FACULTY OF SCIENCE
UNIVERSITY OF COPENHAGEN

Master's thesis

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Crayfish in Denmark

Distribution, identification and eDNA detection of the three known species

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Submitted: 01-09-2015

–Natural History Museum of Denmark–
–University of Copenhagen–

Title
CRAYFISH IN DENMARK - DISTRIBUTION, IDENTIFICATION AND eDNA DETECTION OF THE THREE KNOWN SPECIES

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Duration
SUMMER 2014 - SUMMER 2015

Thesis
MASTER'S THESIS IN BIOLOGY

Department
NATURAL HISTORY MUSEUM OF DENMARK, UNIVERSITY OF COPENHAGEN

Section
SECTION FOR EVOLUTIONARY GENOMICS

Supported
INNOVATIONSFONDEN IN COOPERATION WITH UNIVERSITY OF COPENHAGEN, AMPHICONSULT AND EUROFINS. EVOLUTIONARY GENOMICS MASTER'S THESIS GRANT

Pages
MAIN DOCUMENT 102 PAGES. 117 PAGES INCLUDING APPENDIX

Format
DIGITAL AND PHYSICAL COPY. WRITTEN IN LaTeX. DIGITIAL COPY AVAILABLE VIA DROPBOX AT https://www.dropbox.com/sh/6l75i2wp7h5fo2e/AAAJrX6reWcU5VZrVYCR219ua?dl=0

Contents

1 Preface and acknowledgements

This M.Sc. thesis was conducted at the Section for Evolutionary Genomics, Natural History Museum of Denmark, University of Copenhagen, and was supervised by Peter Rask Møller and Steen Wilhelm Knudsen. The thesis includes two manuscripts and a developed identification key for crayfish species present in Denmark. Through out this study we encountered a lot of misidentification and misunderstanding regarding crayfish species and their distribution in Denmark. Hence, one of the manuscripts and the identification key is written in Danish because it is intended for Danish readers. During this thesis fieldwork and the authors attendance at the European Crayfish Conference (9th - 12th April) was partly funded by the EvoGenomics M.Sc. thesis grand. Laboratory work was funded by Innovationsfonden in collaboration with University of Copenhagen, AmphiConsult and Eurofinns.

Both authors have contributed equally to all parts of this thesis.

We would like to express our appreciation to our supervisors for being dedicated and inspiring, and for valuable guidance and discussion. Especially Peter Rask Møller for his help during night snorkelling in Lake Furesøand Steen Wilhelm Knudsen for guiding us through the challenging world of eDNA. In addition we would like to thank everyone at the Natural History Museum of Denmark, especially Marcus A. Krag, Henrik Carl and Tore Hejl Holm-Hansen for assistance when needed. We would also like to thank Trude Vrålstad and David Strand of The Norwegian Veterinary Institute for collaboration and sharing of knowledge.

During this thesis we have tried to gather crayfish data from a lot of different sources which means that we have reached out to a lot of different people. We are very grateful to everybody who have shared their knowledge and thoughts about crayfish in order to help us. Catching crayfish requires different permissions from each lake owner, and we are very thankful that all authorities provided us with the necessary permissions. Last but not least we would like to thank our friends and families for endless support during this thesis.

2 Thesis abstract

For several hundred years crayfish (Crustacea - Decapoda - Astacidea) have played an important ecological, cultural and culinary role in Europe.

In the beginning of the 20th century the first outbreaks of crayfish plague *Aphanomyces astaci* (Schikora, 1906) occurred on the European continent and during the next hundred years it reached some of the far most corners of Europe, e.g. Finland and Turkey. Because the plague is deadly to native European crayfish, people started to stock American crayfish because of their immunity to the disease, not knowing that the American crayfish were passive carriers and would lead to an increased spread of the disease.

The number of non-native crayfish species now exceeds the number of native species in Europe. Even though recent international initiatives are fighting the invasive species, the future of the European species is still uncertain.

If we are to protect the indigenous species and limit the spread of non-indigenous species, management plans should rely on substantial knowledge on distribution and abundance of both. With human activities being the main force in the spreading of unwanted species, such management plans face major challenges in educating the public. Species identification is an issue and not many people are able to identify crayfish to species, leading to unwanted introductions and questionable data on distributions.

The indigenous European noble crayfish *Astacus astacus* (Linnaeus, 1758) is an annex V species, thus, EU member states are obliged, every sixth year, to determine if the noble crayfish is in a favourable condition or not. However, in Denmark the noble crayfish is not monitored by the national program for monitoring of water and nature (NOVANA) or any other monitoring programs. Neither are the two non-indigenous and invasive species, the narrow-clawed crayfish *Astacus leptodactylus* (Eschscholtz, 1823) and the signal crayfish *Pacifastacus leniusculus* (Dana, 1852).

Future management plans should be based on solid amount of data to be succesful. In Denmark we have very little tradition for crayfish research, so historical and distribution data is limited. Gathering of new data is typically done with traditional methods, such as trapping. These methods are selective and may vary in effectivenes, so newly introduced species in low numbers can avoid detection until established. However, early detection is crucial if impacts of invasive crayfish species are to be controlled. New genetic detection methods have been developed for several other animal groups and environmental DNA (eDNA) is on the verge of becoming an integrated, cost-efficient part of bio-monitoring. The potential of eDNA as a tool for monitoring crayfish is still to be investigated thoroughly, though.

Present master's thesis describes the status of crayfish in Denmark and evaluates future methods of detection.
The results are presented as two manuscripts.

In the first manuscript, we present the first gathering of distribution data on all species, and show updated distribution maps for all species, based on existing data and observations done during the study period. It seems that all species are well established in Denmark, although very little data is available. Furthermore, we present a species field guide, based on a morphological analysis of danish specimens, to secure correct species identification in future surveys and to prevent illegal introductions due to misidentifications.

In the second manuscript, we investigate the potential of eDNA as a monitoring tool for the crayfish species in Denmark, through development of species-specific primer-probe systems and comparison with conventional methods. We were able to develop highly species-specific systems that detected the expected species in field testing experiments. Systems were able to detect eDNA concentrations as low as 80 copy/L.

3 Manuscript one

Krebs i Danmark - opdateret udbredelse og udvikling af guide til artsidentifikation

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Manuskript til Flora og Fauna

Abstract

Crayfish (Decapoda - Astacidea) are key species in freshwater ecosystems and are often referred to as ecosystem engineers. In Europe, the number of non-indigenous species now exceeds the number of indigenous species. If we are to protect the native european species, an overview of both native and non-native species distribution is needed. In Denmark, however, we lack substantial knowledge on crayfish distribution, partly due to lack of certain identification of specimens. The present study is an update of the distribution of the three crayfish species known in Denmark; the noble crayfish *Astacus astacus* (Linneus, 1758), the narrow-clawed crayfish *Astacus leptodactylus* (Eschscholtz, 1823) species complex and the signal crayfish *Pacifastacus leniusculus* (Dana, 1852). From existing observations and observations done during the study period, the distribution is shown as presence within 10x10 km UTM-grids. We found the noble, narrow-clawed and signal crayfish present in 15, 12 and 27 UTM-grids, respectively. Despite a low number of observations, our results show that all three species are well established in Denmark, with the narrow-clawed crayfish only found on the island of Zealand. Furthermore, a morphological study is presented and results are used in development of a simple field guide to assist future species identification.

Keywords: Crayfish, distribution, Denmark, Astacus, astacus, leptodactylus, Pacifasatcus, leniusculus, identification

Resumé

Krebs (Decapoda - Astacidea) er nøglearter i ferskvand og kan påvirke lokale miljøer markant. I Europa overstiger antallet af invasive krebsearter nu antallet af hjemmehørende europæiske krebsearter. Hvis vi vil beskytte de hjemmehørende arter og bekæmpe de invasive, er det essentielt at kende til udbredelsen af dem, men i Danmark mangler vi grundlæggende viden om krebs sammenlignet med andre europæiske lande. Dette studie er en opdatering af udbredelsen af de tre kendte arter fra Danmark; flodkrebs *Astacus astacus* (Linnaeus, 1758), galizisk sumpkrebs *Astacus leptodactylus* (Eschscholtz, 1823) artskompleks og signalkrebs *Pacifastacus leniusculus* (Dana, 1852). Ud fra eksisterende observationer, samt nye observationer foretaget under dette studie, er der gjort status på udbredelsen af alle arter vist som tilstedeværelse i 10x10 km UTM-kvadrater. Vi fandt flodkrebs, galizisk sumpkrebs og signalkrebs til stede i henholdsvis 15, 12 og 27 UTM-kvadrater. På trods af et lavt antal observationer, indikerer vores resultater, at alle tre arter er veletablerede i Danmark. Yderligere præsenteres et morfologisk studie af arterne som danner grundlag for udviklingen af en simpel felt-guide til sikker artsidentifikation.

Nøgleord: Krebs, udbredelse, Danmark, Astacus, astacus, leptodactylus, Pacifastacus, leniusculus, identifikation

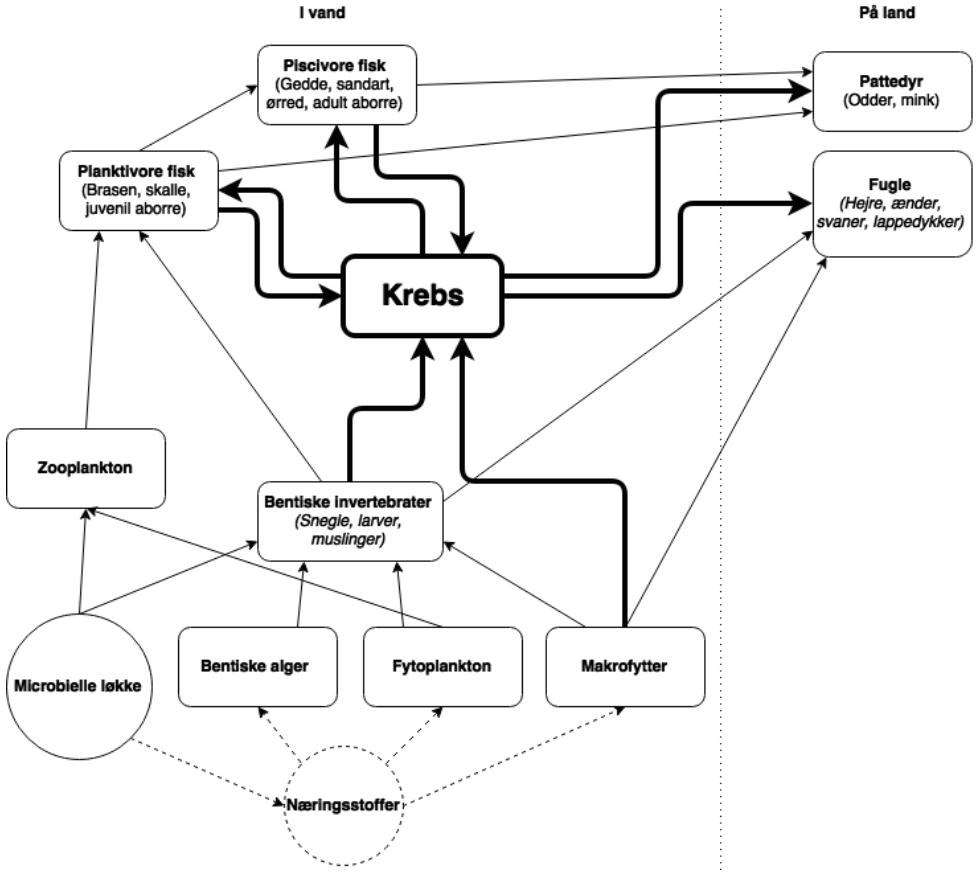
Introduktion

Krebs (Decapoda - Astacidea) hører til blandt de største invertebrater i ferskvand og er ofte beskrevet som nøglearter i limniske systemer (Gherardi og Holdich, 1999; Kouba et al., 2014; Nyström, 1999). Der findes på verdensplan omkring 600 arter opdelt i tre familier; Astacidae ($\sim$ 14 arter), Parastacidae ($\sim$ 170 arter) og Cambaridae ($\sim$ 409 arter) (Souty-Grosset et al., 2006; Crandall og Buhay, 2007).

Fra Europa kendes fem anerkendte, oprindelige arter, samt mindst ti ikke-oprindelige, introducerede arter (Gherardi og Holdich, 1999; Souty-Grosset et al., 2006; Holdich et al., 2009; Reynolds og Souty-Grosset, 2011). De introducerede arter er hovedsageligt kommet til Europa gennem import til opdræt og konsum, akvariehold og som led i reetablering af krebsevande hvor hjemmehørende arter var udryddet eller i tilbagegang. Videre spredning til naturen sker hyppigt gennem menneskelige aktiviteter (Dresser og Swanson, 2013; Gherardi og Holdich, 1999).

Ved introduktion af nye krebsearter til naturen er ikke bare de hjemmehørende krebsearter truet af den direkte konkurrence. Krebs er omnivorer og konsumerer alt fra plantemateriale til makroinvertebrater, hvilket gør dem i stand til også at ændre balancen i økosystemer, hvor der ikke tidligere har levet krebs (Gherardi og Holdich, 1999; Gherardi og Acquistapace, 2007; Souty-Grosset et al., 2006)(figur 1).

Ydermere kan nytilkomne krebs bære på sygdomme som angriber de hjemmehørende arter ekstra hårdt. Ægsporesvampen *Aphanomyces astaci* (Schikora, 1903), populært kaldet krebsepest, kom til Europa i starten af 1900-tallet og er kendt for at have forårsaget udryddelser af lokale bestande af europæiske krebsearter (Makkonen et al., 2012; Harlioğlu, 2004). Særligt i Sverige er flere bestande af flodkrebsen *Astacus astacus* (Linnaeus, 1758) blevet udryddet som følge af konkurrence og spredning af krebsepest via lovlige og ulovlige udsætninger af den invasive signalkrebs *Pacifastacus leniusculus* (Dana, 1852) (Bohman et al., 2006; Bohman og Edsman, 2011).



Figur 1: Krebs position i et typisk limnisk fødenet.

Figure 1: The position of crayfish in a typical limnic foodweb.

Fra Danmark er kendt tre arter af krebs; den hjemmehørende flodkrebs (figur 2a og 3a) samt de to invasive arter; signalkrebs (figur 2b og 3b) og galizisk sumpkrebs *Astacus leptodactylus* (Eschscholtz, 1823) artskompleks (figur 2c og 3c). Yderligere florerer rygter om tilstedeværelse af den amerikanske *Orconectes limosus* (Rafinesque, 1817) (kaldet "kamberkrebs"), men dette er ikke bekræftet. Dog findes der populationer tæt på den dansk/tyske grænse (Anja Dethlefs-Hammes, personlig kommentar, Dec. 2014).

Under arbejdet med udvikling af metoder til eDNA-detektion af krebs (Agersnap og Larsen, in prep.) stod det klart, at udbredelsen af krebs i Danmark er ringe kendt. Kun Naturstyrelsens Winbio database og Danmarks Tekniske Universitets (DTU AQUA) arbejde med signalkrebs (se Skov et al. (2010)) indeholder flere registreringer af krebs fra Danmark. De resterende registreringer er fordelt på mindre, lokale undersøgelser med andre formål end overvågning af krebs (f.eks. Henriksen et al. (2005)). Manglen på målrettet, landsdækkende registrering, skyldes formentlig den mindre interesse for krebs i Danmark sammenlignet med andre lande, som for eksempel Sverige og Finland, hvor krebs er kulturelt, økonomisk og kulinarisk vigtige (Holdich et al., 2009; Souty-Grosset et al., 2006; Skov et al., 2010). Yderligere fin-

des der på nuværende tidspunkt ingen dansk literatur der beskæftiger sig med identifikation af de tre kendte arter. Derfor er registreringer af krebs fra Danmark ofte behæftet med stor usikkerhed og stort set ubrugelige i udbredelsestudier og forvaltning. Særligt er der en udbredt misforståelse vedr. galizisk sumpkrebs, idet den populært er kendetegnet ved lange, smalle klør (deraf navnet "leptodactylus"lang klosaks) (figur 2c). Denne karakter er dog kun gældende for hanner, da hunner har markant kortere klør og derfor minder om flodkrebsen (figur 3a og c) (figur 16, side 24).

Figur 2: Hanner af (a) flodkrebs *Astacus astacus* (ZMUC-CRU-006237), (b) signalkrebs *Pacifastacus leniusculus* (ZMUC-CRU-006236) og (c) galizisk sumpkrebs *Astacus leptodactylus* (ZMUC-CRU-006246). Foto: W. B. Larsen og S. Agersnap.

Figure 2: Males of noble crayfish Astacus astacus *(ZMUC-CRU-006237) (a), signal crayfish* Pacifastacus leniusculus *(ZMUC-CRU-006236) (b) and narrow-clawed crayfish* Astacus leptodactylus *(ZMUC-CRU-006246) (c).* Photo: W. B. Larsen and S. Agersnap.

Flodkrebsen er den eneste oprindelige krebseart i Danmark, men dens udbredelse er ringe kendt og primært baseret på observationer foretaget i forbindelse med undersøgelser med andre formål (Skov et al., 2010; Larsen, 1990). Flodkrebsen findes udelukkende i ferskvand, fra søer til mindre bække (Fiskeriverket og Naturvårdsverket, 2009; Souty-Grosset et al., 2006). Den er optaget på den gældende danske rødliste som akut truet (se Miljø- og Energiministeriet et al. (1997)) og er af IUCN beskrevet som sårbar på baggrund af markante fald i antallet af europæiske populationer (Edsman et al., 2010). Derfor er vi i Danmark forpligtet til at overvåge dens udbredelse. Der foregår dog ikke systematisk overvågning af flodkrebs idag.

Figur 3: Hunner af (a) flodkrebs *Astacus astacus* (ZMUC-CRU-006237), (b) signalkrebs *Pacifastacus leniusculus* (ZMUC-CRU-006236) og (c) galizisk sumpkrebs *Astacus leptodactylus* (ZMUC-CRU-006246). Foto: W. B. Larsen og S. Agersnap.

Figure 3: Females of (a) noble crayfish Astacus astacus *(ZMUC-CRU-006617), (b) signal crayfish* Pacifastacus leniusculus *(ZMUC-CRU-006049) and (c) narrow-clawed crayfish* Astacus leptodactylus *(ZMUC-CRU-006561).* Photo: W. B. Larsen and S. Agersnap.

Signalkrebsen stammer oprindeligt fra det nordlige Amerika og er etableret i den danske natur efter import og udsætninger foretaget i 1960'erne (Skov et al., 2009). Den er uønsket i hele Europa, da den er stærkt invasiv og kan være bærer af krebsepest, som er dødelig for europæiske krebs. Der dog ikke registreret udbrud af krebsepest i Danmark. Signalkrebsen er den eneste krebs i Danmark der bliver overvåget, idet dens udbredelse løbende bliver registreret af Danmarks Tekniske Universitet (DTU Aqua)(se Skov et al. (2009, 2010)).

Den galiziske sumpkrebs er en europæisk art, som er naturligt udbredt i det Østlige Europa i området omkring det Kaspiske Hav og Sortehavet (Kouba et al., 2014). Den blev første gang i 1960'erne eksporteret levende fra Tyrkiet til konsum i store dele af Europa, herunder Danmark (Hamdi et al., 2012). Det er sandsynligvis her introduktionen til den danske natur er sket første gang. Dette er understøttet af et museumseksemplar (ZMUC-CRU-006559) fra Statens Naturhistoriske Museum i København købt fra en fiskehandler i København i 1965. Der foregår stadig import og salg af levende eksemplarer i Danmark, både fra udlandet og fra Danmark (figur 4).

Figur 4: Danskfangede levende galizisk sumpkrebs *Astacus leptodactylus* bliver solgt som danske krebs. Torvehallerne, København K, august 2015. Foto: W. B. Larsen og S. Agersnap.

Figure 4: Live narrow-clawed crayfish Astacus leptodactylus *caught in Denmark are sold as danish crayfish. Torvehallerne, Copenhagen K, August 2015.* Photo: W. B. Larsen and S. Agersnap.

Både galizisk sumpkrebs og signalkrebs er, hvor de er invasive, kendt for at være i stand til at udkonkurrere europæiske arter (Stucki og Romer, 2001; Westman et al., 2002).

For at begrænse spredning af uønskede krebs og beskytte de hjemmehørende arter, er det essentielt at kende til deres udbredelse. På Europæisk plan er der lavet gode kortlægningsprojekter (se Kouba et al. (2014); Souty-Grosset et al. (2006)) gennem internationalt samarbejde (CRAYNET[1]), men i Danmark mangler vi grundlæggende viden om udbredelsen og gode værktøjer til sikker artsidentifikation.

Formålet med nærværende artikel, er således at præsentere en opdateret status for udbredelsen af alle krebsearter i Danmark baseret på literaturgennemgang, videnskabelige samlinger og eget feltarbejde. Yderligere præsenteres et morfologisk studie af danske individer, der danner grundlag for udarbejdelsen af en simpel guide til identifikation af de tre krebs kendt fra Danmark (se vedlagte "Felt-guide til krebs i Danmark"). Vi håber at oversigten og guiden kan danne grundlag for en øget indsats indenfor forskning og forvaltning af krebs i Danmark.

[1]European crayfish as keystone species-linking science, management and economics with sustainable environmental quality (Souty-Grosset, 2005)

Materialer og metoder

Registrering og identifikation

Status for udbredelsen af de tre arter af krebs er opgjort på baggrund af indhentning af eksisterende registreringer samt registreringer foretaget i studieperioden (2014-2015). En registrering er defineret som en observation af et eller flere individer på et veldefineret sted og tidspunkt. Følgende oplysninger om registreringer er, om muligt, inhentet: art, antal, dato, observatør, hvem der har artsbestemt, evt. materiale anvendt til artsbestemmelse, biotop, observationsmetode og artsbestemmelsessikkerhed (for eksempel, se appendix, side 38 figur 19). Bestemmelsessikkerhed er registreret som A eller B, hhv. sikker bestemmelse, verificeret af forfatterne og usikker, ikke-verificeret bestemmelse. Ved registreringer uden oplysninger om art er art angivet som ukendt art. Alle B-bestemte observationer bliver uanset kilde behandlet som ukendt art grundet tidligere nævnte problemer med artsidentifikation. Undtaget fra dette er signalkrebs registreret af fagpersoner, da denne art vurderes til at være lettere genkendelig p.g.a sine unikke karakteristika, som den hvide plet ved basis af klosaksen.

Registreringer i dette studie er typisk verificeret på baggrund af fysisk inspektion af dyret selv eller billedmateriale og er artsbestemt ved hjælp af publikationerne *Atlas of Crayfish in Europe* af Souty-Grosset et al. (2006) samt *Crayfish field guide of Romania* af Pârvulescu (2010) og referenceindivider verificeret ved genetisk analyse som en del af udviklingen af eDNA-metoder til detektion af krebs (Agersnap og Larsen in prep.).

