 during the MONIS 4 project. The *in vivo* test of this assay confirmed the previous records of this species in Danish seas found by conventional monitoring.

Species no. 04: *Pseudochattonella verruculosa:* The developed test system is described and documented in section 3.4. The species-specific assay targeting eDNA from *Pseudochattonella verruculosa* was tested *in vivo* on water samples collected in 16 Danish ports in 2017 during the MONIS 4 project. The *in vivo* test of this assay confirmed the previous records of this species in Danish seas found by conventional monitoring.

Species no. 05: *Karenia mikimotoi:* The developed test system is described and documented in section 3.5. The species-specific assay targeting eDNA from *Karenia mikimotoi* was tested *in vivo* on water samples collected in 16 Danish ports in 2017 during the MONIS 4 project. The *in vivo* test of this assay identified *Karenia mikimotoi* in three out of the 16 samples where two were under Limit of Detection (LOD).

Species no. 06: *Carassius auratus auratus:* The developed test system is described and documented in section 3.6. No eDNA was detected from *Carassius auratus auratus* in the *in vivo* test performed on MONIS 4 water samples from Danish harbours. The absence of eDNA for this species supports the assumption that this non-indigenous species is rare in Danish marine environments.

Species no. 07: *Cyprinus carpio:* The developed test system is described and documented in section 3.7. The *in vivo* tests performed under MONIS 4 supported the presence of *Cyprinus carpio* in Kalundborg and showed low eDNA levels in Aarhus and Aalborg harbour. However, the eDNA levels detected during the *in vivo* test were in all cases below LOD, suggesting that the species was either not present or present in very low numbers. Additional *in vivo* testing of water samples from these three locations may be required for a robust assessment of this species.

Species no. 08: *Colpomenia peregrine:* The developed test system is described and documented in section 3.8. The *in vivo* tests performed under MONIS 4 showed the presence of *Colpomenia peregrine* from several locations where it has not been previously recorded by conventional monitoring approaches. However, the eDNA levels detected during the *in vivo* test were in all but one case below LOD. This suggests that the species was either not present or present in very low numbers. This calls for additional testing of *in vivo* samples coupled with conventional monitoring approaches examining the current occurrence of the species in Danish waters.

Species no. 09: *Neogobius melanostomus:* The developed test system is described and documented in section 3.9. The *in vivo* tests performed showed low levels of eDNA from *Neogobius melanostomus* from localities where it is known to occur. In all cases the eDNA levels were below LOD, which calls for an additional testing of the sensitivity of the primer-probe assay. Nonetheless, the result confirms the general use of the assay for detection of this invasive species.

Species no. 10: *Oncorhynchus mykiss:* The developed test system is described and documented in section 3.10. The *in vivo* tests performed showed the presence of eDNA from *Oncorhynchus mykiss* in four different locations. In all cases the levels were below LOD. The low eDNA levels found from this species confirms the scarcity of this non-indigenous species in Danish marine environments, which is restricted to escaped individuals from fish farms.

Species no. 11: *Oncorhyncus gorbuscha:* The developed test system is described and documented in section 3.11. No eDNA was detected from *Oncorhyncus gorbuscha* in the *in vivo* test performed on water samples from 16 harbours. The absence of eDNA for *O. gorbuscha* confirms the rare occurrence of this non-indigenous species. This calls for an additional in vivo test from waters where this species is known to occur.

Species no. 12: *Crassostrea gigas / Magallana gigas:* The developed test system is described and documented in section 3.12. *Crassostrea gigas* (also known as *Magallana gigas*), the Pacific oyster, is introduced from the Pacific coast of Asia, and has been recorded in along the Western coast of Jylland, in Limfjorden and in Øresund. The *in vivo* tests performed supported the current known distribution of Pacific oyster in Øresund, but the eDNA levels detected during the *in vivo* test for samples from Vesterhavet and Limfjorden are low or absent, which calls for an additional in vivo test for the primer-probe assay targeting this species.

Species no. 13: *Mya arenaria.* The developed test system is described and documented in section 3.13. *Mya arenaria* is common but not considered native in Danish Seas (Strasser, 1999). The assay targeting *Mya arenaria was* tested *in vivo* on water samples collected in summer and autumn 2017.

The assay detected eDNA from several harbours in the inner Danish seas, which match the known distribution of this invasive species.

Species no. 14: *Rhithropanopeus harrisii* : The developed test system is described and documented in section 3.4. The assay targeting *R. harrisii* was tested *in vivo* on water samples collected in summer and autumn 2017. The assay detected eDNA from *R. harrisii* in Køge bugt and Øresund, which match the known distribution of this invasive species.