Eksisterende registreringer

Alle tilgængelige registreringer er medtaget i studiet. Videnskabelige samlinger fra de naturhistoriske museer i Århus og København samt naturstyrelsens registreringsdatabase Winbio (pr. maj 2015). Derudover har naturskoler og privatpersoner bidraget med registreringer. Ved hjælp af internetsiderne `www.google.dk`, `www.youtube.com` og `www.facebook.com` er der søgt efter online rapporteringer om krebs. Som nøgleord er brugt de latinske artsnavne og de gængse, danske navne. Akvakulturer med opdræt af krebs er medtaget i registreringen.

Registrering foretaget i studieperioden

Indsamling og registrering af krebs i studieperioden er foretaget ved hjælp af følgende metoder:

- Visuelle observationer og fangst ved snorkling og med redskaber fra land (rejehov, ketcher o.l.)
- Fældefangst

Undersøgte vande rangerer fra større søer og vandløb, til mindre vandhuller, bække og moser og de bedst egnede metoder er valgt til hver lokalitet.

Visuelle metoder er anvendt i vande hvor sigtbarheden har tilladt det samt i vande med god adgang fra bredden. Om muligt, er indsatsen lagt i de mørke timer af døgnet og koncentreret i områder vurderet

egnet til krebs. Med en kraftig lommelygte er lokaliteterne blevet gennemsøgt for krebs eller tegn på krebs (skalrester). Krebs er enten fanget med hænderne eller med traditionel ketcher og opbevaret i keepnet under indsamling.

Til fældefangst er anvendt agnede fælder af cylindertypen med målene 33 x 60 cm., maskestørrelsen 22 mm og dobbeltindgang (købt i Jagt- og Fiskerimagasinet i København. Som agn er brugt frossen, fersk fisk. Der er blevet fisket med minimum én fælde over minimum én nat. Alle befiskninger er foretaget med tilladelse fra relevante myndighed. Alle fælder blev behandlet med Virkon® S mellem befiskninger for at minimere risiko for spredning af pathogener mellem vande.

Udarbejdelse af felt-guide ud fra morfologiske undersøgelser

I den morfologiske undersøgelser indgår udelukkende individer fra Danmark. Gennem feltarbejde er der indsamlet eksemplarer af begge køn i alle størrelser for de tre arter (tabel 1).

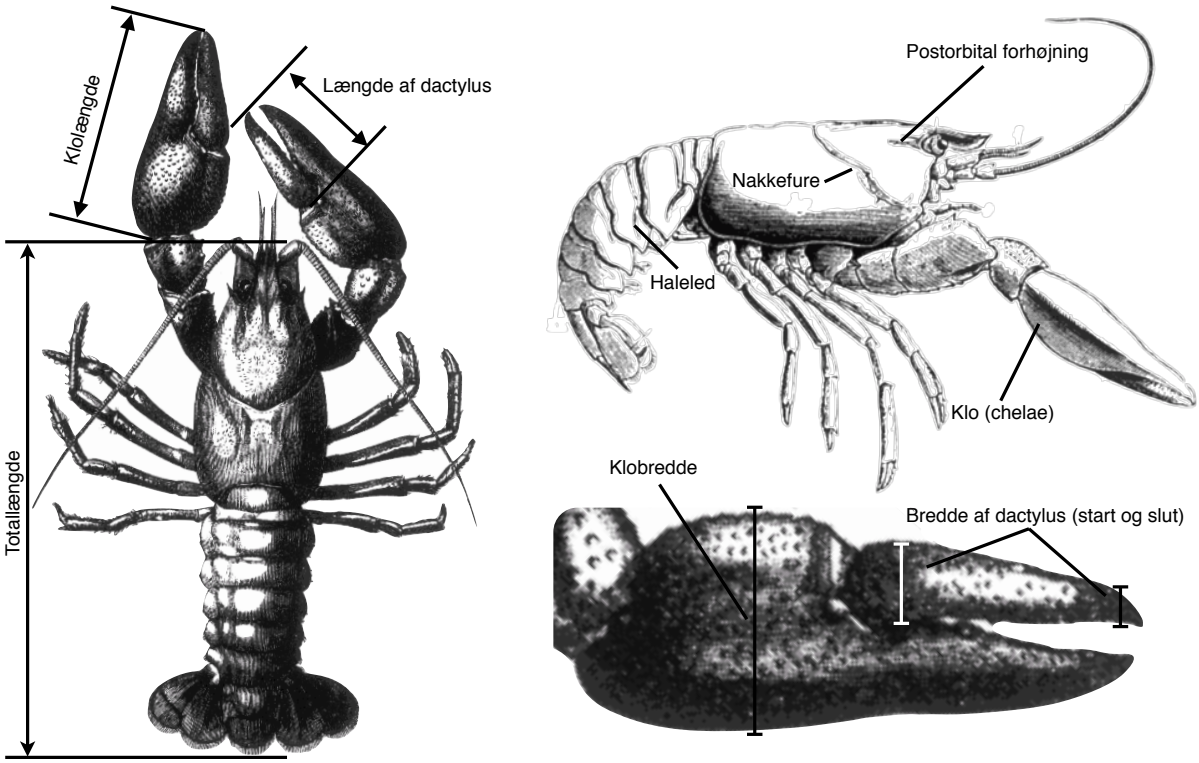
Tabel 1: Antallet af målte individer i den morfologiske undersøgelse.

Table 1: Number of individuals measured in the morphological analysis. Species from the top: Astacus astacus*,* Astacus leptodactylus*,* Pacifastacus leniusculus*.*

Art	Hanner Antal	Hunner Antal
Flodkrebs	31	22
Galizisk sumpkrebs	43	44
Signalkrebs	18	29

Følgende karakterer er anvendt som nøglekarakterer i vedlagte "Felt-guide til danske krebs"(figur 5): Totallængde (TL), klolængde (KL), klobredde (målt på det bredeste sted) (KB), længde af dactylus (kloens bevægelige finger) (DL), bredde af dactylus (middelværdien af bredde ved start og slut), antal mærkbare pigge langs nakkefuren (PN), antal mærkbare pigge på de postorbitale forhøjninger (PPO) og haleleddenes form. Totallængde er målt til nærmeste mm og andre karakterer er målt til nærmeste 0,1 mm. Klolængde- og bredde er målt på den venstre eller mest intakte klo.

Det er blevet vurderet at de nævnte karakterer er tilstrækkelige til at opnåsikker artsidentifikation af krebsene i Danmark.



Figur 5: Karakterer anvendt i udarbejdelse af den vedlagte "Felt-guide til danske krebs". Modificeret fra "The Crayfish"af T. H. Huxley, 1880.

Figure 5: Characteristics used in the included "Felt-guide til danske krebs". Modified from "The Crayfish"by T. H. Huxley, 1880.

Databehandling

Al data er håndteret i Microsoft® Excel® 2008 og kortmateriale er udarbejdet i MapInfo Pro® v. 15.0, Pitney Bowes®. Statitiske analyser er foretaget i SPSS v. 22.0, IBM corp. Morfologiske data blev testet for normalfordeling ved Shapiro-Wilk test, derefter blev variationer mellem arter og køn testet ved ANOVA test og efterfølgende Tukeys test, $\alpha = 0.05$.

Registreringer er stedfæstet så præcist som muligt med geografiske koordinater (længdegrad/breddegrad) og bliver behandlet som til stede/ikke til stede uafhængig af antal individer observeret. Alle registreringer er inddelt efter de geografiske områder; Jylland, Fyn, Sjælland-Lolland-Falster og Bornholm, samt biotoptype; sø, vandhul (defineret som mindre, ofte lavvandede vande), vandløb og brakvand og dato for observation. Der bliver i resultaterne skelnet mellem observationer foretaget før og efter 1960, da det er vurderet, at det er i 1960'erne introduktionen af fremmede krebsearter er sket. Antal registreringer er opdelt i 10 års intervaller og sorteret efter måned. Yderligere er registreringer sorteret efter biotoptype.

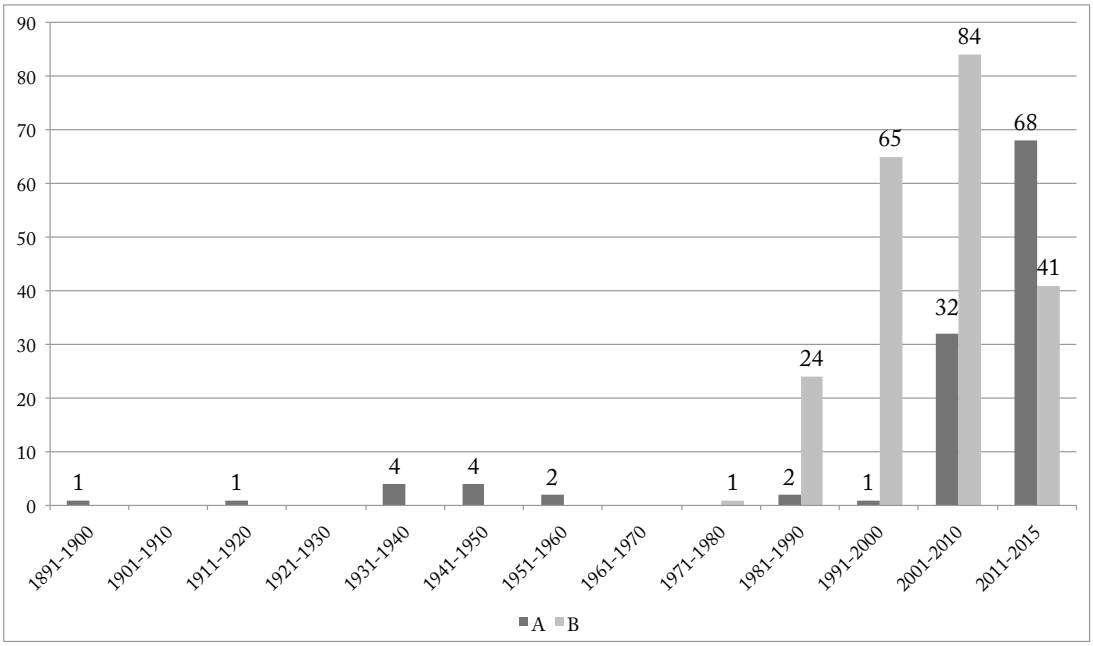
Status for udbredelse er vist på kort som verificeret (A-bestemmelser) tilstedeværelse i 10 x 10 km UTM kvadrater, en metode bl. a. anvendt til Dansk Pattedyratlas af Baagøe og Jensen (2007) og Atlas over Danske Ferskvandsfisk af Carl og Møller (2012). Dette kompenserer for eventuelt klumpende observationer, samt fortrolige oplysninger, da mange ønsker at holde deres krebsevande hemmelige af hensyn til fiskeritrykket.

Kun eksakte registreringer er medtaget, og der er således ikke korrigeret for tilstedeværelse i vandsystemer dækket af flere kvadrater.
Resultater er opgjort på baggrund af registreringer foretaget til og med 24-08-2015.

Resultater

Registrering

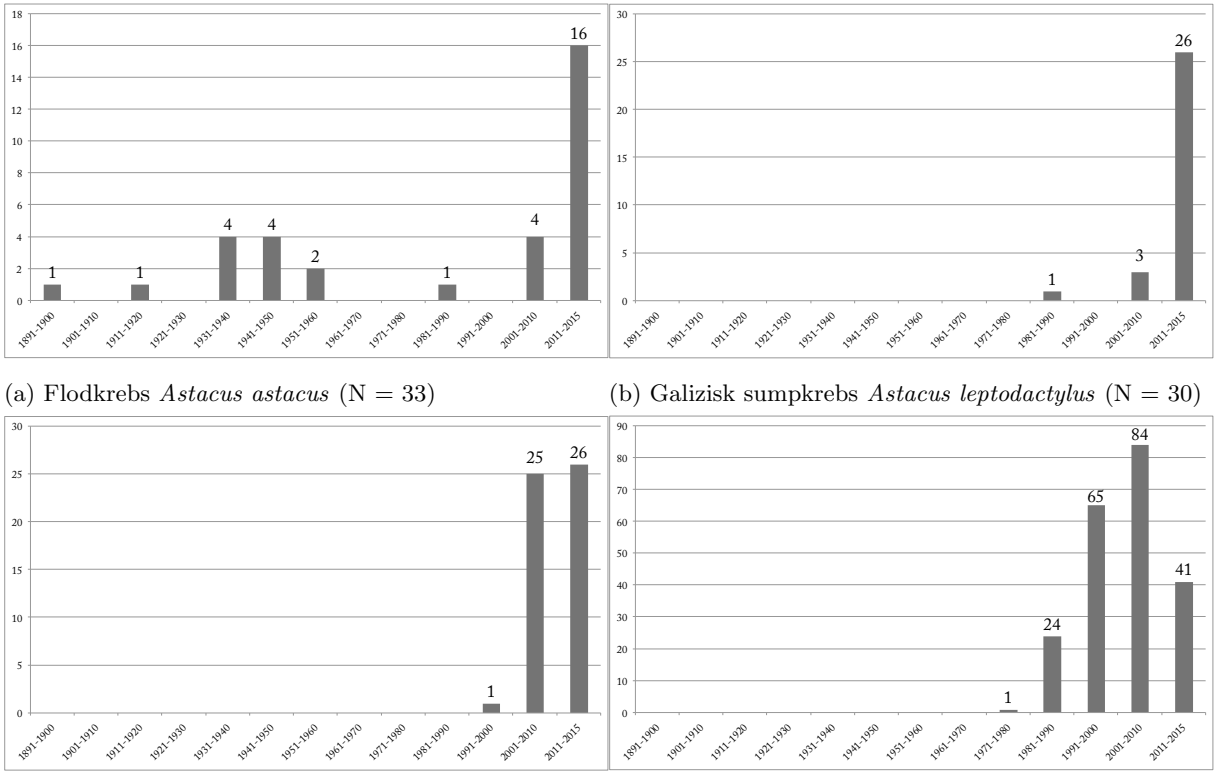
I alt er 330 registreringer af krebs foretaget i hele Danmark mellem 1891 og 2015 (august) medtaget i studiet (figur 6). Antallet af A-registreringer er 115 og antallet af B-registreringer er 215. Størstedelen er foretaget i perioden fra 1980-2015 (august).



Figur 6: Antal registreringer af krebs fra 1891 til 2015 (august) (N = 330). A = A-bestemte registreringer (N = 115). B = B-bestemte registreringer (N = 2015).

Figure 6: Number of crayfish recordings from 1891 till 2015 (August) (N = 330). A = verified observations (N = 115), B = unverified observations (N = 215).

Registreringer af flodkrebs udgør 33, galizisk sumpkrebs 30, signalkrebs 52 og ukendt art 215 (figur 7).



(a) Flodkrebs *Astacus astacus* (N = 33)

(b) Galizisk sumpkrebs *Astacus leptodactylus* (N = 30)

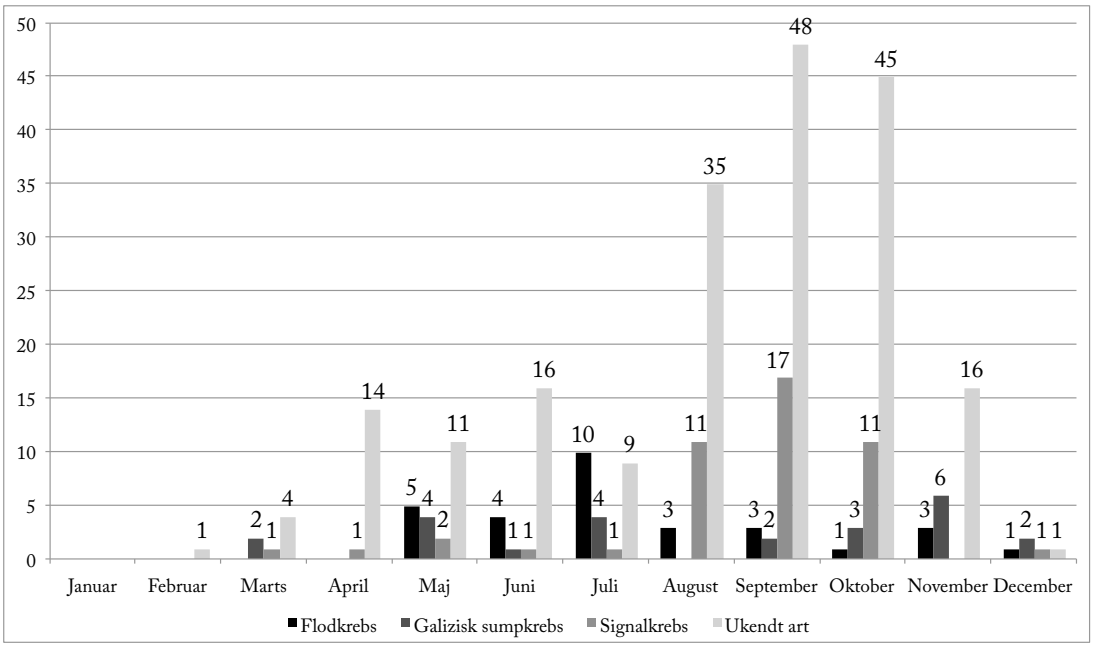
(c) Signalkrebs *Pacifastacus leniusculus* (N = 52)

(d) Ukendt art (N = 215)

Figur 7: Antal A-registreringer for alle arter og registreringer af ukendt art fra 1891 til 2015 (august).

Figure 7: Number of recordings from 1891 till 2015 (August) of (a) noble crayfish Astacus astacus*, (b) narrow-clawed crayfish* Astacus leptodactylus *and (c) signal crayfish* Pacifastacus leniusculus*. (d) Number of unidentified species.*

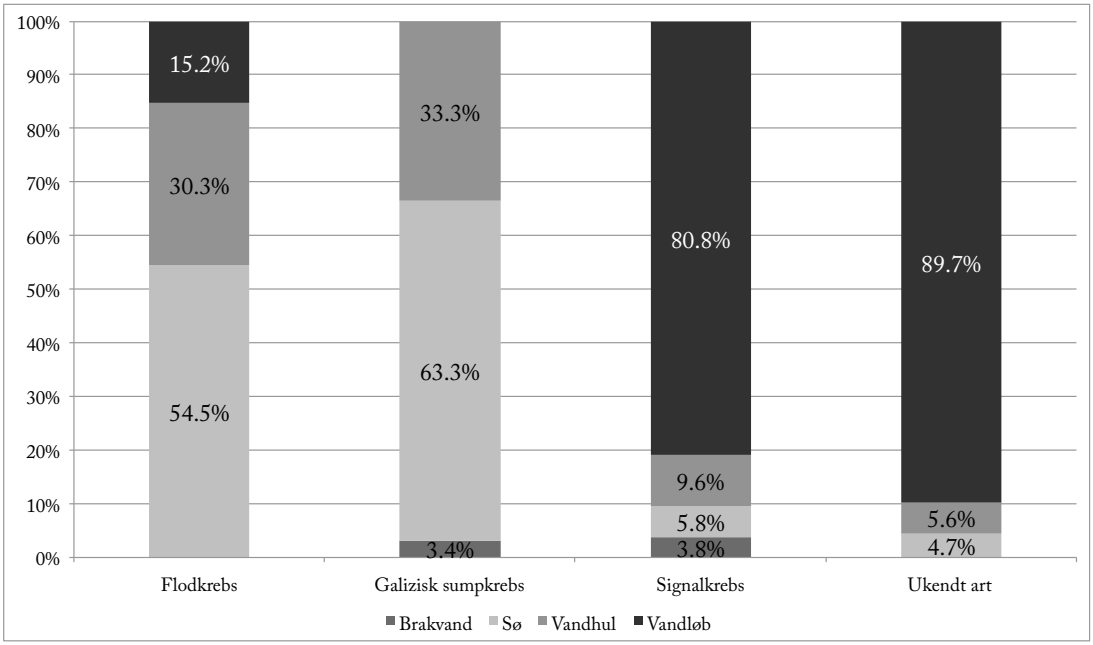
Flest registreringer er foretaget i månederne august, september og oktober, som samlet udgør 60% af alle registreringer med angivelse af måned. Det samlede antal registreringer, indeholdende information om måned, er 300 (figur 8).



Figur 8: Samlet antal registreringer fordelt på måneder, fra 1891 til 2015 (august)(N = 300). Registreringer uden angivelse af måned er udeladt (N = 30).

Figure 8: Total number of recordings per month, from 1981 till 2015 (August). Recordings without registered month are not shown (n = 30). Species from the left: Astacus astacus*,* Astacus leptodactylus*,* Pacifastacus leniusculus*, unknown species.*

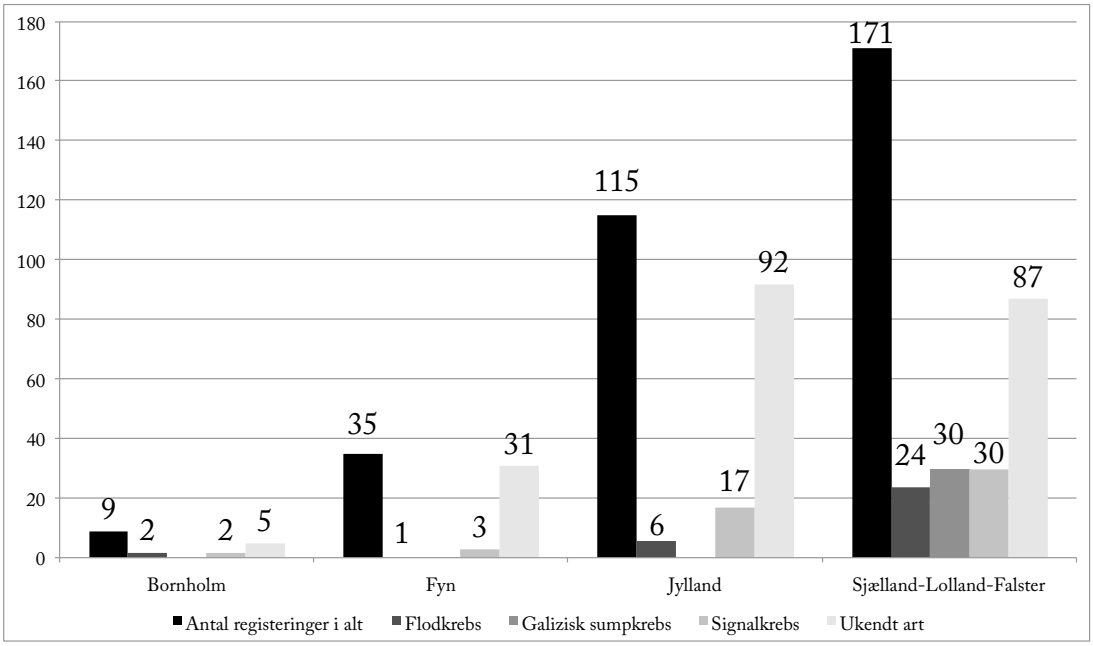
77% af alle registreringer, på tværs af arter, er fra vandløb, 13% er fra søer, 9% er fra vandhuller og 1% er fra brakvand (figur 9).