Species no. 15: *Paralithodes camtschaticus:* The developed test system is described and documented in section 3.14. The species-specific assay targeting eDNA from *Paralithodes camtschaticus* was attempted tested *in vivo* on water samples collected in 16 Danish ports in 2017 during the MONIS 4 project. However, as records of *Paralithodes camtschaticus* are extremely rare in Danish seas, the *in vivo* test was unable to confirm the presence of *Paralithodes camtschaticus* in Danish seas, most likely because *Paralithodes camtschaticus* is absent from the inner Danish seas, as past records using conventional monitoring are scarce and are restricted to the deep parts of the Barents Sea.

Species no. 16: *Eriocheir sinensis.* The developed test system is described and documented in section 3.16. The species-specific assay targeting eDNA from *Eriocheir sinensis* was attempted tested *in vivo* on water samples collected in 16 Danish ports in 2017 during the MONIS 4 project. However, as records of *Eriocheir sinensis* are extremely rare in Danish seas, the *in vivo* test was unable to confirm the presence of *Eriocheir sinensis* in Danish seas, most likely because *Eriocheir sinensis* is absent from the inner Danish seas, as past records using conventional monitoring are scarce and are restricted to the southern part of the North Sea in southern part of United Kingdom and German and Dutch seas.

Species no. 17: *Homarus americanus:* The developed test system is described and documented in section 3.17. The species-specific assay targeting eDNA from *Homarus americanus* was attempted tested *in vivo* on water samples collected in 16 Danish ports in 2017 during the MONIS 4 project. However, as records of *Homarus americanus* are extremely rare in Danish seas, the *in vivo* test was unable to confirm the presence of *H. americanus* in Danish seas, most likely because *Homarus americanus* is absent from the inner Danish seas, as past records using conventional monitoring are scarce and are restricted to Norwegian seas.

Species no. 18: *Cordylophora caspia:* The developed test system is described and documented in section 3.18. No eDNA was detected from *Cordylophora caspia* in the *in vivo* test performed on water samples from 16 harbours. The absence of eDNA for *C. caspia* confirms the rare occurrence of this non-indigenous species.

Species no. 19: *Mnemiopsis leidyi:* The developed test system is described and documented in section 3.19. *Mnemiopsis leidyi* is widely distributed in the inner Danish waters over the late part of the summer and the autumn but disappears from the inner Danish waters during the winter. A re-invasion of the inner Danish seas then starts out over the spring from the North Sea, where *M. leidyi* is present all year round. The *in vivo* test of this assay over the early part of summer 2017 and the late part of autumn 2017 confirmed this distribution with eDNA from *M. leidyi* being absent from the south-eastern parts of the Danish seas in the spring, and eDNA being found in high levels at all ports sampled in the inner Danish seas.

Species no. 20: *Acipenser baerii:* The developed test system is described and documented in section 3.20. The species-specific assay was in vivo tested in both the sea and a lake 'Poppelsøen' on Sjælland during 2017-2018. Lake 'Poppelsøen' is known to be inhabited by a few (less than 10) individuals. Conventional monitoring has yet to record *A. baerii* from marine waters in Denmark. The water

samples collected from 'Poppelsøen' returned positive detection of *A. baerii*, confirming the ability of this assay to detect eDNA from *A. baerii*. The water samples collected from the 16 Danish ports did not return any positive detection. Hybridization between *Acipenser baerii*, *Acipenser gueldenstaedtii* and *Acipenser ruthenus* is known from aquaculture farming, and can result in an *Acipenser* fenotype that carries a different mt-genome as genotype – i.e. an *Acipenser gueldenstaedtii* fenotype can carry an *Acipenser ruthenus* genotype for the mt-genome, and vice versa. This makes it extremely difficult to distinguish between the different non-indigenous species of *Acipenser* in Danish waters when only mt-genome variation is examined. However, all three species of *Acipenser* (i.e. *Acipenser baerii*, *A. gueldenstaedtii* and *A. ruthenus*) are considered non-indigenous in Danish waters, and a quick comparison of mt-genome variation among these three species of *Acipenser* on NCBI GenBank, suggested that less than 10% of the sequences deposited on NCBI GenBank are hybrids.

Species no. 21: *Acipenser gueldenstaedtii:* The developed test system is described and documented in section 3.21. The primer-probe combination targeting *Acipenser gueldenstaedtii* was found to amplify *in vitro* on other species of non-indigenous species of *Acipenser* in Danish seas – *i.e.* the primers will also amplify DNA from *Acipenser baerii*, *A. gueldenstaedtii*, *A. ruthenus* and *Acipenser stellatus*. The assay is therefore capable of detecting eDNA from non-indigenous species of *Acipenser* in Danish seas, but is not capable of distinguishing between the four non-indigenous species of *Acipenser – i.e.* not able to distinguish between *Acipenser baerii*, *A. gueldenstaedtii*, *A. ruthenus* and *Acipenser stellatus.* Compare with the note for the system for species 20 in this section.