Figur 9: Andelen af registreringer i procent fra habitattype for flodkrebs, galizisk sumpkrebs, signalkrebs og ukendt art.

Figure 9: Percentage of recordings of species per habitat type (brackish water, lake, pond, stream). Species from the left: Astacus astacus*,* Astacus leptodactylus*,* Pacifastacus leniusculus*, unknown species.*

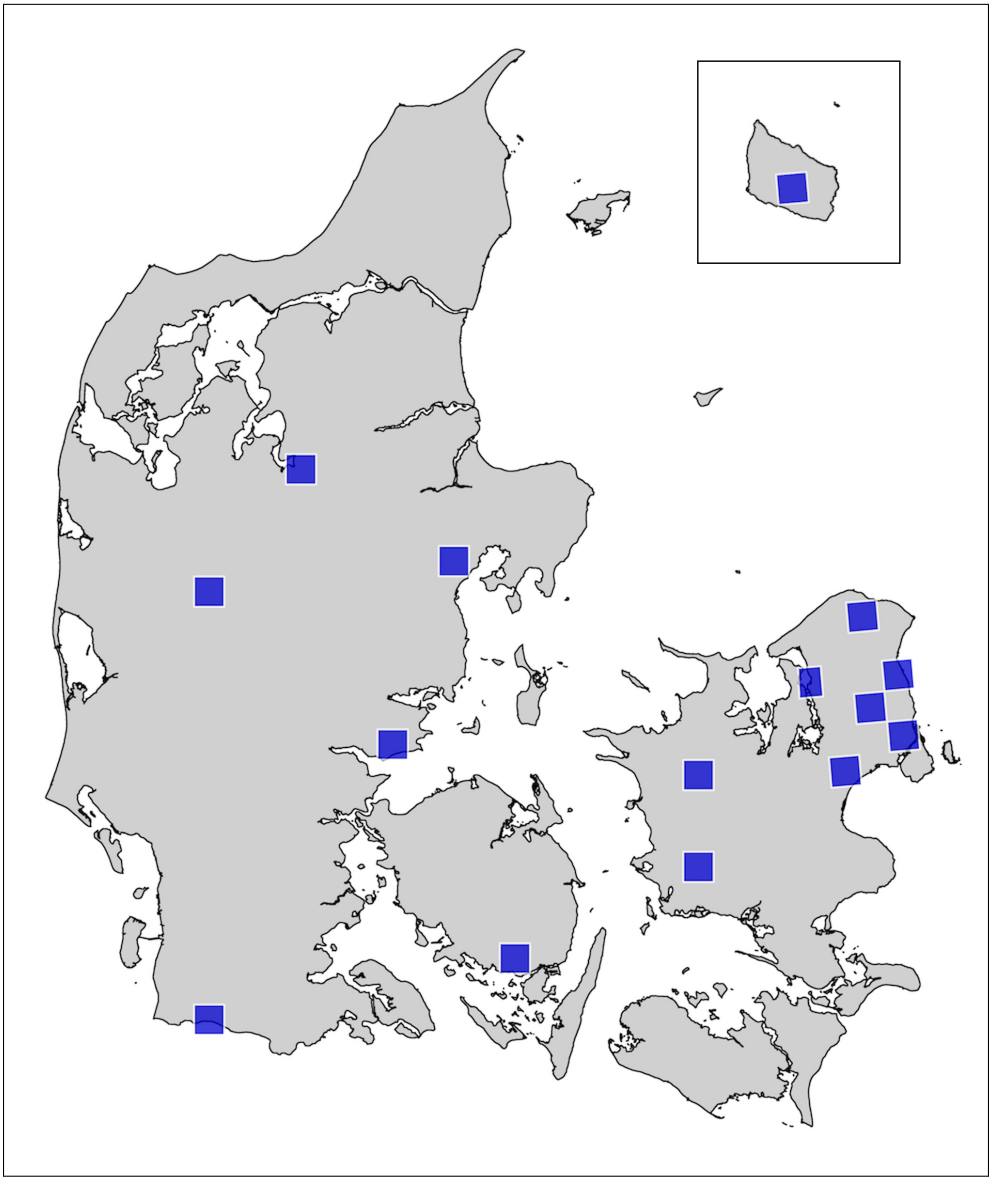
171 registreringer er foretaget på Sjælland-Lolland-Falster, 115 i Jylland, 35 på Fyn og 9 på Bornholm (figur 10).



Figur 10: Antal registreringer pr. geografisk område.

Figure 10: Number of recordings from geographical regions. From the left: Total recordings, Astacus astacus*,* Astacus leptodactylus*,* Pacifastacus leniusculus*, unknown species.*

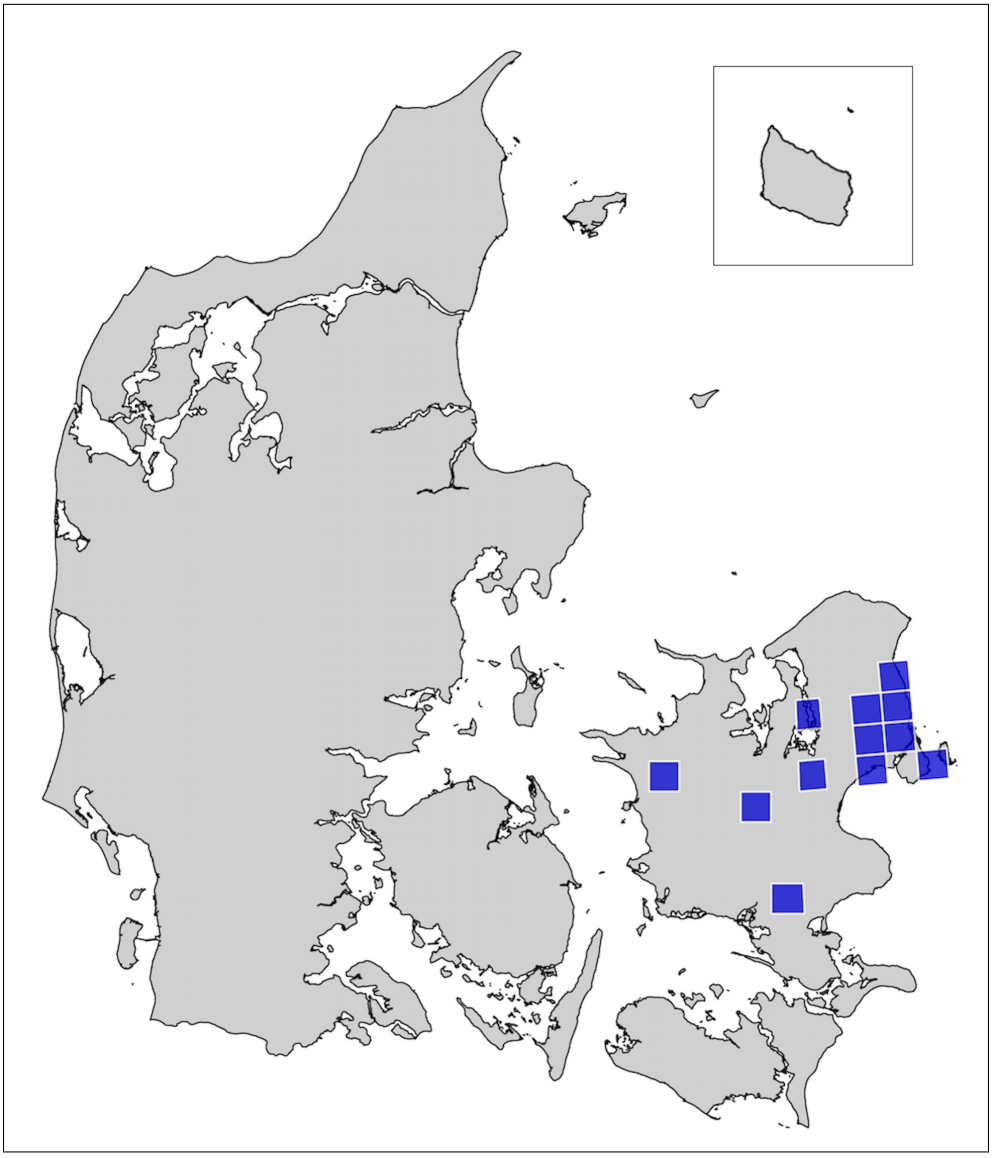
Status for udbredelse af krebs i Danmark



Figur 11: Status for udbredelsen af flodkrebs *Astacus astacus* efter 1960 vist som tilstedeværelse i 10x10 km UTM-kvadrater (N = 15).

Figure 11: Noble crayfish Astacus astacus *distribution after 1960 shown as presence within 10x10 km UTM-grids (N = 15).*

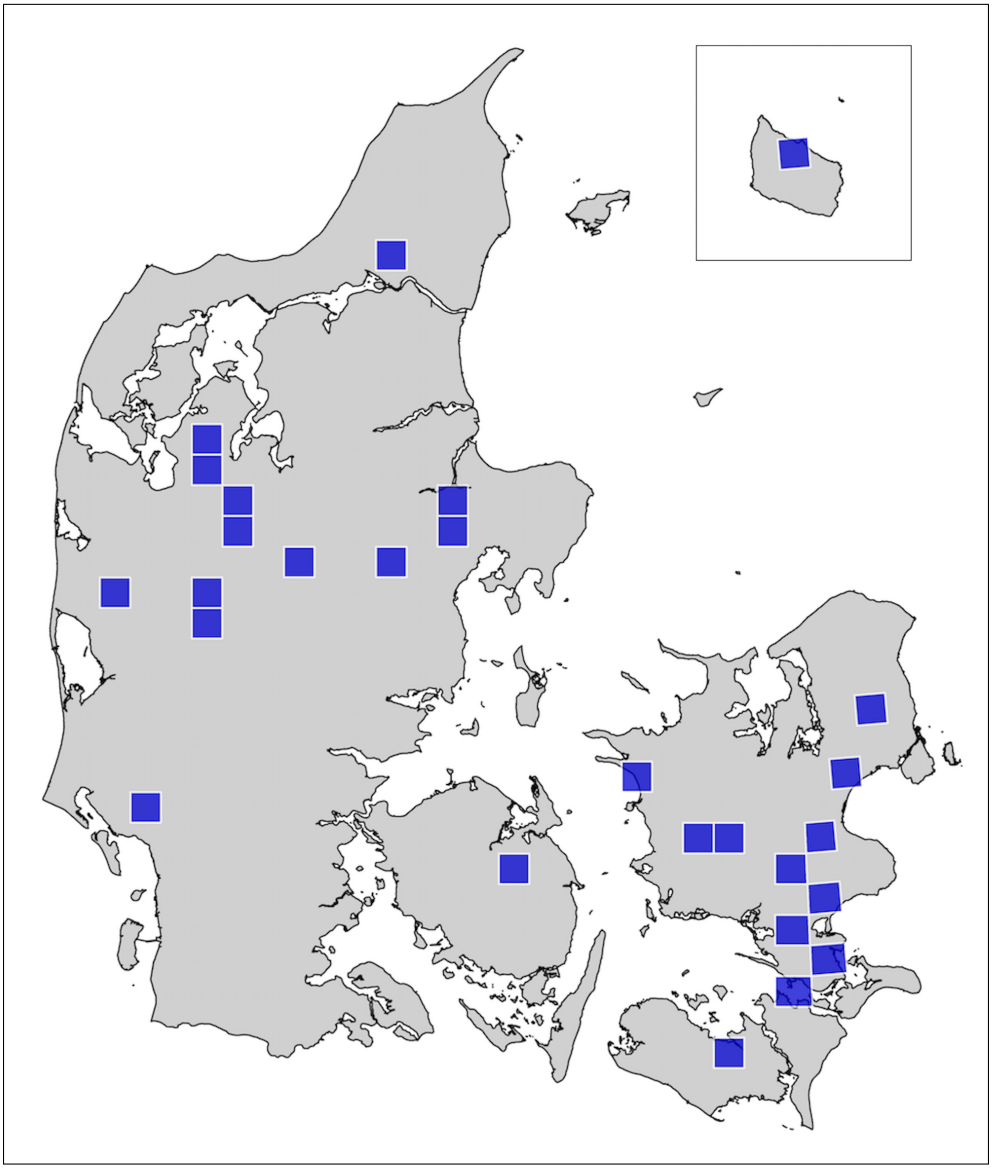
Tilstedeværelse af flodkrebs er verificeret i 15 kvadrater (figur 11). Flodkrebsen er ikke efter 1960 registreret i Nordjylland, på Lolland-Falster samt mindre øer.



Figur 12: Status for udbredelsen af galizisk sumpkrebs *Astacus leptodactylus* vist som tilstedeværelse i 10x10 km UTM-kvadrater (N = 12).

Figure 12: Narrow-clawed crayfish Astacus leptodactylus *distribution shown as presence within 10x10 km UTM-grids (N = 12).*

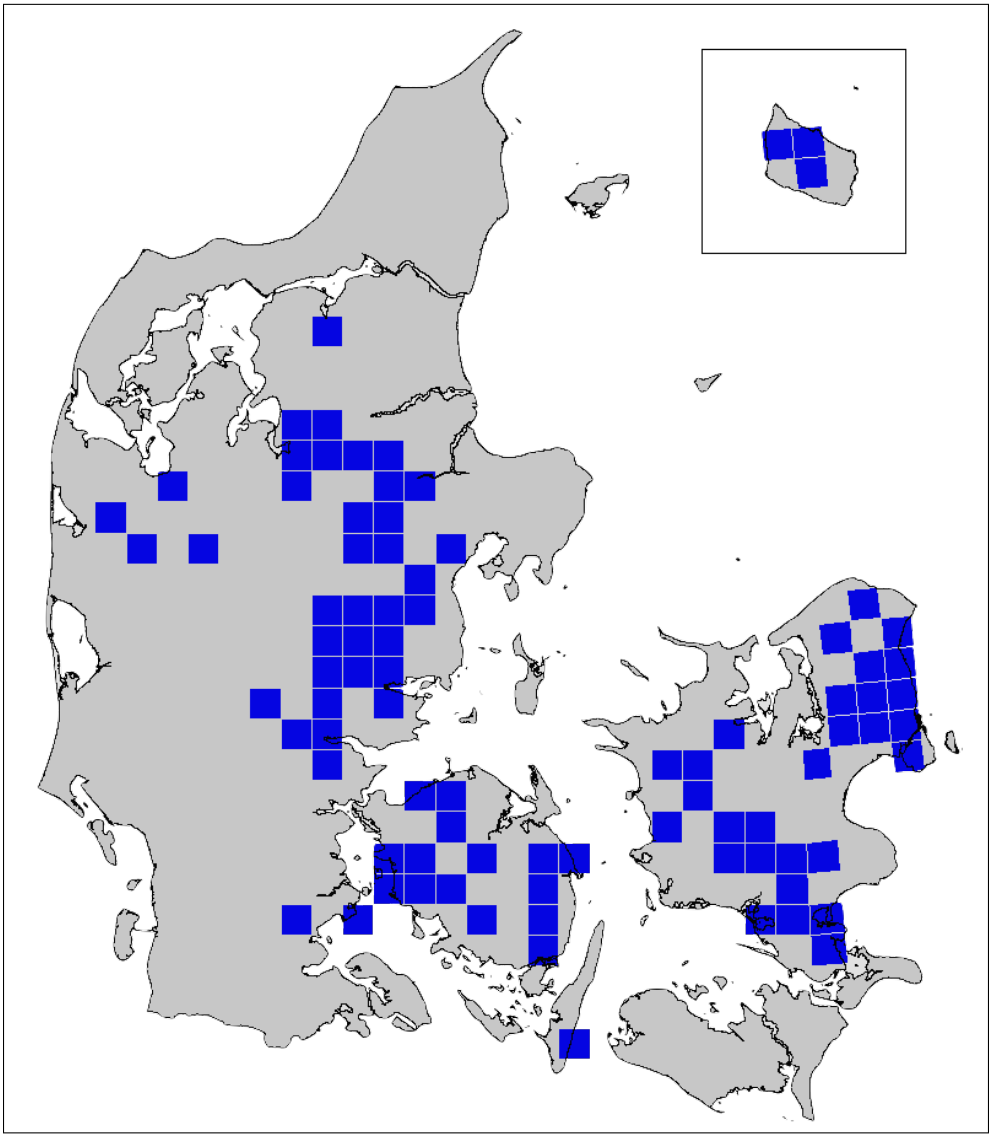
Den verificerede udbredelse af galizisk sumpkrebs begrænser sig til Sjælland, hvor den er udbredt i 12 kvadrater (figur 12). Én registrering er fra brakvand i Kastrup Havn (2014).



Figur 13: Status for udbredelsen af signalkrebs *Pacifastacus leniusculus* vist som tilstedeværelse i 10x10 km UTM-kvadrater (N = 27).

Figure 13: Signal crayfish Pacifastacus leniusculus *distribution shown as presence within 10x10 km UTM-grids (N = 27).*

Signalkrebsen er registreret i 27 kvadrater fra hele landet (figur 13). Yderligere er to observationer foretaget i brakvand, én i Storstrømmen ved Vordingborg (2013) og én i Køge bugt ud for Mosede Havn (2014).



Figur 14: 10x10 km UTM-kvadrater indeholdende registreringer af ukendt art (N = 86).

Figure 14: 10x10 km UTM-grids showing recordings of unknown species (N = 86).

I alt er der registreret ukendt art i 86 kvadrater (figur 14).

Udarbejdelse af felt-guide ud fra morfologiske undersøgelser

Resultatet af alle målinger og tællinger er vist i tabel 3.

Der er ingen forskel i klolængde relativt til totallængde mellem hanner af alle arter (F=1,65, p=0,199) eller mellem hunner af alle arter (F=2,26, p=0,111) (figur 15).

For alle arter gælder, at hanner har længere klør relativt til totallængden end hunner (flodkrebs: F=39,96, p<0,001; galizisk sumpkrebs: F=31,10, p<0,001; signalkrebs: F=30,98, p<0,001) (tabel 2, figur 16). Ligeledes har hanner bredere klør relativt til totallængden (flodkrebs: F=22,36, p<0,001, galizisk sumpkrebs: F=16,7, p<0,001, signalkrebs: F=23,86, p<0,001).

Hunner har bredere klør relativt til klolængden end hanner, gældende for flodkrebs (F=4,44, p=0,04) og galizisk sumpkrebs (F=20,23, p<0,001) (tabel 2, figur 17a, b). Der er ingen signifikant forskel på hanner og hunner for signalkrebs (F=1,56, p=0,218) (figur 17c).

For galizisk sumpkrebs gælder, at hanner har længere dactylus relativt til klolængden end hunner (F=6,64, p=0,013) (tabel 2). For signalkrebs har hunner længere dactylus relativt til klolængde end hanner (F=5,30, p=0,026). Der er ingen signifikant forskel i længde af dactylus for flodkrebs (F=1,11, p=0,297).

Tabel 2: Resultater af ANOVA test på effekten af køn på målte karakterer indenfor de tre arter. KL/TL: klolængde som procent af totallængde, KB/TL: klobredde som procent af totallængde, KB/KL: klobredde som procent af klolængde, DL/KL: længde af dactylus som procent af klolængde, DB/DL: bredde af dactylus som procent af længde af dactylus.

Table 2: Results from ANOVA test on the effect of sex on morphometric measures in all three species. Species from the left; Astacus astacus*,* Astacus leptodactylus*,* Pacifastacus leniusculus*. KL/TL: Claw length as percentage of total length, KB/TL: claw width as percentage of total length, KB/KL: claw width as percentage of claw length, DL/KL: length of dactylus as percentage of claw length, DB/DL: dactylus width as percentage of dactylus length.*

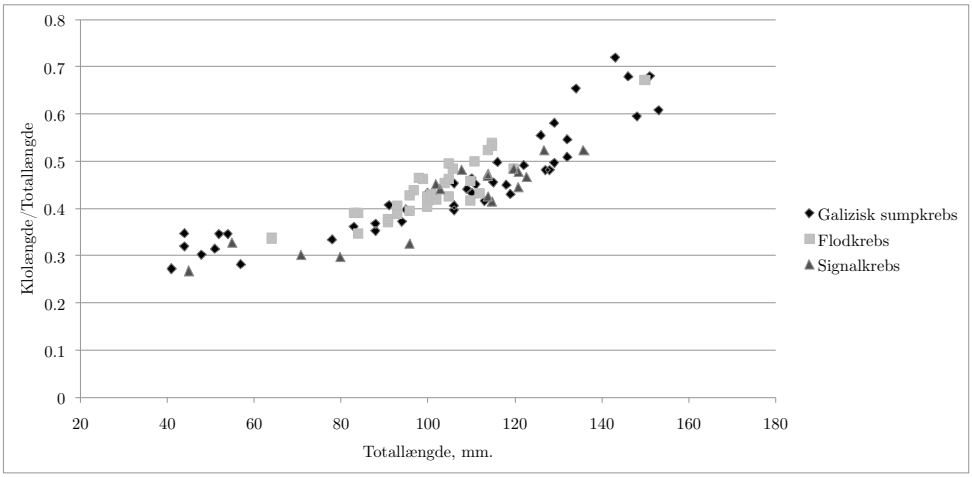
	Flodkrebs		Galizisk sumpkrebs		Signalkrebs	
Karakter	Han	Hun	Han	Hun	Han	Hun
KL/TL	44,31***	34,64***	47,19***	33,56***	42,10***	32,74***
KB/TL	16,73***	13,61***	16,58***	13,58***	18,63***	14,02***
KB/KL	37,75*	39,22*	35,76***	40,62***	43,89	42,71
DL/KL	56,88	57,52	57,31*	55,45*	55,98*	57,60*
DB/DL	15,26*	15,93*	15,20**	17,80**	16,53	16,74

***: p<0,001. **:p<0,01. *:p<0,05.

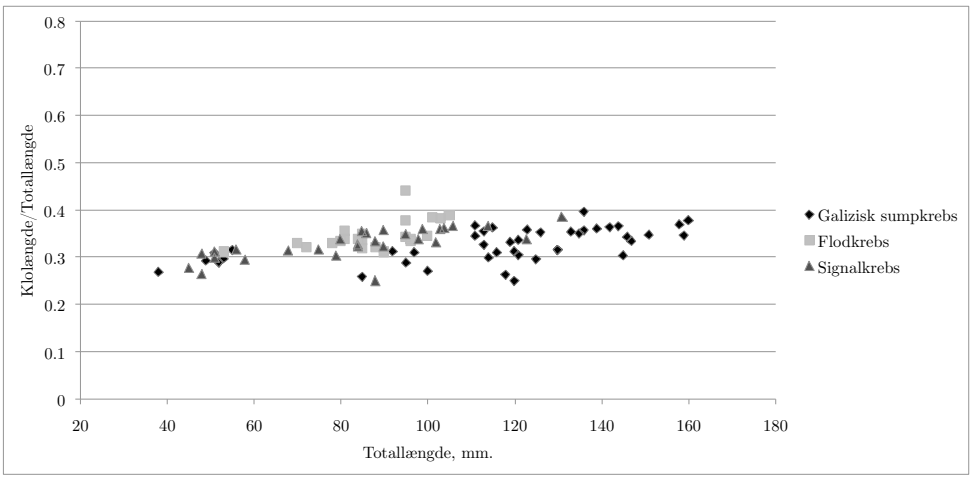
Tabel 3: Morfologiske karakterer målt og talt i den morfologiske undersøgelse. KL/TL: klolængde som procent af totallængde, KB/TL: klobredde som procent af totallængde, KB/KL: klobredde som procent af klolængde, DL/KL: længde af dactylus som procent af klolængde, DB/DL: bredde af dactylus som procent af længde af dactylus. Alle værdier er vist som middelværdi med interval og standardafvigelse i parentes. Tællinger er vist med interval og median i parentes. PN: mærkbare pigge langs nakkefuren, PPO: mærkbare pigge på postorbitale forhøjninger.

Table 3: Measurements and countings used in the morphological analysis. KL/TL: Claw length as percentage of total length, KB/TL: claw width as percentage of total length, KB/KL: claw width as percentage of claw length, DL/KL: length of dactylus as percentage of claw length, DB/DL: dactylus width as percentage of dactylus length. All values are shown as means with range and standard deviation in parenthesis. Countings are shown with range and median in parenthesis. PN: number of notable spines along cervical groove, PPO: number of notable spines on postorbital ridges.

	Flodkrebs		Galizisk sumpkrebs		Signalkrebs	
Karakter	Han (n=31)	Hun (n=22)	Han (n=43)	Hun (n=44)	Han (n=18)	Hun (n=29)
TL	101,74 (64-150, 14,67)	87,18 (53-105, 12,35)	103,44 (41,00-153,00, 31,96)	113,55 (38,00-160,00, 32,96)	103,61 (45,00-136,00, 25,20)	83,76 (45,00-131,00, 23,13)
KL/TL	44,31 (33,67-67,28, 6,67)	34,64 (31,09-44,17, 3,10)	44,96 (27,19-71,99, 11,14)	32,39 (25,00-39,65, 3,54)	42,10 (26,76-52,39, 8,14)	32,74 (24,99-38,62, 3,22)
KB/TL	16,73 (12,64-25,09, 2,71)	13,61 (11,23-18,76, 1,77)	15,78 (10,53-24,22, 3,41)	12,97 (10,20-15,30, 1,37)	18,63 (10,93-23,62, 4,49)	14,02 (8,97-17,88, 1,91)
KB/KL	37,75 (32,95-43,51, 2,46)	39,22 (34,96-44,69, 2,54)	35,59 (26,98-43,95, 3,95)	40,25 (30,61-50,00, 4,07)	43,89 (34,91-48,53, 3,81)	42,71 (35,88-49,45, 2,67)
DL/KL	56,88 (53,35-62,05, 2,11)	57,52 (53,72-62,19, 2,24)	58,25 (50,00-68,75, 3,74)	56,43 (50,00-70,00, 3,38)	55,98 (51,14-60,34, 2,42)	57,60 (53,94-63,66, 2,30)
DB/DL	15,26 (13,48-17,86, 1,20)	15,93 (14,36-18,42, 0,99)	15,20 (11,36-22,07, 2,99)	17,80 (13,56-22,81, 2,24)	16,53 (13,58-19,10, 1,47)	16,74 (14,19-19,96, 1,50)
PN	1-4 (1)	1-3 (1)	1-3 (2)	1-3 (2)	0	0
PPO	1-2 (1)	1-2 (2)	2	2	1-2 (1)	2



(a) Hanner



(b) Hunner

Figur 15: Klolængde/totallængde plottet mod totallængde for (a) hanner og (b) hunner af alle arter.

Figure 15: Length of claw/total length plotted against total length for (a) males and (b) females of all species.

Der er ingen forskel i klobredde relativt til totallængde hos hanner (F=2,16, p=0,123) eller hunner (F=0,62, p=0,542).

Der er signifikant forskel i klobredde relativt til klolængde mellem arter for både hanner (F=29,30, p<0,001) og hunner (F=9,17, p<0,001) (figur 18). Post hoc test (Tukeys) viser at hunner af signalkrebs har bredere klør relativt til klolængde end hunner af flodkrebs (p<0,001) og hunner af galizisk sumpkrebs (p=0,025) (figur 18b). Hanner af signalkrebs har bredere klør end hanner af flodkrebs (p<0,001) og hanner af galizisk sumpkrebs (p<0,001) (figur 18a).

Hunner har bredere dactylus relativt til længden af dactylus for flodkrebs (F=4,64, p=0,036) og galizisk sumpkrebs (F=12,91, p=0,001) (tabel 2). Der er ingen forskel i bredden af dactylus for hunner af signalkrebs (F=0,213, p=0,647).

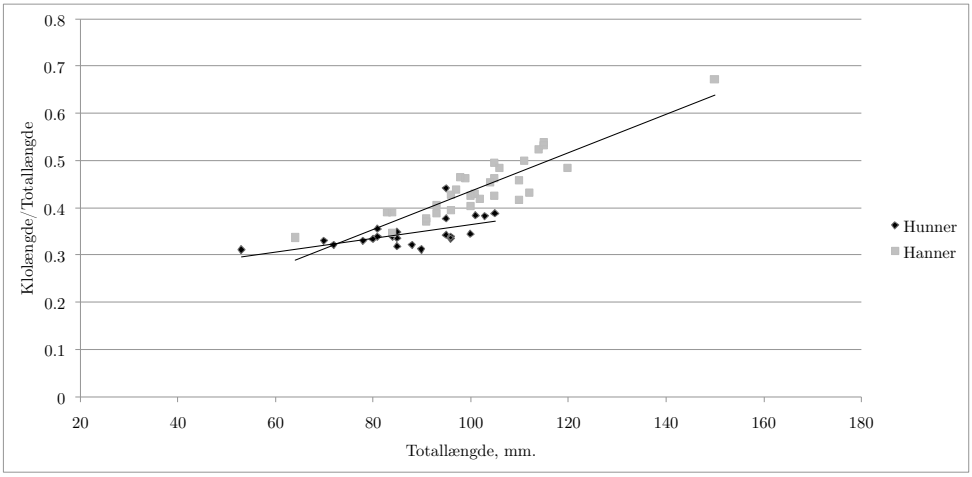
Der er ingen forskel i længde af dactylus relativt til klolængde hos hanner (F=1,50, p=0,231). Der er ingen forskel i bredde af dactylus relativt til længde af dactylus for hanner (F=2,76, p=0,070).

Længde af dactylus relativt til klolængde er signifikant forskellig hos hunner (F=7,82, p=0,001). Post hoc test (Tukeys) viser at hunner af galizisk sumpkrebs har signifikant kortere dactylus relativt til klolængde end hunner af flodkrebs (p=0,006) og hunner af signalkrebs (p=0,002). Der er ingen forskel mellem hunner af flodkrebs og hunner af signalkrebs (p=0,990).

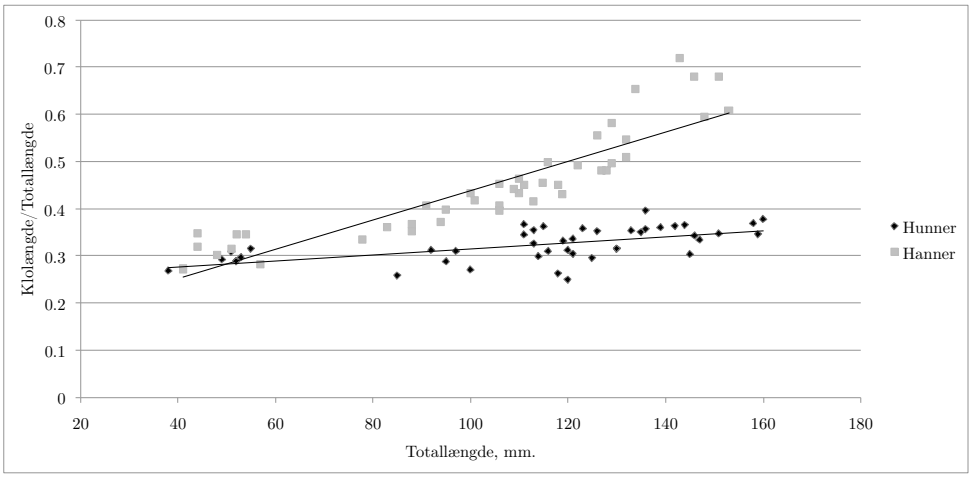
Bredde af dactylus relativt til længde af dactylus er signifikant forskellig hos hunner (F=7,59, p=0,001). Post hoc test (Tukeys) viser at den relative bredde af dactylus for galizisk sumpkrebs er bredere end for flodkrebs (p=0,001), men ikke bredere end signalkrebs (p=0,056). Der er ingen forskel mellem flodkrebs og signalkrebs (p=0,213).

Der er ikke observeret forskelligheder mellem køn i undersøgelsen af haleleddenes form. Samtlige galizisk sumpkrebs havde symmetriske haleled med tydelig pig for enden. Både flodkrebs og signalkrebs havde alle tydeligt asymmetriske haleled, ofte uden mærkbar pig.

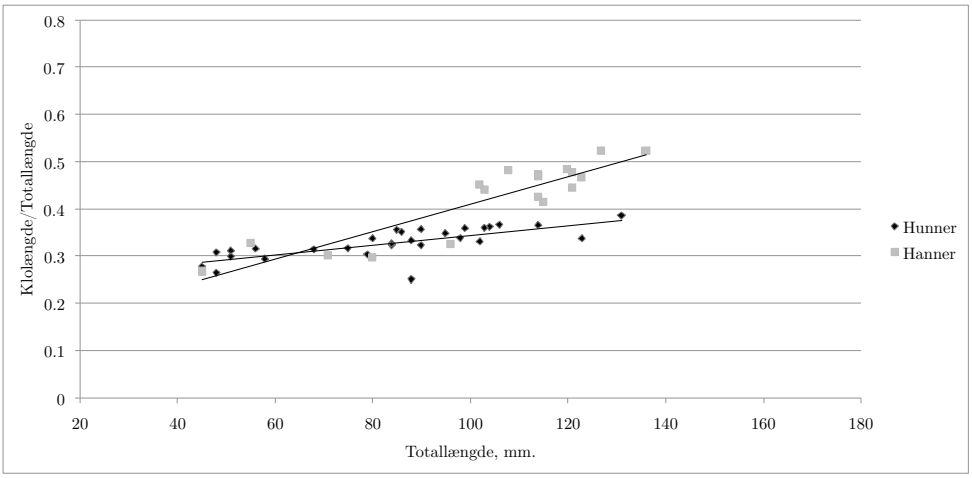
Der er under vejs i studiet observeret store variationer i farver, også indenfor samme art. Kun signalkrebsen adskiller sig fra de andre ved den hvide plet ved basis af klosaksen (dactylus) (figur 2). Flodkrebs og galizisk sumpkrebs er observeret i mange forskellige farver og kan ikke med sikkerhed skelnes på farver. Der er dog en tendens til, at flodkrebsen er rødlig på undersiden af klør, hvor den galiziske sumpkrebs ofte er farveløs.



(a) Flodkrebs *Astacus astacus*



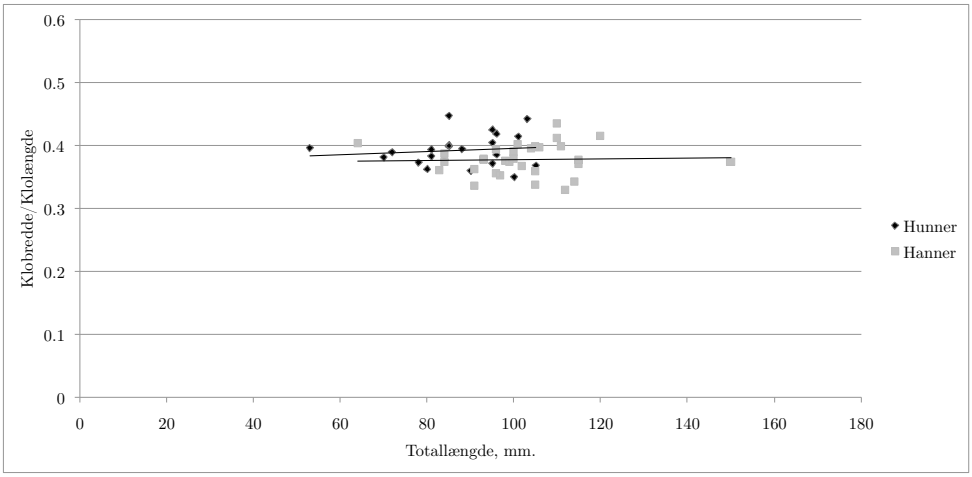
(b) Galizisk sumpkrebs *Astacus leptodactylus*



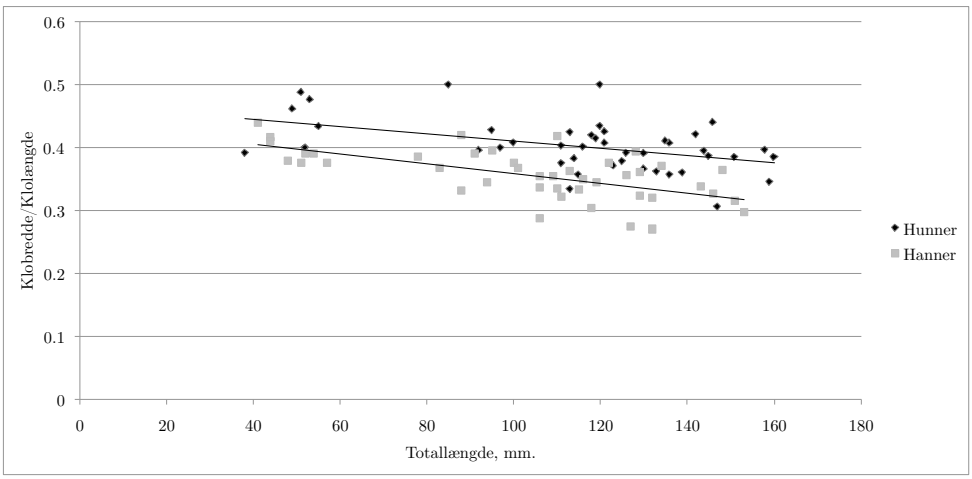
(c) Signalkrebs *Pacifastacus leniusculus*

Figur 16: Klolængde/totallængde plottet mod totallængde for (a) flodkrebs, (b) galizisk sumpkrebs og (c) signalkrebs. For alle arter gælder, at hanner har signifikant længere klør.

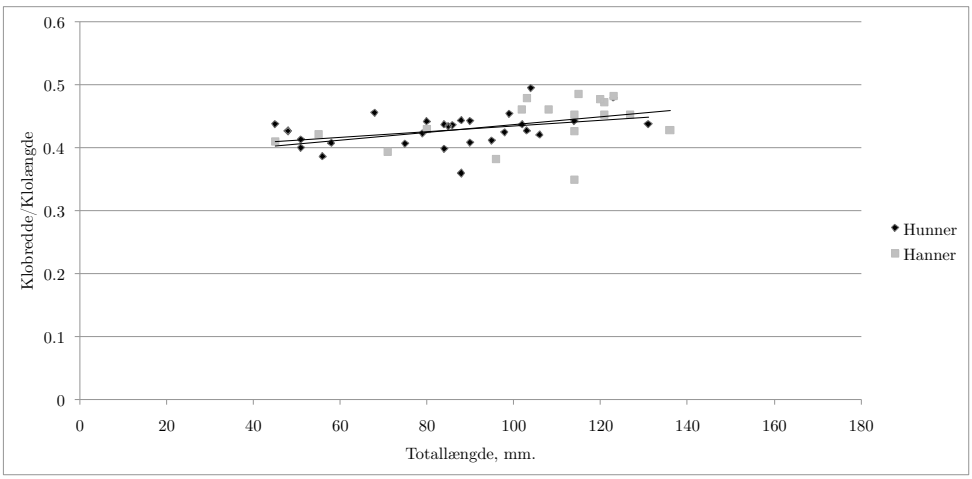
Figure 16: Length of claw/Total length plotted against total length. (a) Astacus astacus*, (b)* Astacus leptodactylus *og (c)* Pacifastacus leniusculus*. Males of all species have significant longer claws.*



(a) Flodkrebs *Astacus astacus*



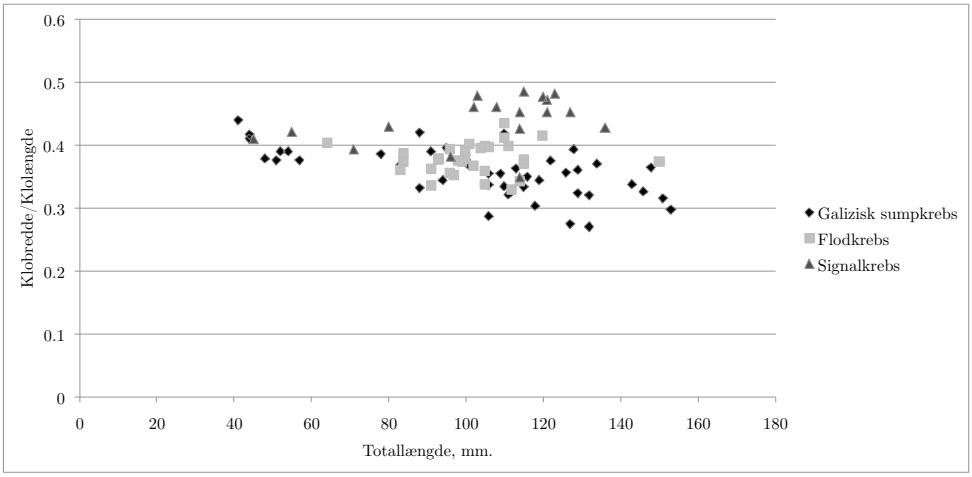
(b) Galizisk sumpkrebs *Astacus leptodactylus*



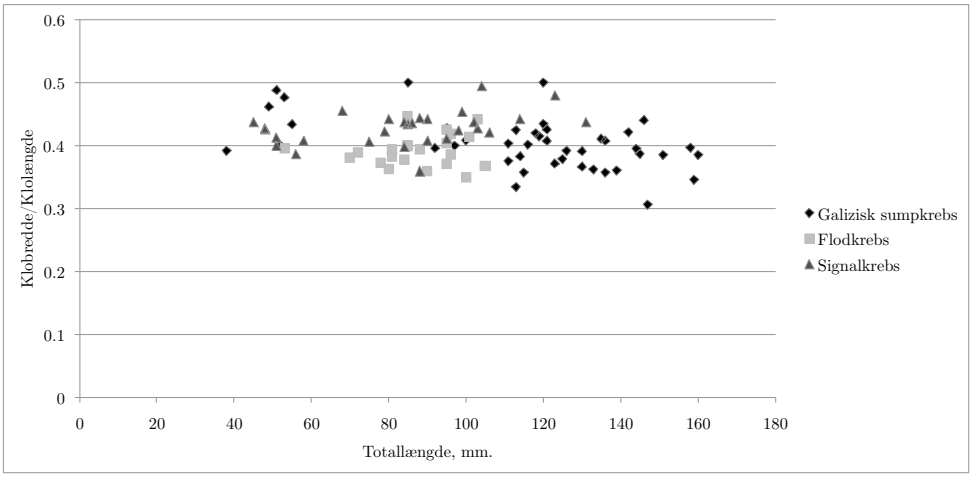
(c) Signalkrebs *Pacifastacus leniusculus*

Figur 17: Klobredde/Klolængde plottet mod totallængde for hunner og hanner af (a) flodkrebs, (b) galizisk sumpkrebs og (c) signalkrebs. Hunner har signifikant bredere klør end hanner for flodkrebs og galizisk sumpkrebs. Der er ingen kønsforskelle for signalkrebs.

Figure 17: Width of claw/length of claw plotted against total length. Females and males of (a) Astacus astacus*, (b)* Astacus leptodactylus *and (c)* Pacifastacus leniusculus. *Females of* Astacus astacus *and* Astacus leptodactylus *have significant wider claws than males. There is no difference between male and female signal crayfish.*



(a) Hanner



(b) Hunner

Figur 18: Klobredde/Klolængde plottet mod totallængde for (a) hanner og (b) hunner af alle arter. Signalkrebs har signifikant bredere klør end flodkrebs og galizisk sumpkrebs.

Figure 18: Width of claw/Length of claw plotted against total length for (a) males and (b) females of all species. Pacifastacus leniusculus *have significant wider claws than both* Astacus astacus *and* Astacus leptodactylus.

Diskussion

Registrering

Det forholdsvis lave antal registreringer af krebs i Danmark er et udtryk for det manglende fokus og interesse på området. Kun signalkrebsen har været mål for overvågning. Siden 1980'erne, hvor der i lovgivningen kom fokus på danske vådområder, er antallet af registreringer øget gennem naturstyrelsens arbejde med vandløbsovervågning og lignende. De første officielle registreringer af invasive krebs fra naturen er observationerne af galizisk sumpkrebs i 1988 (Observeret af Jesper Andersen) og signalkrebs i 1996 (Styrishave, 1996) på trods af, at de formentlig kom til Danmark allerede i 1960'erne. De sene registreringer af invasive arter kan skyldes manglende kompetencer ud i artsbestemmelse af krebs. Der hersker stadig idag stor usikkerhed om artbestemmelse af krebs, selv blandt særligt interesserede. Dermed har eventuelle observationer af invasive krebs ikke vakt særlig opsigt og de har derfor kunnet sprede sig i naturen.

Der har dog uden tvivl været et vis kendskab til krebsene blandt specielt interesserede i tiden inden de første officielle registreringer fra dette studie.

Antallet af registreringer per måned afspejler, at sø- og vandløbsundersøgelser typisk bliver gennemført i forår og sommer, samt at sæsonen for krebsefangst er i sensommermånederne, jævnfør de gældende fredningstider for flodkrebs (hanner:1/10 - 31/3, hunner:1/10 - 31/7) (se http://m.fisketegn.dk/krebs.aspx?ID=45955) (Johansson og Lauridsen, 2014). Der er i dette studie registreret krebs i alle årets måneder undtagen januar. Dog svinger metodernes effektivitet med flere faktorer, bl. a. temperatur, hvilket der skal tages højde for ved effektivisering af registreringsindsatser (Richards et al., 1996; Somers og Stechey, 1986).

Inddelingen af registeringer i A- og B-bestemmelser sikrer, at den aktuelle status for udbredelsen er veldokumenteret.

Særligt for flodkrebsen, er flere registreringer blevet vurderet til at være B-bestemmelser på baggrund af mange fejlbestemmelser opdaget løbende gennem studiet. Her er det særligt hunner af galizisk sumpkrebs, som er forvekslet med flodkrebs grundet flere ligheder og udbredte misforståelser. Alle registreringer af flodkrebs fra naturstyrelsens winbio-database er således vurderet til at være B-bestemmelser, da der ikke foreligger yderligere dokumentation.

Den større andel af A-registreringer blandt galizisk sumpkrebs skyldes, at størstedelen er foretaget i studieperioden og derfor er veldokumenterede. Der er ikke registreret galizisk sumpkrebs i Winbio-databasen.