Species no. 22: *Acipenser ruthenus:* The developed test system is described and documented in section 3.22. The primer-probe combination targeting *Acipenser ruthenus* was found to amplify *in vitro* on other species of non-indigenous species of *Acipenser* in Danish seas – *i.e.* the primers will also amplify DNA from *Acipenser baerii*, *A. gueldenstaedtii*, *A. ruthenus* and *Acipenser stellatus*. The assay is therefore capable of detecting eDNA from non-indigenous species of *Acipenser* in Danish seas, but is not capable of distinguishing between the four non- indigenous species of *Acipenser* – i.e. not able to distinguish between *Acipenser baerii*, *A. gueldenstaedtii*, *A. ruthenus* and *Acipenser stellatus.* Compare with the note for the system for species 20 in this section.

With the exemption of the three primer-probe systems developed for *Acipenser*, species 20, 21 and 22, all primer-probe systems were found to be highly species-specific in the *in vitro* tests performed. The first *in vivo* tests performed on water samples collected over summer-2017 and autumn-2017 strongly indicates that the ability of these primer-probe systems to detect eDNA from the non-indigenous species can provide a reflection of the known occurrence and distribution of these 22 marine non-indigenous species.

The fact that the *Acipenser* systems (targeting eDNA from species 20, 21 and 22) are incapable of species-specificity between the non-indigenous species of *Acipenser* can be considered irrelevant, as all three systems are capable of distinguishing between indegionus and non-indigenous species of *Acipenser*. This means that the three systems developed for monitoring non-indigenous species of *Acipenser* will not return a false-positive detection in the presence of indigenous *Acipenser*.

The primer –probe systems presented in this study should still be optimized for concentrations in qPCR set ups, before performing continuous monitoring using these species-specific systems. Each of the primers and each probe will perform better (i.e. be better at detecting low eDNA concentrations) if the optimal qPCR setup reaction concentrations have been inferred prior for each primer and probe, similar to the optimization described by Agersnap *et al.* (2017), Jensen *et al.* (2018) and Knudsen *et al.* (2018b). Such optimization will ensure a more sensitive and more precise monitoring.

With the inclusion of the systems described in this report targeting the very rare marine non-indigenous species such as *Paralithodes camtschaticus*, *Eriocheir sinensis*, and *Homarus americanus*, it will possible to monitor early indications of the presence of non-indigenous species. Among these three species of Decapoda, only *E. sinensis* has been recorded and only in an extremely low number of individuals. Using the systems presented here on multiple water samples collected regularly provides the opportunity of detecting very early invasions from these non-indigenous species. Also, the neighbouring countries around Denmark (e.g. Holland, Germany, Norway and United Kingdom) will most likely be very interested in seeing these three systems used on waters in their vicinity, as these countries are more plagued by these three non-invasive species of Decapoda than Denmark is.

Using these 22 assays in a large scale and continuous monitoring project will most likely give the best picture of the distribution and occurrence of non-indigenous species in Danish marine seas ever prepared. We strongly recommend that water samples are collected regularly and for as many locations as possible along the Danish coastline to ensure a detailed and precise monitoring of marine non-indigenous species can be insured.

4.2 Where are we going?

Based on the work carried out and the results achieved, we conclude the following: 1) Technical Guidance for monitoring under the NOVANA programme has been published, 2) a total of 22 eDNA-based test systems have been developed, 3) the systems have been tested as part of the MONIS 4 project and are thus validated. In order to follow up on these achievements by the MONIS projects and partners, we propose undertaking the following activities:

- Implementation of eDNA-based monitoring in NOVANA monitoring and assessment activities, first of all under the marine sub-programme.
- Development of additional species-specific eDNA-based test systems according to the national Target Species List.
- Updating of the Technical Guidance based on lessons learned from NOVANA monitoring and from the MONIS 4 project.

All in all, we conclude that the MONIS projects have contributed significantly to the development of not only a national Danish strategy for monitoring af non-indigenous species in marine waters, but also to the advancement of using biomolecular methods (eDNA) routinely in monitoring assessment activities.

These results demonstrate that the MONIS projects have lived up to the expectations from funding institution and project partners over the past 4 years. An important next step is to make use of the eDNA-based test systems and to include the results as part of the annual reporting of the national NOVANA monitoring programme.

Another important step to be taken is to improve coordination with similar development activities in neighbouring countries, in the short-term with Sweden and Germany and in the longer term, with Norway and Poland. Exchange of information and lessons learned is key, but so too is avoidance of duplication of efforts with regard to development of eDNA-based test systems.

5 References

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NIVA Denmark is the name, water is our game

NIVA Denmark Water Research is a regional office of the Norwegian Institute for Water Research (NIVA) and has just recently been established to resolve environmental issues concerning the freshwater and marine systems that relate to Denmark.

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NIVA Denmark is a a place for practice, observation, testing and synthesis. Key research and test areas include eutrophication, hazardous substances, biodiversity, and ecosystem health as well as the implications of multiple human activities in marine waters and in streams, rivers and lakes. We develop indicators, monitoring methods and tools to assess the state of an ecosystem in order to carry out analyses and contribute to evidence based and sustainable solutions to the challenges we and the environment face.

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