For signalkrebs skyldes den større andel af A-registreringer, at arten er vurderet til at være lettere at identificere, grundet sine karakteristiske træk (f.eks. den hvide plet ved basis af klosaks (dactylus)) samt at der har været mere fokus på den gennem naturstyrelsen og DTU Aquas arbejde med registrering og bekæmpelse.

Status for udbredelse

Flodkrebsens udbredelse i Danmark er dårligt dokumenteret. Antallet af ikke-verificerede registreringer overstiger antallet af verificerede, og der er i høj grad brug for mere data for at kunne konkludere om flodkrebsen lider samme skæbne i Danmark som i resten af Europa. Det er dog sandsynligt at flodkrebsen også er i tilbagegang i Danmark som resultat af introduktion af invasive arter, forurening, vandløbsudretning og anden habitatforringelse (Skurdal et al., 1999).

Den galiziske sumpkrebs udbredelse er kun verificeret på Sjælland, hvor den er vidt udbredt. Den er ifølge andre kortlægningsprojekter (se Souty-Grosset et al. (2006); Kouba et al. (2014)) også registreret i Jylland, men grundet manglende dokumentation og flere tilfælde af forvekslinger med flodkrebs er disse ikke medtaget. Udbredelse af galizisk sumpkrebs har hidtil blot været raporteret fra få vande på Sjælland, her i blandt Furesøen ved København (Skov et al., 2009), men er i studieperioden blevet registreret på mange lokaliteter, særligt i Storkøbenhavn, hvor den både findes i mindre parksøer og større søer, f.eks. Damhussøen.

Signalkrebsen er udbredt i alle landsdele på nær mindre øer. Særligt på det sydlige Sjælland og i Jylland er der flere registreringer. Mange registreringer er fra større vandløb f.eks. Susåen på Sjælland og Gudenåen i Jylland.

Signalkrebsen og den galiziske sumpkrebs er begge registreret i brakvand omkring det sydlige og østlige Sjælland. Begge arter tolererer brakvand og har dermed stort spredningspotentiale i Østersøområdet (Gherardi og Holdich, 1999).

Antallet af kvadrater indeholdende registreringer af ukendt art overstiger langt antallet af sikre artsbestemmelser. På trods af manglende verificering af art, kan denne viden om udbredelsen dog godt bruges da samtlige registreringer af krebs har interesse for fremtidige kortlægningsprojekter.

Der er kun på én lokalitet blevet observeret mere end én art. Her var det galizisk sumpkrebs og flodkrebs, der blev fanget ved samme indsats. Denne sameksistens kan ved nærmere undersøgelser bidrage til vigtig viden om interaktioner mellem invasive og hjemmehørende krebs. Det vides fra andre studier, at både signalkrebs og galizisk sumpkrebs er i stand til at udkonkurrere andre hjemmehørende arter (Stucki og Romer, 2001; Westman et al., 2002).

Udarbejdelse af felt-guide ud fra morfologiske undersøgelser

Ud fra målte og talte morfometriske karakterer er der fundet forskelle mellem både arter og køn, der kan anvendes til sikker artsbestemmelse af krebs i Danmark.

Vigtigst er, at den gængse opfattelse af, at galizisk sumpkrebs er kendetegnet ved lange, smalle klør ikke er sand. Dette studie viser at der ikke er signifikant forskel i længden af klør for de tre arter. Det er derimod kønnet der har den største effekt på klolængden, da hunner generelt har kortere klør end hanner.

Signalkrebsen markerer sig ved at have bredere klør end flodkrebsen og den galiziske sumpkrebs. Bredden af klør kan ikke alene adskille flodkrebs og galizisk sumpkrebs.

Klolængden og klobredden er ikke alene brugbare karakterer til sikker bestemmelse af arter i felten.

Analysen af haleleddenes form viser, at denne karakter er et vigtigt kendetegn der, i denne undersøgelse, kunne adskille galizisk sumpkrebs fra de to andre arter, på tværs af køn. Flodkrebs og signalkrebs kan ikke sikkert adskilles på denne karakter.

Tællinger af pigge langs nakkefuren viser, at signalkrebsen kan kendes fra de to andre arter ved manglen på pigge. Alle individer af flodkrebs og galizisk sumpkrebs havde én eller flere pigge langs nakkefuren. Dette er et kendetegn der, sammen med den ofte tydelige hvide eller blålige plet ved basis af dactylus, kan adskille signalkrebsen fra de andre arter.

Det anbefales dog, at farver generelt anvendes med forsigtighed i artsbestemmelse af krebs.

Perspektivering

Ifølge Füreder og Machino (2002), bliver særligt krebs ofte fejlbestemt. For at øge mængden og kvaliteten af fremtidige registreringer er det nødvendigt at udbrede kendskabet til krebs, samt at udvikle let anvendelige værktøjer, der kan gøre alle i stand til at identificere krebs (Souty-Grosset et al., 2006). På den måde kan krebs blive en del af faunaregistreringen i forbindelse med ferskvandsundersøgelser og Citizen Science projekter som f. eks. Naturstyrelsens projekt Naturtjek.

Yderligere er der behov for udvikling af mere moderne overvågningsmetoder som eksempelvis detektion ved hjælp af miljø-DNA (eDNA). Dette er et felt i stadig udvikling, men der er begrænset fokus på krebs i udviklingen af detektionssystemer. Der findes på nuværende tidspunkt blot ét publiceret studie som tester detektion af krebs med eDNA (se Tréguier et al. (2014)), men flere er på vej.

Et godt kendskab til arterne og deres udbredelse er essentielt ved bekæmpelse af invasive arter. Menneskelig aktivitet er en vigtig årsag til spredning af uønskede krebs og bunder ofte i misforståelser og misidentifikationer, men med god information og lovgivning på området kan den menneskelige spredning begrænses (Dresser og Swanson, 2013). Med en øget opmærksomhed på krebs vil fremtidige invasioner måske kunne bremses før vi ser nye veletablerede populationer af uønskede arter i Danmark.

Det er anerkendt, at veletablerede bestande af invasive krebs er noget nært umulige at udrydde uden anvendelse af stærkt invasive metoder som f. eks. giftstoffer (Gherardi et al., 2011; Peay et al., 2006). Signalkrebsen er forsøgt udryddet lokalt i Alling Å i Jylland (et tilløb til Randers Fjord), dog uden succes (Iversen et al., 2011). Det kan alligevel godt betale sig at bekæmpe etablerede invasive krebs, da fjernelse af dele af bestandene kan medføre en forøgelse af diversiteten af mindre invertebrater og dermed også højne kvaliteten af vandsystemer (Moorhouse et al., 2014).

Konklusion

Ud fra i alt 330 registreringer af krebs fra Danmark, er udbredelsen af de tre arter, flodkrebs, galizisk sumpkrebs og signalkrebs blevet opdateret pr. august 2015. Alle tre arter er veletablerede i Danmark, dog begrænser den verificerede udbredelse af galiziske sumpkrebs sig til Sjælland.

Mere systematisk registrering af krebs i fremtiden er nødvendig for at danne et brugbart overblik over udbredelse, til anvendelse i fremtidig forvaltning af krebs i Danmark.

Den udarbejde guide til danske krebs er et værktøj der kan være medvirkende til at højne kvaliteten af fremtidige registreringer og rette op på generelle misforståelser omkring identifikation af krebs i Danmark.

Tak

Der skal lyde en stor tak til alle der har bidraget til projektet med registreringer og anden hjælp. Særlig tak til Peter Rask Møller, Marcus A. Krag, Henrik Carl, medlemmer af Dansk Krebseavlerforening (særligt Birthe Lindberg), involverede kommuner og Naturstyrelsen.

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Appendix A

Art_Latin	Antal krebs	Antal_nøjagtighed	Artsbestemt_af	Bestemmelsessikkerhed	Materiale	Obs_dg	Obs_Md	Obs_År	Observatør	Område	Nøjagtig_lokalitetsangivelse	Biotop	Observationsmetode	Bemærkninger	Lgd_grad	Brd_grad	Zone
Astacus leptodactylus	5	Præcis	William Brenner Larsen, Sune Agersnap	A	I hånden	1	7	2015	William Brenner Larsen, Sune Agersnap	Sjælland-Lolland-Falster	Agersø, Rude Skov	Sø	Fælde		12.482874	55.84167	Fersk
Astacus astacus	33	Præcis	William Brenner Larsen, Sune Agersnap	A	I hånden	1	7	2015	William Brenner Larsen, Sune Agersnap	Sjælland-Lolland-Falster	Agersø, Rude Skov	Sø	Fælde		12.482874	55.84167	Fersk
Astacus leptodactylus	1	Arten registreret	William Brenner Larsen, Sune Agersnap	A	I hånden	1	7	2015	William Brenner Larsen, Sune Agersnap	Sjælland-Lolland-Falster	Fuglsangsø, Klampenborg	Sø	Fælde		12.563903	55.772787	Fersk
Astacus astacus	13	Præcis	William Brenner Larsen, Sune Agersnap	A	I hånden	1	7	2015	William Brenner Larsen, Sune Agersnap	Sjælland-Lolland-Falster	Løje sø, Rude skov	Sø	Fælde		12.478715	55.832589	Fersk
Astacus leptodactylus	6	Præcis	William Brenner Larsen, Sune Agersnap	A	I hånden	1	7	2015	William Brenner Larsen, Sune Agersnap	Sjælland-Lolland-Falster	Damhussøen, Valby	Sø	Fælde		12.480795	55.672899	Fersk
Astacus leptodactylus	3	Præcis	William Brenner Larsen, Sune Agersnap	A	I hånden	12	7	2015	Marcus A. Krag	Sjælland-Lolland-Falster	Skuldelev grusgrav	Sø	Snorkling		12.02799	55.766463	Fersk
Astacus leptodactylus	1	Præcis	William Brenner Larsen, Sune Agersnap	A	I hånden	7	6	2015	Marcus A. Krag	Sjælland-Lolland-Falster	Springdam, N for Ubberød dam, Hørsholm	Vandhul	Rejehov		12.501727	55.869452	Fersk
Pacifastacus leniusculus	2	Præcis	William Brenner Larsen, Sune Agersnap	A	I hånden	22	3	2015	William Brenner Larsen, Emil Frost og Jens Frost	Fyn	Davinde Sø	Sø	Håndfanget		10.539086	55.332318	Fersk
Pacifastacus leniusculus	146	Præcis	William Brenner Larsen, Sune Agersnap	A	I hånden	1	7	2015	William Brenner Larsen, Sune Agersnap	Sjælland-Lolland-Falster	Nydam, Bagsværd	Vandhul	Fælde		12.442784	55.769896	Fersk
Astacus astacus	1	Arten registreret	William Brenner Larsen, Sune Agersnap	A	I hånden	27	5	2015	William Brenner Larsen	Jylland	Elev grusgrav	Sø	Snorkling		10.186593	56.237111	Fersk

Figur 19: Eksempel på indtastning af registreringer.

Figure 19: Exampel of how registrations were typed in.

4 Manuscript two

Development and field testing of genetic markers for eDNA detection and quantification of three crayfish (Decapoda - Astacidea) species in Denmark

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Manuscript for Management of Biological Invasions

Abstract

During the last century, European crayfish populations have faced major declines. Anthropogenic disturbances together with the introduction of several non-indigenous crayfish species, have severely reduced population sizes and distributions of indigenous crayfish all over Europe. In Denmark three species of crayfish are present; the indigenous, threatened noble crayfish *Astacus astacus* (Linnaeus, 1758) and the two non-indigenous, invasive species; signal crayfish *Pacifastacus leniusculus* (Dana, 1852) and narrow-clawed crayfish *Astacus leptodactylus* (Eschscholtz, 1823) species complex. In Europe, the number of non-indigenous crayfish species now exceeds the number of indigenous species. Knowledge on crayfish distribution and early detection of non-indigenous crayfish are crucial elements in successful protection of indigenous crayfish and for eradication of non-indigenous crayfish. The use of environmental DNA (eDNA) has proved to be a powerful tool for detection of species in aquatic environments, but macro-invertebrates, such as crayfish, have received little attention. In the present study, molecular markers were developed and used to investigate distribution and abundance of crayfish in Denmark by quantitative PCR analysis of eDNA extracted from water samples. Results were compared to conventional trapping, in order to assess the reliability of the eDNA method and to relate the number of DNA copies in water samples to a relative abundance, using catch per unit of effort (CPUE) and mark-recapture estimations of population size.

eDNA was successfully detected and quantified for all three targeted crayfish species. The estimated limit of detection and quantification was one copy/μL extract for all three species (equal to 80 copies/L of water filtered).

Keywords: Crayfish, eDNA, qPCR, Astacus, astacus, leptodactylus, Pacifastacus, leniusculus

Introduction

Freshwater ecosystems are heavily threatened and affected by anthropogenic activities (e.g channelization, pollution and translocation of non-indigenous species) (Dudgeon et al., 2006; Reynolds and Souty-Grosset, 2011). Knowledge on current and past distribution, population size and biodiversity patterns of species is crucial to the management of threatened and non-indigenous species and for efforts to mitigate consequences of biological shifts in freshwater systems (Diaz-Ferguson and Moyer, 2014).

Monitoring of biodiversity has conventionally depended on physical detection and identification of species, ranging from auditive and visual detection, of e.g harbour porpoises (Carstensen et al., 2006), to collection of individuals through different trapping methods, e.g baited traps and gill nets (Bolat et al., 2011; Kristensen et al., 2014). These detection methods are often highly selective and can rarely be applied across taxonomic groups. Furthermore, these methods require field experience and taxonomic expertise. Both skills can be challenging to learn and can, when absent, result in flaws and false conclusions regarding the absence or presence of species (Thomsen and Willerslev, 2015). Additionally, threatened species and newly introduced non-native species are often present in low densities and traditional detection of species at low densities is particularly difficult in aquatic environments.

Monitoring of freshwater crayfish (Astacidea) is carried out in several countries in Europe, including Germany, France and Austria (Souty-Grosset et al., 2006). However, Denmark has not adopted any monitoring programs for any of the three species of crayfish present in the country; the native and threatened noble crayfish *Astacus astacus* (Linnaeus, 1758) and the two non-native species, signal crayfish *Pasfi-castacus leniusculus* (Dana, 1852) and narrow-clawed crayfish *Astacus leptodactylus* (Eschscholtz, 1823) species complex (Souty-Grosset et al., 2006). Invasive crayfish such as the signal crayfish can be carriers of the crayfish plague *Aphanomyces astaci* (Schikora, 1906), which is lethal to native European crayfish (Makkonen et al., 2012). Thus, there is a need for early detection of non-indigenous crayfish species. Crayfish in Denmark are only registered if they are unintentionally caught during monitoring of stream health and fish communities, and identification of species is not necessarily verified. Crayfish species can be difficult to identify morphologically, even for museum specialists and crayfish enthusiasts. Additionally, some researchers working with crayfish can only determine and distinguish crayfish species by genetic markers (Füreder and Machino, 2002). Using molecular markers requires detailed knowledge about phylogenetic structures (Avise, 1994).

Even though *Astacus leptodactylus* is described as a species complex its taxonomy has often been conveniently simplified (Souty-Grosset et al., 2006). A recent study by Maguire et al. (2014) shows two distinct genetic linages of *A. leptodactylus*, one European and one Asian. In Turkey Akhan et al. (2014) find three different clades within the Turkish population of *A. leptodactylus*: clade I, II and III. *Astacus leptodactylus* is native to the south-eastern Europe and the Caspian sea area, but is now present in many countries outside its native range.

It is possible to obtain fragments of DNA, covering ancient to present time and micro to macro organisms, in several different environments (e.g ice, water, soil and sediments) (Pedersen et al., 2015; Thomsen and Willerslev, 2015). Environmental DNA (eDNA) detection in aquatic habitats depend on the persistence of short DNA fragments and the continuous excretion of DNA (e.g faeces, urine, mucus and epidermal cells) from organisms (Takahara et al., 2012). Concentrations of DNA in the environment are believed to correlate with species body size, density, amounts of released DNA and DNA degradation (Diaz-Ferguson and Moyer, 2014; Thomsen(b) et al., 2012).

Species detection using eDNA requires the development of specific genetic markers. Sites in mitochondrial DNA (mtDNA) are preferred over nuclear DNA (nDNA), because mtDNA is more abundant in the cell than nDNA (Thomsen and Willerslev, 2015). These markers are used to target specific taxa or whole taxonomical groups (Diaz-Ferguson and Moyer, 2014). Genetic markers have been narrowly applied to some taxa in freshwater ecosystems, and the focus has mainly been on vertebrates, e.g amphibians and fish (e.g. Ficetola et al., 2008; Rees et al., 2014; Takahara et al., 2012; Thomsen(b) et al., 2012), using water samples as the source for eDNA. Only few studies have investigated the detection of eDNA from invertebrates (e.g. Deiner and Altermatt, 2014; Roussel et al., 2015; Thomsen(b) et al., 2012; Tréguier et al., 2014).

Tréguier et al. (2014) are so far the only ones to investigate the potential of eDNA to detect crayfish, and they succesfully detect the invasive red swamp crayfish *Procambarus clarkii* (Girard, 1852) in several lakes in France.

eDNA has in some studies improved detection of freshwater species relative to conventional methods (e.g. Dejean et al., 2011; Ficetola et al., 2008), and in some cases a correlation between the density of target organisms and DNA concentration has been shown (e.g. Takahara et al., 2012; Thomsen(a) et al., 2012; Thomsen(b) et al., 2012). However, both Tréguier et al. (2014) and Thomsen(b) et al. (2012) find that eDNA is not always detectable in waterbodies where the target species have been caught with conventional tools in the same period of time.

The application of eDNA analysis across taxonomic groups has some possible pitfalls regarding field sampling and laboratory analysis, these are reviewed and discussed by Roussel et al. (2015) and Rees et al. (2014, 2015).

When recognizing the limitations of eDNA, the method is still a cost-effective tool with potential for monitoring threatened and non-indigenous species, and has been widely investigated through more than a decade (Ficetola et al., 2008; Rees et al., 2015; Pedersen et al., 2015; Takahara et al., 2012; Thomsen and Willerslev, 2015). It is believed that ever-improving laboratory and sampling methodology will reduce the uncertainties and enhance the success rate of eDNA detection systems (Rees et al., 2014).

In this study, we investigate the potential of eDNA as a tool for detecting the three crayfish species found in Denmark; noble crayfish, narrow-clawed crayfish and signal crayfish. Species-specific primer-probe systems targeting a region in the mtDNA are developed from DNA extracted from tissue samples and sequences acquired from the National Center for Biotechnology Information (NCBI Genbank®, www.ncbi.nlm.nih.gov) (hereafter Genbank). Their effectiveness is tested through quantitative PCR

analysis on waters known to house populations of crayfish. We quantify eDNA from the targeted species and compare results with results from conventional methods, e.g. trapping and mark-recapture experiments, in means of catch per unit of effort (CPUE) and catchable population size in numbers.

Material and methods

Field work

Estimation of catchable population size with mark/recapture

Mark/recapture experiments were carried out in Lake Furesø, which holds a population of the narrow-clawed crayfish, and in Lake Nydam, which houses the signal crayfish, in June and October 2014, respectively.

Lake Nydam (figure 2) is a 0.02 km^2 lake located in the city of Bagsværd north of Copenhagen. It is surrounded mainly by private homes and is only accessible to the public on an approximately 70 m stretch along road Skovaléen. The lake, which was stemmed in 1670, is now owned by the private association Søejerlauget Nydam Sø (Gladsaxe Kommune, 2007). It drains the surrounding land including the forest Aldershvile skov and also receives water from a rainwater reservoir in Gladsaxe municipality during heavy rain falls. Drainage is through a pipeline leading to Lake Bagsværd, which is part of the Mølleå water system (Gladsaxe Kommune, 2007).

Lake Furesø is located approximately 20 km north of Copenhagen and is surrounded by the municipalities of Furesø, Virum, Holte, Birkerød and Værløse. Like Lake Bagsværd it is a part of the Mølleå water system and with its 9.41 km^2 it is also the biggest lake in the system and the seventh biggest in Denmark (Furesø Kommune, 2015). The lake consists of two parts; the main basin with a maximum depth of $\approx$ 38 m and a mean depth of $\approx$ 16.5 m and a smaller part, called Store Kalv, with a maximum depth of $\approx$ 4.5 m and a mean depth of $\approx$ 2.5 m.

Figure 1: Satellite photo showing the magnified location of the stone reef in Lake Furesø. The tip of the arrow points to the stone reef. Source: Krak.dk. December 2014

In this study, we focused on a stone reef located in the south eastern corner of the lake, near the drain to Mølleå (figure 1). The reef is artificially made to support the water way leading into the Mølleå. It consists of rocks in sizes ranging from a few inches to $\approx$ 1 m in diameter. The rocks make up good potential shelters for crayfish of all sizes. The surrounding substrate is muddy and sandy with scattered vegetation and debris (e.g. logs, sticks and common trash).

The mark/recapture experiment done in Lake Nydam included three capture events (figure 2, table 4, page 19). Specimens were measured in total length (tip of rostrum to end of tail) (TL) and weighed to the closest 0.1 g and the sex was determined. Furthermore, it was noted if females were carrying eggs. Marking was done immediately after the animals were caught and the crayfish were released evenly in the lake.

The traps used for catching crayfish were of the cylindrical spring type, and were bought from Jagt- og Fiskerimagasinet in Copenhagen, Denmark. Measurements were 60x33 cm with a mesh size of 11 mm. The traps were baited with different kinds of feed, including fish and mixtures of fish feed. In Lake Nydam, 30 traps evenly distributed on three 30 m long strings, were used, giving a 3 m gap between traps (figure 3). Each trap was attached to the main line with a snap hook to ease handling during deployment and emptying.

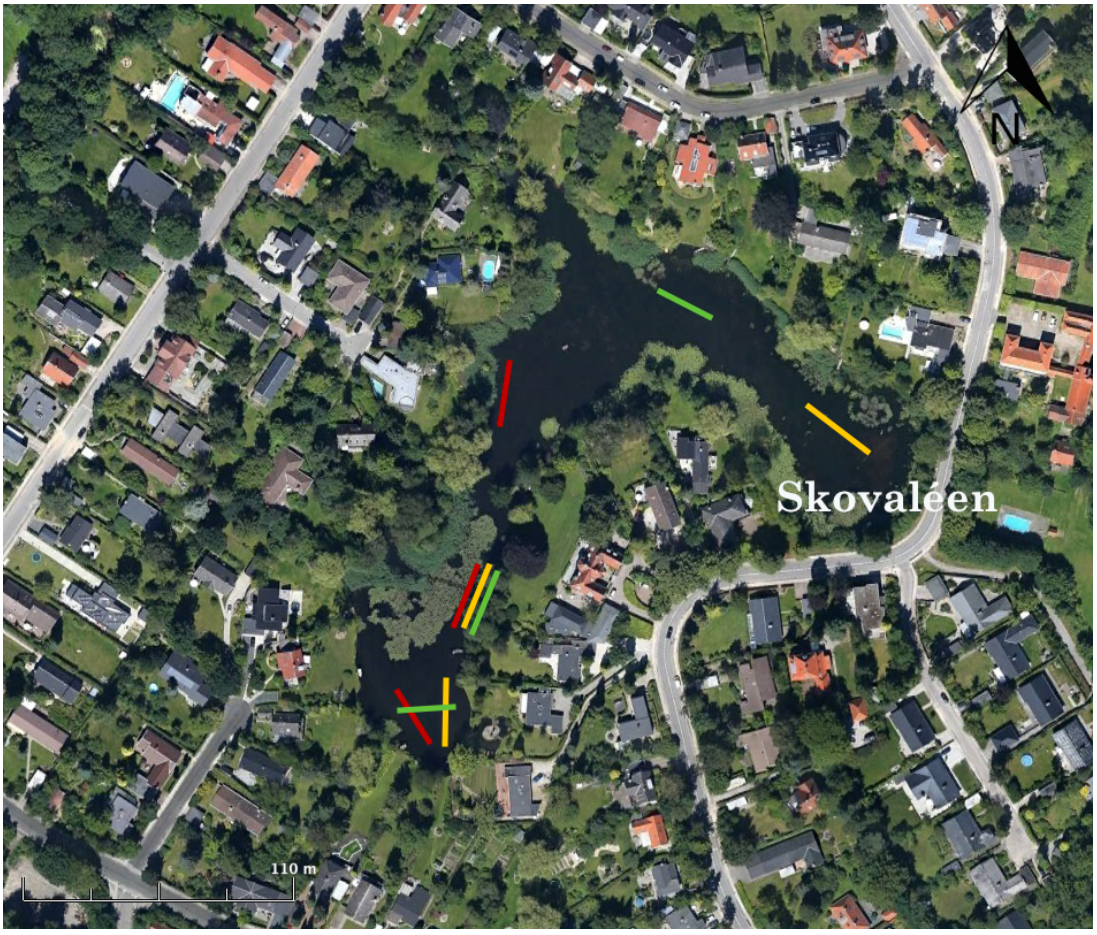


Figure 2: Satellite photograph of Lake Nydam with trap lines plotted. The trap lines were deployed Oct. 12th, 2014 (marked as yellow), Oct. 18th, 2014 (red) and Oct. 30th, 2014 (green). Note: Skovaléen is the only accessible stretch around the lake. Source: Google Earth. December 2014

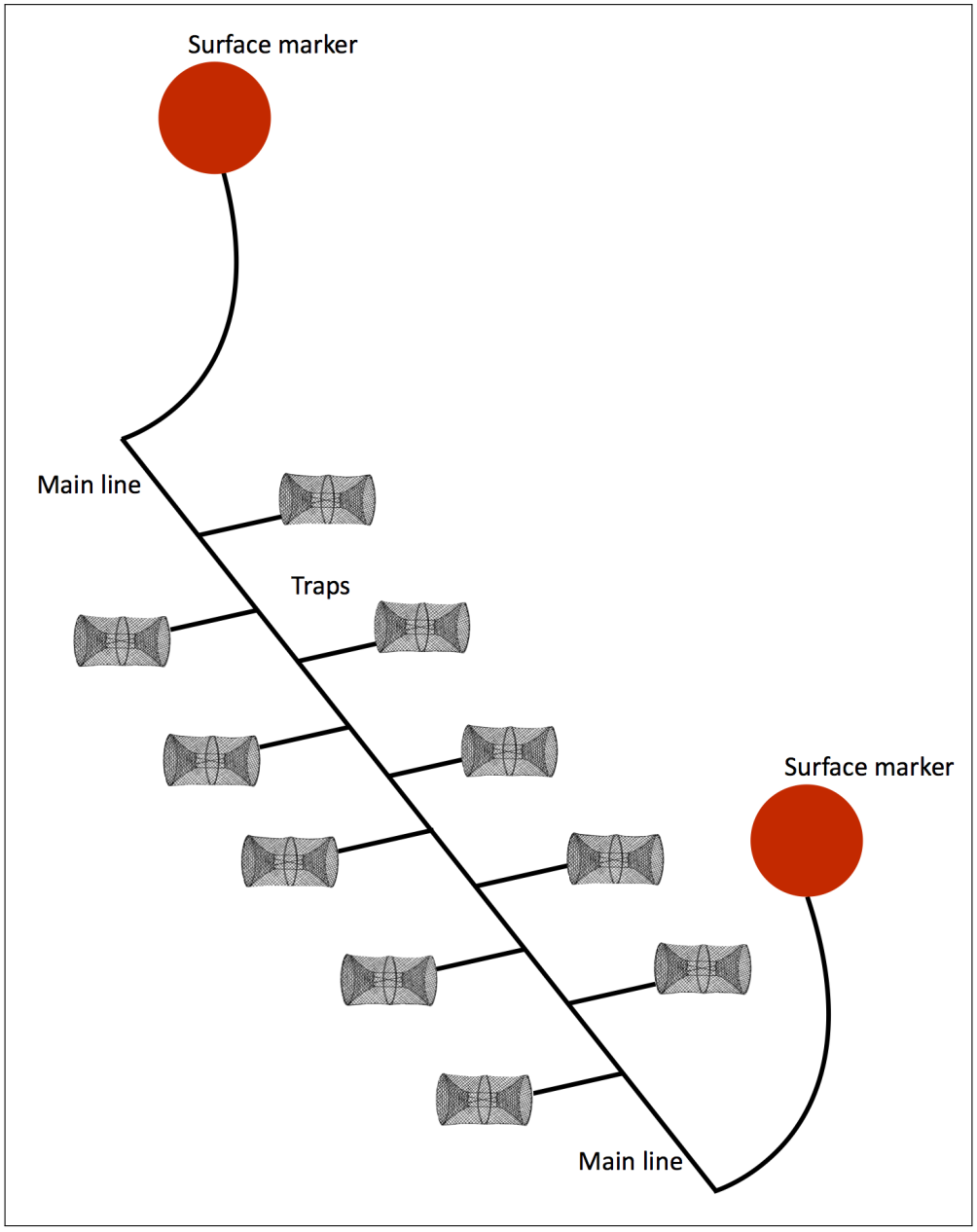


Figure 3: Illustration of a 30 m long trap line placed on the bottom. Traps are connected to the main line with snaphooks, and a floating surface marker is connected to each end.

The mark/recapture experiment in lake Furesø included five capture events (table 6, page 20). Crayfish were caught by hand while snorkelling at night. Specimens were measured in post-orbital carapace length (from the back of the orbit to the end cephalothorax) (POCL) and the sex was determined. Furthermore, it was noted if females were carrying eggs. All crayfish were marked just after capture and released evenly on the stone reef.

Visible implant elastomer tags (VIE) bought from *Northwest Marine Technology*^{TM} were used to mark crayfish in both experiments. VIE tags are small and inexpensive two component, colour fluorescent tags that are easily injected into the animal, where they will solidify. Hence, they are suitable for marking a large number of individuals (Haddaway et al., 2011). It has been shown that tagging does not affect death rates and molting in crayfish and that retention rates are high, up to 100%, through several molts (Clark and Kershner, 2006; Mazlum, 2005). Tag visibility is high, but may decrease over time, making these tags ideal for short-term studies (Brown et al., 2003).

Crayfish were tagged ventrally in the abdomen, just beneath the exoskeleton. Here the exoskeleton is transparent which eases tag detection. Also, when tagged in this region, the tag is visible in females carrying eggs (figure 4).

Figure 4: VIE tag in female signal crayfish carrying eggs. Photo: W. B. Larsen and S. Agersnap.

Population size was calculated with the Schnabel method (formulas 1 and 2) (Schnabel, 1938) which assumes closed populations. Formula 2 accounts for a relatively low number of recaptures (less than 10% of population).

The crayfish population in Lake Nydam is considered closed. The population inhabiting the stone reef in Lake Furesø is assumed to be closed, because of a small time span between capture events.

$$\boxed{N = \frac{\sum_{i=1}^{m} M_i * C_i}{\sum_{i=1}^{m} R_i}} \quad (1)$$

$$\boxed{N = \frac{\sum_{i=1}^{m} M_i * C_i}{\sum_{i=1}^{m} R_i + 1}} \quad (2)$$

Where: M_i = number of marked individuals before current sample, C_i = number of individuals caught in sample i, R_i = number of marked individuals caught in sample i, N = estimated population size, m = number of the sample in the series.

Measuring catch per unit of effort (CPUE)

The activity of crayfish measured as CPUE (crayfish/trap/hour) was investigated for all three crayfish species, from March till July, 2015.

To determine the effect of temperature on CPUE, the following experiments were carried out in the lakes Lake Sankt Jørgens Sø; a 0.066 km^2 lake with a maximum depth of 5 m and a mean depth of 4.2 m, Lake Damhussøen; a 0.46 km^2 lake with a maximum depth of 2.4 m and a mean depth of 1.6 m and Lake Nydam. These lakes are known to house populations of noble crayfish, narrow-clawed crayfish and signal crayfish, respectively. The exact same location in every lake was fished using three traps baited with fish and temperature was measured 40 cm above the bottom. For all crayfish caught, TL was measured, sexes determined and females carrying eggs were noted. Immediately after handling, all individuals were released into the same area where they were caught.

To estimate a relative activity (CPUE) of crayfish for later comparison with eDNA, three traps baited with dead fish were set at the same location and on the same day as water samples were taken. The traps were left overnight and when emptied, all specimens were counted and sexed.

Traps that were moved between water bodies, were disinfected with Virkon® S.

Design of species-specific primer-probe systems

Sampling of crayfish for DNA extraction

Eight specimens of each of the three study species were collected for DNA extraction to represent different populations (figure 5, table 1). Crayfish specimen sampling was carried out on several events in the period between September and December, 2014. Various methods were used to collect crayfish, e.g. snorkelling, baited traps and hand nets covering lakes, streams and coastal areas. All collected specimens were preserved in 96% ethanol until DNA extraction.

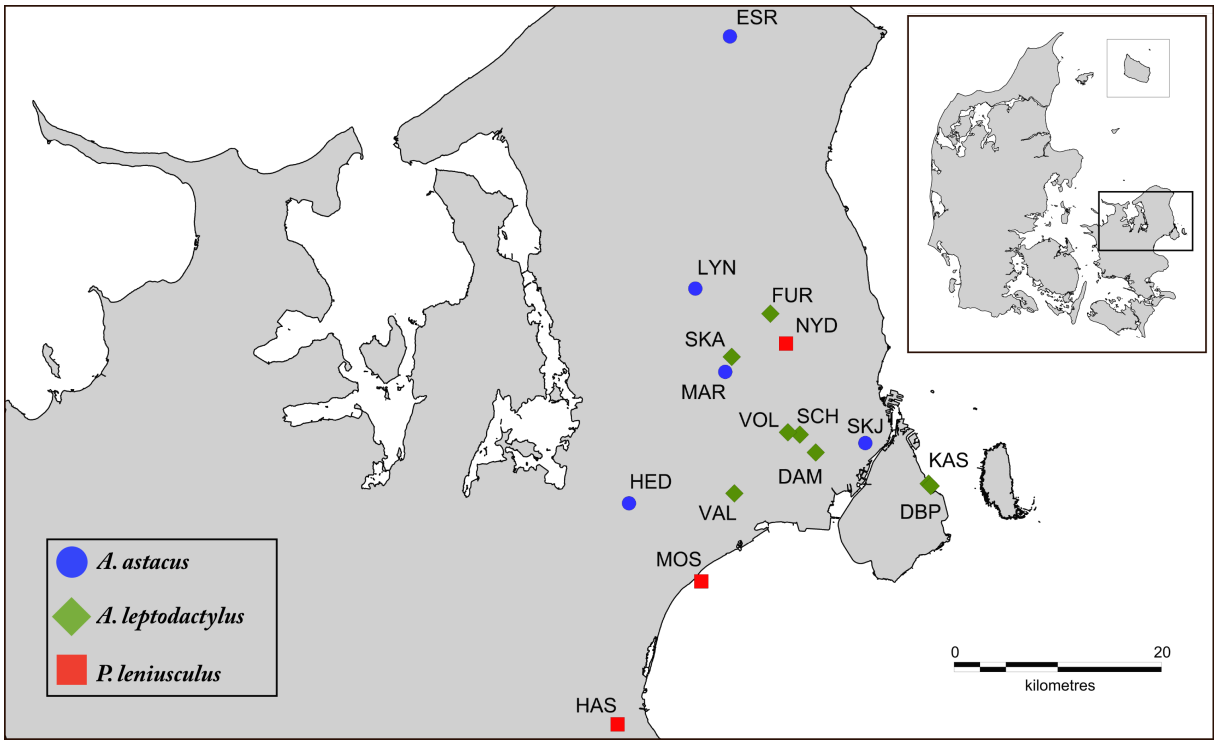


Figure 5: Crayfish tissue sampling sites, Zealand, Denmark. DAM = Lake Damhussøen, DBP = Pond, Den BlåPlanet, ESR = Stream Esrum å, FUR = Lake Furesø, HAS = Lake Hastrup, HED = Lake Hedeland, KAS = Kastrup harbor, LYN = Lynge Put 'N' Take, MAR = Lake Marks, MOS = Køge bay, Mosede harbor, NYD = Lake Nydam, SCH = Pond, Schweizerdalsparken, SKA = Lake Skallemosen, SKJ = Lake Sankt Jørgens Sø, VAL = Lake Tueholm, VOL = Vestvolden.

Table 1: Overview of tissue samples from *Astacus leptodactylus*, *Astacus astacus* and *Pacifastacus leniusculus*, showing sampling site, site code (explained in fig. 5) and museums ID number (Natural History Museum of Denmark).

Species	Sample site (longitude-latitude)	Code	Type	Museum ID.
A. leptodactylus	12.438907-55.691911	VOL	Channel	ZMUC-CRU-004773
A. leptodactylus	12.457318-55.689385	SCH	Pond	ZMUC-CRU-004847
A. leptodactylus	12.357194-55.760575	SKA	Pond	ZMUC-CRU-004777
A. leptodactylus	12.653911-55.640496	KAS	Brackish	ZMUC-CRU-005976
A. leptodactylus	12.480795-55.672899	DAM	Lake	ZMUC-CRU-005674
A. leptodactylus	12.351023-55.640567	VAL	Lake	ZMUC-CRU-005495
A. leptodactylus	12.657066-55.638314	DBP	Pond	ZMUC-CRU-005680
A. leptodactylus	12.420731-55.796842	FUR	Lake	ZMUC-CRU-005502
A. astacus	12.378904-56.042448	ESR	Stream	ZMUC-CRU-005649
A. astacus	12.558574-55.681184	SKJ	Lake	ZMUC-CRU-004845
A. astacus	12.305374-55.822068	LYN	Lake	ZMUC-CRU-004810
A. astacus	12.186253-55.636285	HED	Lake	ZMUC-CRU-005699
A. astacus	12.345997-55.7477	MAR	Lake	ZMUC-CRU-004844
A. astacus	12.305374-55.822068	LYN	Lake	ZMUC-CRU-004809
A. astacus	12.558574-55.681184	SKJ	Lake	ZMUC-CRU-004846
A. astacus	12.378904-56.042448	ESR	Stream	ZMUC-CRU-005695
P. leniusculus	12.442784-55.769896	NYD	Lake	ZMUC-CRU-005691
P. leniusculus	12.442784-55.769896	NYD	Lake	ZMUC-CRU-005684
P. leniusculus	12.442784-55.769896	NYD	Lake	ZMUC-CRU-005683
P. leniusculus	12.152816-55.442244	HAS	Lake	ZMUC-CRU-005682
P. leniusculus	12.442784-55.769896	NYD	Lake	ZMUC-CRU-005684
P. leniusculus	12.442784-55.769896	NYD	Lake	ZMUC-CRU-005685
P. leniusculus	12.442784-55.769896	NYD	Lake	ZMUC-CRU-005691
P. leniusculus	12.293014-55.564369	MOS	Brackish	ZMUC-CRU-006036

DNA extraction and PCR amplification

Tissue samples were taken from the tail musculature under sterile conditions. All tools were treated with 96% ethanol and heated in a flame between samples. Samples were stored in 96% ethanol at $-18°$C until extraction.

A 1.0 mm^3 piece of tail tissue was comminuted and transferred to a 2.0 mL eppendorff tube. Genomic DNA was isolated using the commercial Qiagen® DNeasy® blood & tissue kit following the quick-start protocol, with modifications (see appendix B, page 46, figure 11).

Protein coding mitochondrial cytochrome c oxidase subunit 1 (COI) codes for a fundamental enzyme in the oxygen metabolism of eukaryotes and has a relatively fast mutation rate, which usually makes

it possible to distinguish between closely related species (Hebert et al., 2003). In this study, COI was targeted, using the COI-broad range invertebrate primers HCO2198 and LCO1490 developed by Folmer et al. (1994). Polymerase chain reaction (PCR) was performed with a reaction mixture of 25.0 μL containing 2.0 μL DNA-template, 0.1 μL of 5.0 U/μL AmpliTaq polymerase, 2.5 μL of 2 mM dNTP, 2.5 μL of 10x Buffer (ampliTaq), 1.0 μL of 2mM $MgCl_2$, 1.0 μL of 10.0 mg/mL BSA, 1.25 μL of each 10.0 μM primer and 15.4 μL of double distilled water (ddH_2O). The applied PCR temperature cycling settings were: [Phase 1] Initial denaturation: 60 s at 95°C. [Phase 2] Denaturation: 10 cycles of 95°C for 30 s. Annealing: 60°C for 30 s and decreasing 1°C per cycle, 72°C for 90 s followed by 30 cycles of 30 s at 95°C, 30 s at 50°C and 90 s at 72°C. [Phase 3] Extension: 5 minutes at 72°C. Store: 10°C until removed.

For better amplification of some samples, settings from Maguire et al. (2014) were modified and applied (appendix A, page 41, table 11 and 12). Analysis of PCR-products was performed using gel-electrophoresis on 2% agarose gel for 30 minutes at 130 V and 130 A, and visually examined with infrared photography, to verify lengths of the amplified products. Purification and sequencing was performed by Macrogen Europe (Amsterdam, The Netherlands, www.macrogen.com). Separated laboratories were used for pre and post PCR procedures, and throughout the study, extraction and PCR blanks were used to monitor contamination.

Analysis of sequences and design of species-specific primer-probe systems

Geneious v. 7.1.7.(Biomatters Ltd.) (Kearse et al., 2012) was used to trim amplified sequences and to remove low quality reads within the sequences. Forward and reverse sequences were aligned and visually inspected and consensus sequences were then aligned with crayfish COI sequences from Genbank using the MAFFT algorithm (Katoh et al., 2002). Possible primer and probe sites were visually inspected and primer-probe systems were designed to target the region targeted by Tréguier et al. (2014) in *Procambarus clarkii*.

Primer-probe systems were named after the three first letters in the family and species name of the target species, as follows: *Astacus astacus*, Astast; *Pacifastacus leniusculus*, Paclen. For *Astacus leptodactylus* the primer-probe system is named after another accepted synonym; *Pontastacus leptodactylus*, Ponlep, and the number, I or III, refers to clade number (table 7).

Phylogenetic analysis

Phylogenetic relationships were reconstructed based on the mtDNA COI region. COI is an $\approx$ 1500 bp long region, however, there were great variations in the lengths of COI sequences available in Genbank for *A. astacus*, *P. leniusculus* and *A. leptodactylus* (350 - 658 bp). Sequences from sampled crayfish from Denmark were aligned to sequences from Genbank resulting in a final alignment length of 350 bp.

DNA mutations, such as base substitutions, are complex and happen at different rates. For instance, the interchanges between the two-ring purines, Adenine (A) and Guanine (G), are less likely to occur than interchanges between the one-ring pyrimidines, Cytosine (C) and Thymine (T) (Palumbi, 1996). The specific substitution rate within a specific region can be tested using different programs, however

this is beyond the aim of this study. The base frequency substitution model HKY85, that accounts for some differences in transition and transversions of bases (Hasegawa et al., 1985), was chosen for all analyses.

A sub-optimal, but simple and fast neighbour joining (NJ) tree (Saitou and Nei, 1987) containing all three species was performed in Geneious. The neighbour joining analysis was performed with 1000 bootstrap pseudoreplicates and *P. leniusculus* as an outgroup. For the *A. leptodactylus* species complex (Maguire et al., 2014; Akhan et al., 2014), the stronger maximum likelihood (ML) analysis (Felsenstein, 1981) was performed using PhyML online (Guindon et al., 2010). Eight random independent tree starts were performed, with branch support calculated from 1000 bootstraps. For an optimized tree topology, both Nearest Neighbour Interchange (NNI) (DasGupta et al., 1997) and Subtree Pruning and Regrafting (SPR) (Hein et al., 1996) were selected. The default settings at PhyML-online (Guindon et al., 2010) were chosen for other parameters. For the ML analysis, *A. astacus*, *A. pallipes* and *A. torrentium* was used as the outgroup, with accession numbers JN254675, JF293432 and AY667112, respectively (figure 8).

Testing and optimizing primer-probe systems

Primer-probe system specificity was tested on DNA extracted from tissue samples from all three crayfish species present in Denmark (*Astacus astacus*, *Astacus leptodactylus* and *Pacifastacus leniusculus*). Primers were first tested on PCR using the same PCR settings and gel-electrophoresis verification as described in section "DNA extraction and PCR amplification" (page 12). Then the combined primer-probe systems specificity was tested on a Stratagene® Mx3005P qPCR, with a reaction volume of 25.0 μL, containing 2.0 μL DNA template, 10.0 μL of 2.0 U/μL TaqMan® Environmental Master Mix 2.0 (Life Technologies®), 1.0 μL of each 10.0 μM primer, 1.0 μL of 2.5 μM probe and 10.0 μL ddH_2O. Thermal conditions: [Phase 1] 50°C for 5 minutes then 10 minuets at 95°C. [Phase 2] 50 cycles of 95°C for 30 s and 60°C for 1 minute.

According to Souty-Grosset et al. (2006) and Kouba et al. (2014) 15 crayfish species are present i Europe. Two sequences from each species were downloaded from Genbank and tested in Geneious against our primer-probe systems to assess the risk of cross-species amplification.

Primer and probe concentrations were optimized for the Astast and Paclen qPCR systems (table 7). Different forward and reverse primer concentrations of 0.2 μM and 1.2 μM were tested on positive DNA tissue extracts. As described by Bustin et al. (2009) the concentrations resulting in the lowest cycle threshold value (C_t) for each system were considered the optimal setting. These optimized primer concentrations were used when testing the optimal probe concentration for the systems. Probe concentrations between 0.1 μM and 1.2 μM were tested on positive DNA tissue extracts. Results were extrapolated to both Ponlep I and III systems (table 7) and adopted into the working process (table 9).

Determining limit of detection (LOD) and limit of quantification (LOQ)

To determine LOD and LOQ, a standard curve (C_t vs. concentration (copies/μL extract)) was made for all species-specific primer-probe systems, except Ponlep I (table 7).

DNA extracted from tissue samples of each crayfish species was PCR amplified using the same primers and probes as for qPCR. Samples were purified using the Qiagen® QIAquick PCR purification kit and the applied protocol. Concentrations of double stranded DNA (ds-DNA) were measured on a Qubit 2.0 Fluorometer (Life Technologies) and the number of target copies was calculated from the specific molecular weight of each 65 bp sequence (equation 3) (Kibbe, 2007).

$$\boxed{C = \frac{C_s}{M} * N_A} \tag{3}$$

Where: C = stock concentration in copies/μL, C_{stock} = stock concentration in ng/μL, M = molecular weight of ds-DNA, N_A = the Avogadro constant.

From calculated stock concentrations, dilution series of known concentrations from 10^{-1} copies/μL increasing tenfold to 10^8 copies/μL were made for each species.

qPCRs with optimized settings were run with three replicates of each dilution. LOD is determined as the lowest concentration giving minimum one positive replicates. LOQ is determined as the concentration at which C_t values of all three positive replicates are clustered (Biggs et al., 2015).

Field testing of primer-probe systems

Study sites

Field testing of the developed primer-probe systems was performed at nine lakes on the island of Zealand, Denmark (figure 6). Knowledge on crayfish abundance was acquired through previous field work (described in section "Field work", page 5). Two lakes contained signal crayfish; Lake Lerchenborg and Lake Nydam. Three lakes contained narrow-clawed crayfish; Lake Damhussøen, Lake Fuglesangssø and Lake Furesø. Three lakes contained noble crayfish; Lake Sankt Jørgens Sø, Lake Løjesø and Lake Agersø. Lake Botanisk Have is not known to contain crayfish.

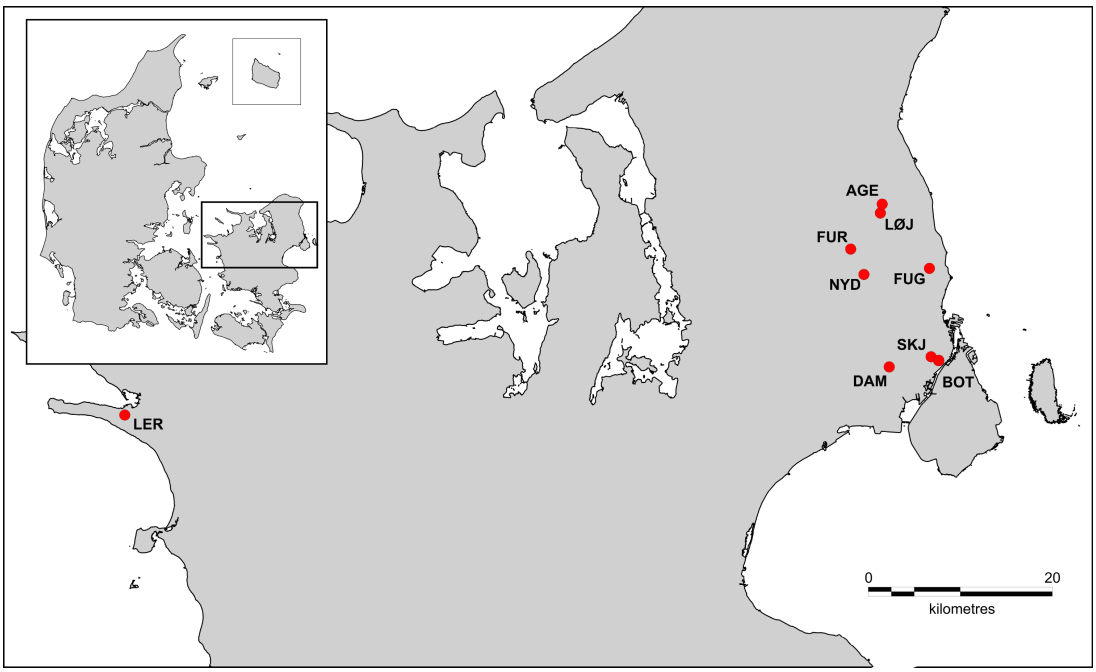


Figure 6: eDNA sampling sites. AGE = Lake Agersø, BOT = Lake in Copenhagen Botanical Garden, DAM = Lake Damhussøen, FUG = Lake Fuglssangssø, FUR = Lake Furesø, LER = Lake Lerchenborg, LØJ = Lake Løjesø, NYD = Lake Nydam, SKJ = Lake Sankt Jørgens Sø.

Water sampling

Water sampling was performed in April and June, 2015. Three samples with identical volume between 0.5 L and 1.5 L, were collected from each water body, and filtered in the field, using HSW Soft-Ject® 60 mL syringes and sterivex™ filter unit with a pore size of 0.22μm. Samples were collected from the shore approximately 40 cm above the bottom substrate, with Lake Furesø as the exception. In Lake Furesø, water was collected and filtered during snorkelling on the stone reef described earlier. During sampling temperature was measured 40 cm above the bottom. Filters were immediately stored in dark conditions and kept on ice during transport to the laboratory, where they were stored at -18°C. Extraction was done within 24 h after sampling to minimize DNA degradation. Field work and laboratory work was not

carried out the same day to minimize the risk of contaminating samples. Before and after laboratory work all surfaces and instruments were cleaned with bleach and 70% ethanol.

eDNA extraction from filters

Prior to extraction, the outside of the filters were bleached. Extraction was done using Qiagen® DNeasy® Blood & Tissue kit and the applied protocol was followed with modifications (see appendix B, figure 12, page 47).

Detection and quantification with qPCR

Species-specific primer-probe systems were tested on eDNA extracted from filters. One qPCR reaction of 25.0 μL contained 10.0 μL TaqMan® Environmental Master Mix 2.0 (Life technologies), 7.0 μL ddH_2O, 1.0 μL of each primer and probe (1.2 μM and 0.1 μM, respectively) and 5.0 μL of extracted DNA. Thermal settings: [Phase 1] Initial denaturation: 5 minutes at 50°C followed by 10 minutes at 95°C. [Phase 2] Denaturation: 50 cycles of at 95°C for 30 s followed by annealing at 60°C for 1 minute [Phase 3] Final extension: 10 minutes at 72°C. The final extension time was increased to enhance possible future cloning of qPCR products. For each primer-probe system, four reactions were performed per lake. Standard dilutions with four replicates of 10^8-10^{-1} copies/μL, two positive DNA extraction controls, four negative PCR controls and two negative extraction controls were included in all qPCR runs.

From standard curves based on a dilution series of known concentrations, the concentration of eDNA in copies/L of filtered water was calculated from formula 4.

$$\boxed{C_L = \frac{C_r * (\frac{V_e}{V_r})}{V_w}} \tag{4}$$

Where: C_L = copies/L, C_r = copies/reaction, V_e = volume of extract in μL , V_r = volume of extract in reaction in μL, V_w = water volume filtered in L.

Detection is considered reliable if at least two of four qPCR replicates show positive results. When calculating final eDNA concentrations, replicates with no detection of eDNA were treated as zero (0), as recommended by Ellison et al. (2006).

Results

Field work

Estimation of catchable population size with mark/recapture

In Lake Nydam, the catchable population size of signal crayfish is estimated to be 3827 individuals (table 2). The length of caught crayfish ranged from 6.7 cm to 14.7 cm TL (table 3).

Table 2: Catchable population estimate in numbers.

Location	Method	Population estimate	95% C.I.
Lake Nydam	Schnabel	3827	2511-6506
Lake Furesø	Schnabel	4334	2730-9770

Table 3: Weight and length of male (n = 150) and female (n = 65) signal crayfish caught with traps in Lake Nydam.

	Males		Females	
	TL (cm)	Weight (g)	TL (cm)	Weight (g)
Mean	10.8	51.9	10.4	40.6
SD	1.5	24.3	1.4	15.4
Min	6.7	9.0	7.0	12.0
Max	14.7	153.0	13.2	79.0

Table 4: Mark/recapture from Lake Nydam. Recaptures are not included in the total catch.

Date	Total catch	No. of recaptures	Newly marked	No. of ind. marked before sampling
Oct. 12th, 2014	159	0	159	0
Oct. 18th, 2014	225	7	218	159
Oct. 30th, 2014	98	12	86	377
Total	482	19		

In Lake Furesø, the catchable population size of narrow-clawed crayfish on the stone reef in Lake Furesø is estimated to be 4334 individuals (table 2). Sizes range from 1.8 cm to 6.1 cm POCL (table 5).

Table 5: Weight and length (post-orbital carapace length) of male (n = 43) and female (n = 25) narrow-clawed crayfish caught with snorkelling in Lake Furesø.

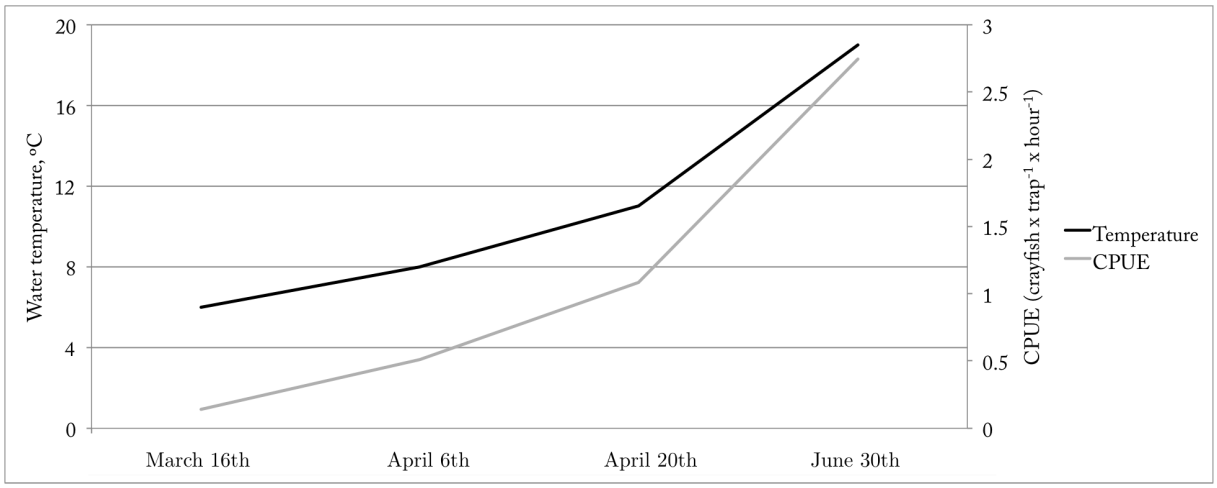
	Males		Females	
	POCL (cm)	Weight (g)	POCL (cm)	Weight (g)
Mean	4.0	43.7	4.3	50.6
SD	0.9	26.9	1.0	24.1
Min	1.8	4.0	3.0	16.0
Max	6.1	138.0	5.6	101.0

Table 6: Mark/recapture from stony reef in Lake Furesø. Recaptures are not included in the total catch.

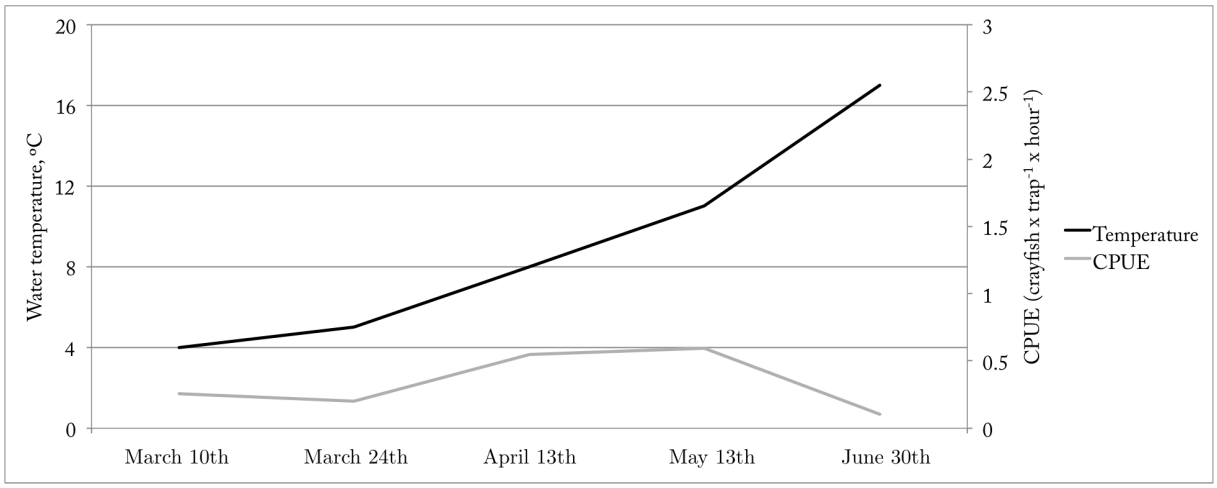
Date	Total catch	No. of recaptures	Newly marked	No. of ind. marked before sampling
May 13th, 2014	100	0	100	0
May 22nd, 2014	92	3	89	100
May 25th, 2014	33	3	30	189
May 29th, 2014	70	2	68	219
July 6th, 2014	74	3	71	287
Total	358	11		

Measuring CPUE

There is a moderate positive correlation between water temperature and CPUE when combining data for the signal crayfish and the narrow-clawed crayfish ($r = 0.653$, $p = 0.056$, $N = 9$)(figure 7). No noble crayfish were caught in Lake Sankt Jørgens Sø, although specimens were observed from the shore during the same period.



(a) Signal crayfish *Pacifastacus leniusculus* from Lake Nydam.



(b) Narrow-clawed crayfish *Astacus leptodactylus* from Lake Damhussøen.

Figure 7: Relationships between water temperature and catch per unit of effort (crayfish/trap/hour). (a) Signal crayfish *Pacifastacus leniusculus* from Lake Nydam, (b) narrow-clawed crayfish *Astacus leptodactylus* from Lake Damhussøen.

Design of species-specific primer-probe systems

DNA extraction and PCR amplification

Three DNA extracted tissue samples from signal crayfish and eight from narrow-clawed crayfish were succesfully amplified and sequenced.

All blank controls showed negative results.

Development of primer-probe systems

A 65 base pair species-specific primer-probe system for the mtDNA COI region was developed targeting the same region applied by Tréguier et al. (2014) for crayfish *Procambarus clarkii*. In order to cover the genetic variations of the crayfish present in Denmark, the results from the phylogenetic analysis (see section "Phylogenetic analysis", page 22) were used to create four species-specific primer-probe systems (table 7). Development of a primer-probe system for *A. astacus* was based solely on sequences from Genbank. The primer-probe systems for *P. leniusculus* and *A. leptodactylus* were based on sequences from Genbank and sequences acquired from sampled specimens of the species.

Table 7: Species-specific primer-probe systems are named after the first three letters in family name and species name. *Astacus leptodactylus* primers are named after the accepted synonym *Pontastacus leptodactylus* and clade number as in Akhan et al. (2014). Fam = reporter dye, BHQ-1 = quenching dye.

Species specificity	Primer/Probe	Sequence (5'-3')	Number of bases
A. astacus	Astast_COI_F0336	GATTAGAGGAATAGTAGAGAG	21
	Astast_COI_P0357	Fam-AGGAGTAGGGACAGGATGAACT-BHQ-1	22
	Astast_COI_R0397	CTGATGCTAAAGGGGGATAA	20
P. leniusculus	Paclen_COI_F0336	AACTAGAGGAATAGTTGAAAG	21
	Paclen_COI_P0357	Fam-AGGAGTGGGTACTGGATGAACT-BHQ-1	22
	Paclen_COI_R0397	CCGCTGCTAGAGGAGGATAA	20
A. leptodactylus	PonlepI_COI_F0336	AACTAGGGGTATAGTAGAGAG	21
clade I	PonlepI_COI_P0357	Fam-AGGAGTAGGGACCGGATGAACT-BHQ-1	22
	PonlepI_COI_R0397	CTGATGCTAAAGGGGGATAA	20
A. leptodactylus	PonlepIII_COI_F0336	AACTAGAGGTATAGTAGAGGG	21
clade III	PonlepIII_COI_P0357	Fam-GGGTGTAGGAACTGGATGAACC-BHQ-1	22
	PonlepIII_COI_R0397	CTGATGCTAGGGGAGGATAA	20

Phylogenetic analysis

Sequences from Genbank represent mostly populations from Europe, but Armenia, Russia and the United States are also represented (appendix A, page 42, table 13). The performed neighbour joining tree illustrates that both noble crayfish and signal crayfish sequences from Genbank clustered together in a single clade for each species, while narrow-clawed crayfish sequences were separated into several clades

(appendix A, page 44, figure 9). Our three signal crayfish sequences are located in the middle of the signal crayfish clade, whereas our eight narrow-clawed crayfish sequences were separated into two of the three clades recognized by Akhan et al. (2014), one in clade I and seven in clade III (appendix A, page 45, figure 10).

Testing and optimizing of primer-probe systems

All primer-probe systems were tested using PCR and qPCR, on 24 DNA extracted tissue samples and successfully amplified the species or clade it was targeting, without any amplification products for any other species or clade.

In Genbank two sequences were chosen to represent each crayfish species. The number of mismatches between the sequences and the species-specific primer-probe systems ranged between 6 and 15 base pairs (table 8).

Table 8: Number of single base pair mismatches between different crayfish species and four different 65 bp long species-specific primer-probe systems. Two sequences from each species were obtained from Genbank. The most conservative average of mismatches is shown.

	Primer-probe systems				
Species	Astast	Paclen	Ponlep I	Ponlep III	Genbank no.
Astacus astacus	0	11	8	12	JN254670 ; JN254671
Pacifastacus leniusculus	11	0	12	11	JF437995 ; JF437995
Astacus leptodactylus Clade I	8	12	0	6	JQ421471 ; JQ421471
Astacus leptodactylus Clade II	8	14	7	6	JQ421478 ; JQ421479
Astacus leptodactylus Clade III	11	11	6	1	JQ421489 ; JQ421490
Astacus pachypus	N/A	N/A	N/A	N/A	
Austropotamobius torrentium	13	14	12	13	AY667128 ; AM180946
Austropotamobius italicus	11	14	10	15	HM622614 ; AY121127
Austropotamobius pallipes	10	13	11	14	AY667114 ; AY667115
Cherax destructor	15	15	13	13	KM039112 ; KJ950555
Orconectes immunis	16	14	15	15	JF438005 ; JF438006
Orconectes limosus	13	12	13	15	JF437992 ; JF437993
Orconectes virilis	12	10	14	15	FJ608577 ; EU442743
Orconectes rusticus	16	13	15	17	AY701248 ; AY701249
Procambarus clarkii	11	11	9	13	JN000900 ; JN000901
Procambarus sp. (Marmorkrebs)	11	10	9	9	HM358011 ; KF033123

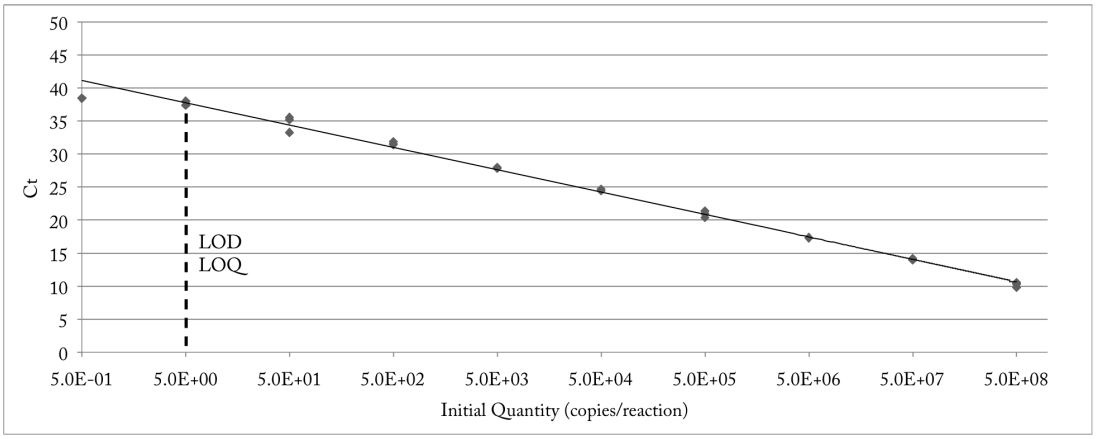
Optimization of primer and probe concentrations showed the same results for both Astast and Paclen systems. Both forward and reverse primers had the lowest C_t value at a reaction concentration of 1.2 μM. The probe performed best at a reaction concentration of 0.1 μM. These results were extrapolated to both primer-probe systems Ponlep I and III (table 9).

Table 9: Optimized settings for qPCR. Primers F and R are species-specific primers corresponding to the targeted species.

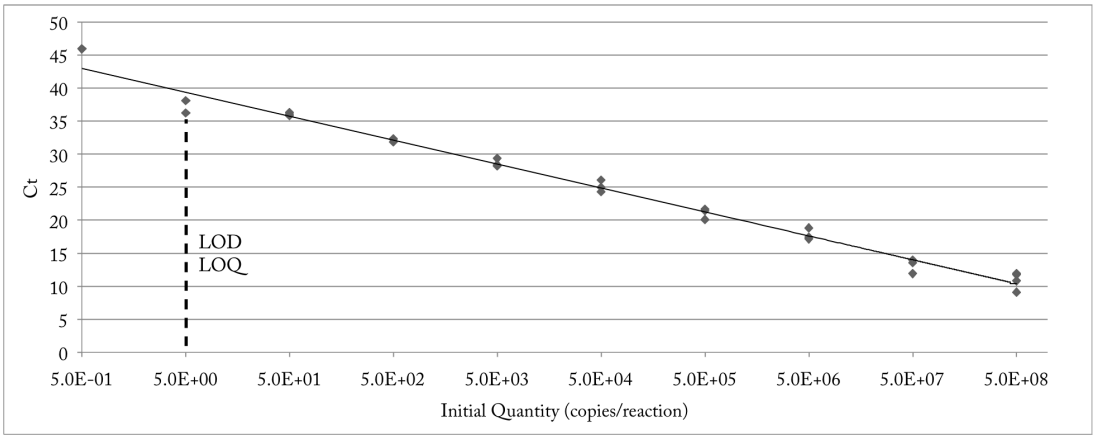
Materials	Vol / well (μL)	Concentration / well
EM Mix 2.0	10.0	0.8 U/μL
Primer F	1.0	1.2 μM
Primer R	1.0	1.2 μM
Probe	1.0	0.1 μM
H_20	7.0	
DNA-template	5.0	
	Total volume 25.0	

Determining LOD and LOQ

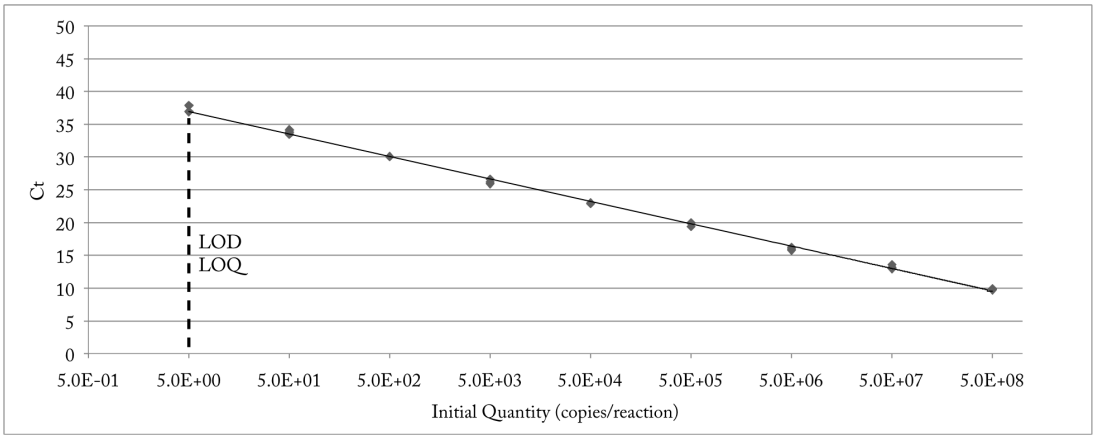
Determination of LOD and LOQ is based on a dilution series of amplified and purified DNA, which was run on qPCR with optimized settings. LOD and LOQ for all three species was found to be 5 copies/reaction equal to 1 copy/μL extract (figure 8).



(a) Noble crayfish *Astacus astacus*.



(b) Narrow-clawed crayfish *Astacus leptodactylus*.



(c) Signal crayfish *Pacifastacus leniusculus*.

Figure 8: C_t values plotted against concentration (copies/reaction), giving standard curves based on three replicates, the dotted line represents both limit of detection (LOD) and limit of quantification (LOQ) for (a) noble crayfish *Astacus astacus*, (b) narrow-clawed crayfish *Astacus leptodactylus* clade III and (c) signal crayfish *Pacifastacus leniusculus*. Only two replicates were successfully amplified from concentrations of 5 copies/reaction in all species.

Field testing of primer-probe systems

The species-specific primer-probe systems successfully detected the expected crayfish species in water samples from six out of eight lakes (table 10). All results with positive qPCR replicates are shown, however we only assume a positive detection when two replicates are above LOD, to minimize the risk of accepting false-positive results. All together, the systems detected crayfish eDNA in nine out of eleven water samples tested (table 10). It was not possible to detect *Pacifastacus leniusculus* eDNA in the last water sample from Lake Nydam, even though a high CPUE was measured. Similarly, it was not possible to detect *Astacus leptodactylus* eDNA from Lake Furesø despite knowledge of their presence in high numbers from the mark/recapture experiment. In Lake Agersø, both *A. leptodactylus* and *A. astacus* were caught during the CPUE experiment. However their presence was not clearly shown by eDNA, as *Astacus leptodactylus* was detected in two qPCR replicates and *Astacus astacus* was only detected in one replicate. Based on trapping done by Peter Rask Møller on several occasions and a trapping event performed during this study, the lake in Copenhagen Botanical Garden does not house any crayfish. However our Ponlep I system show four clear positive replicates. Similarly, in Lake Damhussøen, *Pacifastacus leniusculus* was detected by eDNA, however this has not been confirmed by conventional methods.

DNA concentrations above the LOQ were quantified, and estimated to be between 48 and 87552 average DNA copies/L. We were not able to compare eDNA concentrations to CPUE due to the lack of data (table 10). CPUE data from *A. leptodactylus* was excluded because it is not possible to distinguish between clade I and III morphologically.

All PCR and extraction blanks were negative except one extraction blank. This extraction blank showed positive amplification with the Ponlep III system in one out of two replicates. The blank was run again and was positive in one out of four replicates. Despite this, results from Ponlep III are still included in our analysis.

Table 10: Results from watersamples and CPUE. AGE = Lake Agersø, BOT = Lake in Copenhagen Botanical Garden, DAM = Lake Damhussøen, FUG = Lake Fuglsangssø, FUR = Lake Furesø, LER = Lake Lerchenborg, LØJ = Lake Løjesø, NYD = Lake Nydam, SKJ = Lake Sankt Jørgens Sø.

Location	Species	Water filtered (mL)	Positive qPCR replicates	Avg. DNA copies/L (SD)	Species presence confirmed	Detection methods	Catchable pop. size estimate in numbers (95% C.L.)	Average CPUE (crayfish/trap/hour)
AGE	*A. astacus*	3x1000	1[c]	126	Yes	Visual, trapping	N/A	0.34
AGE	*A. leptodactylus* III	3x1000	2	902 (1100)	Yes[e]	Trapping	N/A	N/A
BOT	*A. leptodactylus* I	3x1500	4	N/A	No		N/A	N/A
DAM	*A. leptodactylus* III	3x1000	4	87552 (47557)	Yes[e]	Visual, trapping	N/A	N/A
DAM	*A. leptodactylus* I	3x1000	3	N/A	Yes[e]	Visual, trapping	N/A	N/A
DAM[a]	*A. leptodactylus* III	3x1000	4	2654 (1593)	Yes[e]	Visual, trapping	N/A	N/A
DAM[a]	*P. leniusculus*	3x1000	2	16 (22)[d]	No		N/A	0
FUG	*A. astacus*	3x500	1[c]	6[d]	No		N/A	0.00
FUG	*A. leptodactylus* I	3x500	1[c]	N/A	Yes[e]	Visual, trapping	N/A	N/A
FUG	*A. leptodactylus* III	3x500	4	4893 (1443)	Yes[e]	Visual, trapping	N/A	N/A
FUR	*A. leptodactylus*	3x500	0	N/A	Yes[e]	Visual, trapping	4334 (2730-9770)[g]	N/A
LER	*P. leniusculus*	3x1000	4	251 (150)	No[f]	Trapping[f]	N/A	N/A
LER	*A. leptodactylus* III	3x1000	1[c]	7[d]	No		N/A	N/A
LØJ	*A. astacus*	3x750	4	203 (73)	Yes	Visual, trapping	N/A	0.23
NYD[b]	*P. leniusculus*	3x1000	4	39 (15)[d]	Yes	Visual, trapping	3827 (2511-6506)	1.08
NYD[b]	*A. leptodactylus* I	3x1000	1[c]	N/A	No		N/A	0
NYD[b]	*A. leptodactylus* III	3x1000	1[c]	48	No		N/A	0
NYD	*P. leniusculus*	3x1000	0	N/A	Yes	Visual, trapping	3827 (2511-6506)	2.75
SKJ	*A. astacus*	3x1000	4	103 (139)	Yes	Visual	N/A	N/A

[a]: Sampled on the 13th of April. [b]: Sampled on the 20th of April. [c]: Below limit of replicates for positive detection. [d]: Below limit of detection. [e]: Species present but clade unknown. [f]:Presence confirmed in 2008 by Peter Rask Møller. [g]: Estimated pop. size on reef, June 2014.

Additional results

In collaboration with David Strand and Trude Vrålstad from the Norwegian Veterinary Institute and the TARGET project (project number NFR/243908), the Astast system was tested on water samples from Norwegian lakes. The Astast system successfully detected noble crayfish eDNA in Norwegian lakes, where noble crayfish are known to be present.

Furthermore, DNA extracted from tissue samples of three signal crayfish (ZMUC-CRU005691, ZMUC-CRU005682, ZMUC-CRU006036) and one narrow-clawed crayfish (ZMUC-CRU005674) were analysed, together with eDNA extracted from water samples from Lake Lerchenborg, Lake Damhussøen and Lake Nydam, for the presence of crayfish plague. All seven samples were analysed by David Strand as described in Vrålstad et al. (2009) and all showed negative results.

In collaboration with DNA & LIV, a high-school citizen science based educational program in collaboration with the Natural History Museum of Denmark, students used the Astast and Paclen systems (table 7) and successfully detected *Pacifastacus leniusculus* eDNA in Lake Damhussøen.

Discussion

Field work

Mark/recapture

Mark/recapture is a widely applied method for estimating the catchable population size of crayfish (e.g. Bolat et al., 2011; Maguire et al., 2004). Despite a bias towards large males, baited funnel traps are still the most common capturing technique (Bolat et al., 2011; Parkyn, 2015). Pilotto et al. (2008) successfully used snorkelling to catch *Orconectes limosus* (Ranesque, 1817) in Lake Varese in Italy. It seems that results from active methods generally represent true population structure better than those of passive methods (Dorn et al., 2005). Due to sex and size bias, the results presented in this study (table 6 and 4) only represent the catchable population size and we would expect the actual population size to be larger (Parkyn, 2015). It is not possible to compare mark/recapture results between Lake Nydam and Lake Furesø, due to the difference in sampling method and in the measure of length. The assumption that the stone reef in Lake Furesø is a closed population, is convenient but might overestimate the catchable population size inhabiting the reef, because migration and immigration is not acounted for (Pilotto et al., 2008).

Marked crayfish were regularly recaptured up to ten months after marking, presumably spanning at least one moulting event, and VIE-tags were still visible. Pilotto et al. (2008) find that VIE-tags were visible after 80 days and one moult.

CPUE

CPUE is often used as a measure of crayfish activity and abundance (e.g. Dorn et al., 2005; Richards et al., 1996), but values obtained are affected by many factors, such as water temperature, bait type and lunar phase (Richards et al., 1996; Somers and Stechey, 1986). This makes it difficult to compare CPUE values from different waters with eDNA quantifications. The basic assumptions behind the comparison of CPUE to the amounts of eDNA in the water, are that CPUE is a reliable measure of abundance and activity and that abundance and activity is correlated with DNA excretion rate as described by Takahara et al. (2012) for fish. However, Tréguier et al. (2014) make the same assumptions even though this has not been investigated for crayfish.

Our results show that CPUE across species is positively correlated with water temperature, as also shown in previous studies by Richards et al. (1996) and Somers and Stechey (1986).

Design of species-specific primer-probe systems

DNA extraction and PCR amplification

Only 11 of 24 DNA extracted tissue samples were successfully amplified and sequenced with the Folmer primers. We did not manage to sequence any DNA extracted from tissue samples from *A. astacus* and only a limited number (three out of eight) of the *P. leniusculus* DNA extracted from tissue samples. Thus, only a narrow representation of the Danish crayfish species and the genetic variation within populations was obtained.

Schrimpf et al. (2014) analysed the mtDNA site COI in *A. astacus* and found that the European populations are relatively genetic homogeneous, owing to anthropogenic translocations. However, Schrimpf et al. (2014) show an explicit genetic structure with endemic haplotypes in two *A. astacus* populations; one in the Eider catchment west of Kiel, and another in northern Germany and Rhineland-Palatinate in south-west Germany. However the population structure of Danish noble crayfish and their genetic position in the European population remains unknown. If the Astast system or any of the primer-probe systems, is to be used elsewhere in Europe, local positive samples should be tested in order to ensure local specificity.

Phylogenetic analysis

Genetic trees were a necessary tool to resolve the genetic position of some of the Danish specimens of signal crayfish and narrow-clawed crayfish, especially the positions in the narrow-clawed species complex, and the need for different primer-probe systems for each clade. Knowledge on the narrow-clawed crayfish clades in Denmark can further provide an understanding of the invasion history because the clades represent different areas in Turkey.

A neighbour joining analysis was chosen for an overview of the tree topology, due to the fast data handling time and simple settings of this method. In order to increase the strength of the analysis we selected the maximum likelihood analysis which can include more parameters. For the ML analysis the model must be specified correctly otherwise it can lead to poor statistical properties (Yang and Rannala, 2012). Bootstrap values in the ML analysis showed several zero values next to high values, which might be due to non-specified settings in the ML model.

Development of primer-probe systems

Development of species-specific primer-probe systems requires explicit knowledge about phylogenetic relationships between species and subspecies, to ensure steady species detection. In this study we confirmed the developed systems specificity on PCR and qPCR positives, tested the cross-species amplification on other species, compared the primer-probe systems to all previous published sequences in Genbank (appendix A, table 13) and analysed the number of mismatches between the developed primer-probe systems and crayfish species present in Europe. We had high primer-probe specificity to sequences available in Genbank, and observed no cross-species amplification of primer-probe systems even within different clades

of the same species, with six base pair mismatches as the minimum. These experiences entail that each primer-probe system is specific and effectively amplifies target species eDNA in an aquatic environment.

The genetic region targeted by Tréguier et al. (2014), and used in this study, is species-specific for at least four different crayfish species and can distinguish clades within the same species. These findings might be a good starting point for future development of crayfish primer-probe systems targeting eDNA in the aquatic environment. However, the development of primer-probe systems should also rely on a high number of sequences in order to reveal inter- and intraspecific gene variation, from the geographical region where they are designed to be species-specific. This study demonstrates that at least two clades from the *A. leptodactylus* species complex are present in Denmark, and emphasizes the need for a high number of sequences to develop species-specific primer-probe systems.

Testing primer-probe systems

Testing the primer-probe systems on DNA extracted from tissue samples might display the actual specificity of the systems. In this study, it is expected that the DNA extractions only represent a small portion of the inter- and intraspecific genetic variation within crayfish in Denmark. Thus, the specificity of our primer-probe system is only tested on a limited number of crayfish populations in Denmark. A further reduction of the potential risk of cross-species amplification would require a better understanding of the genetic diversity of crayfish species throughout their native and non-native range. However, this is beyond the scope of this study. The Astast system (table 7) is solely developed based on sequences from Genbank (appendix A, table 13), but testing performed on DNA extracted from tissue samples showed compatibility with all eight samples. Furthermore, the Astast system was tested in collaboration with the Norwegian Veterinary Institute on water samples from Norwegian lakes and the system was able to detect *Astacus astacus* eDNA, which confirmes specificity of the system outside Denmark.

The Ponlep I and III systems (table 7) were tested on DNA extractions and results supported the genetic analysis (appendix A, figure 9 and 10) in the assumption that the Ponlep I system was only specific to one sample, whereas the Ponlep III system showed specificity to all samples except the one specific to Ponlep I.

In order to determine whether the primer-probe system could potentially amplify other species of crayfish and thereby cause false-positive results, base pair mismatches between the systems and native and non-native crayfish species present in Europe were analysed. The lowest number of base pair mismatches was six for both Ponlep I and III within the species complex of *A. leptodactylus* and Ponlep I and Ponlep III only showed specificity towards its own clade. Thus we anticipate that with atleast six or more mismatches, cross-species amplification is highly unlikely to occur. According to Wilcox et al. (2013) mismatches in the primers influence the specificity more than mismatches in the probe, however, this was not investigated in the present study.

Optimization was only done for the Astast and Paclen systems. They showed the same optimal concentrations for both primer and probes and since all primer-probe systems target the same region of COI, the optimized concentrations were extrapolated to both Ponlep I and Ponlep III. Primers and probes

were only tested to a certain concentration, and elevated primer concentrations could be tested in the future. In order to be able to run several water samples with different primer-probe system at the same time, temperature settings in the qPCR were not optimized, but fixed at a standard temperature setting. Additionally, during the testing of the Astast system in Norway, David Strand from the Norwegian Veterinary Institute discovered a better amplification when the annealing temperature was lowered from 60°C to 56°C. This indicates that other temperature settings can be applied to enhance detection. However, a change in settings should not be incautiously applied before tested properly, because an increased temperature might increase over all affinity of the primer-probe system resulting in false-positive results.

We find a theoretical LOD and LOQ at 1 copy/μL extract (equal to 80 copies/L water filtered) for all systems, which is close to the LOD at approximately 62 copies/L seawater that Thomsen(a) et al. (2012) find for the fish species *Platichthys flesus* (Linnaeus, 1758) and *Gasterosteus aculeatus* (Linnaeus, 1758).

For noble crayfish and narrow-clawed crayfish, one of three replicates amplified at concentrations of 0.1 copy/μL, and thus it would be possible to set LOD at this level. But it is not possible for a reaction to contain less than one copy of DNA, these positive replicates must stem from the chance of transfer one of the few DNA copies present in the 10^{-1} dilution. High amount of DNA template, such as the 5 μL used in this study, can enhance the possibility of detection at concentrations below 1 copy/μL.

There is no standardized method to estimate theoretical LOD and LOQ, even though successful quantification of eDNA is the key to a higher level of understanding of eDNA in the aquatic environment. Several studies, such as Tréguier et al. (2014); Biggs et al. (2015) and Diaz-Ferguson et al. (2014) use pure genomic DNA to estimate LOD and LOQ in ng/μL, thus, quantifying the amount of genomic DNA and not the targeted region. Further, some studies (e.g. Takahara et al., 2012) correctly display quantified eDNA in copies/μL but do not give en explanation on how these values were estimated. We conclude, that the most correct way to estimate LOD and LOQ is from a dilution made from purified PCR product, as described in both Wilcox et al. (2013) and Thomsen(b) et al. (2012). This lack of a standardized protocol to estimate LOD and LOQ, makes it difficult to compare quantification of eDNA between studies. We recommend that future attempts to quantify eDNA should be based on dilution series made from purified PCR products as described in this study.

Alternative methods for eDNA quantification are being developed. An alternative platform of qPCR, might be the digital droplet PCR (ddPCR)(Jones et al., 2014), where the reaction mixture is made into 20,000 drops that are analysed individually to enhance detection of low number of targeted copies and to avoid inhibition from non-targeted DNA fragments. Splitting the reaction mixture into 20,000 drops should also make it possible to quantify the amount of eDNA. However, according to Jones et al. (2014) this is not yet fully resolved but could improve future detection and quantification without the risk of contaminating samples during the creation of dilution series.

Field testing of primer-probe systems

The positive extraction blank occurred in one out of two and in one out of four replicates. Thus, we believe it is a single contamination during preparation of samples. When creating the dilution series with targeted DNA fragments, extreme large amounts of DNA fragments are present, creating a high potential for contamination. In the study by Wilcox et al. (2013) dilution series were made from synthesised DNA fragments, hence it could be labelled and possible contamination could be recognized. In this study, the eDNA detection using the Ponlep III system must be seen in the perspective of the positive extraction blank.

Anyhow, we successfully detect and quantify eDNA with species-specific primer-probe systems. Our sample size was to small too give an indication of the relationship between CPUE and eDNA.

In Lake Nydam we detected eDNA from signal crayfish in one out of two water sampling events, even though our mark/recapture (table 2) and CPUE results (figure 7) showed that signal crayfish were present in high number. It is unknown why some results were negative. In Lake Furesø, water filtering was done during an algae bloom and samples were taken while snorkelling over the stone reef. Particles in the water column such as algae are known to inhibit the detection of eDNA (Turner et al., 2014).

It was not possible to compare eDNA copies/L to CPUE for *A. leptodactylus* clade I and III, because there is no knowledge of the ratio between clade I and III present in the lakes investigated. If a dilution series for the Ponlep I system was present, the quantified eDNA copies/L for both Ponlep I and III could be added together and could then be compared with CPUE for *A. leptodactylus* as one species.

The amount of eDNA and the chances of positive eDNA detection will vary depending on several variables, e.g. volume of water filtered, density and activity of the study organism, and DNA excretion rate. In this study between 500 and 1500 mL of water was filtered. The amount of eDNA released from crayfish might be lower compared to fishes and amphibians because of the crayfish exoskeleton and lack of mucus. The benthic environment provides further complications because suspension of eDNA to the water column can be physically limited.

Transport of eDNA in and between connected water systems can result in false positives. In a recent study by Deiner and Altermatt (2014) they are able to detect eDNA from two lake living invertebrates up to $\approx$ 10 km downstream the river. In Copenhagen, several lakes are connected through underground channels e.g. Lake Damhussøen, Sankt Jørgens Søand the lake in the Botanical Garden (Liboriussen et al., 2007). This emphasize the need for a detailed investigation of possible water system connections when using eDNA. This might further explain the detection of eDNA from signal crayfish in Lake Damhussøen and the detection of the narrow-clawed crayfish clade I in the lake in the Botanical Garden. However, further investigation of eDNA transport is needed to understand these processes that potentially can lead to false positive detection.

The abiotic conditions of a lake affect the degradation of eDNA present. In a tank experiment, Strickler et al. (2014) find that cold, alkaline water bodies with low UV-B exposure contained a higher amount of bullfrog tadpole *Lithobates catesbeianus* (Shaw, 1802) DNA, than warm, neutral to acidic water with a high UV-B exposure. A recent study by Turner et al. (2015) on bigheaded asian carp *Hypophthalmichthys*

spp, find that the top 2 cm of the sediment contained 8 to 1800 times higher concentrations of carp eDNA than water samples. This might be due to the settling of eDNA from the water column. Further, Turner et al. (2015) detect carp eDNA in sediment up to 132 days after carp removal. It is possible that crayfish eDNA will follow the same pattern, since crayfish are benthic organisms. Tréguier et al. (2014) were aware of this issue and collected water samples close to the bottom after gentle circulation movements to resuspend eDNA fragment. Several studies find a relationship between species size and abundance and eDNA present. However this might change through different life events. Tréguier et al. (2014) detect *P. clarkii* more often if there are juveniles present. Since juveniles are known to moult more frequently than adult crayfish, Tréguier et al. (2014) hypothesize that a large amount of DNA is excreted during moulting. Hence, it is important to know what time of year will give the best detection probabilities.

Conclusion and Perspectives

In this study, we successfully designed and tested four species-specific primer-probe systems for *Astacus astacus*, *Pacifastacus leniusculus* and two clades within the *Astacus leptodactylus* species complex. All four systems were tested and optimized before they were used to detect eDNA in water samples from nine different lakes. Our primer-probe systems were able to detect eDNA from the known present species in six out of eight lakes. Further we were able to quantify the number of eDNA copies/L of filtered water, however it was not possible to statistically compare the quantified amount of eDNA with the number of crayfish caught during CPUE and mark-recapture experiments. Additionally we discovered that two genetic distinguishable clades of *A. leptodactylus* are present in Denmark, presumably further studies can reveal new knowledge about the invasion history of *A. leptodactylus* and increase our understanding of the dynamics driving invasions.

According to Parkyn (2015) crayfish research needs to develop new and standardized methods to monitor and quantify the abundance of crayfish. The results of this study provide a basis for detecting noble, narrow-clawed and signal crayfish by eDNA and thus enhance the opportunity for future improvement of eDNA detection and quantification of crayfish.

Acknowledgements

We would like to thank Peter Rask Møller for supervision. Thanks to Steen Wilhelm Knudsen for assisting in the laboratory. Thanks to søejerlauget Lake Nydam and especially Knud Flensted and Peter Theander for allowing access to the lake. Thanks to Marcus A. Krag, Henrik Carl and Tore Hejl-Holm Hansen assisting in various situations. Thanks to David Strand and Trude Vrålstad from the Norwegian Veterinary Institute for including us into the TARGET project, and for the testing of Astast system and screening for crayfish plague. Thanks to all the local municipalities and the Danish Nature Agency for giving us permission to catch crayfish in their lakes. This study was partly funded by Innovationsfonden in collaboration with University of Copenhagen, AmphiConsult and Eurofins.

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1 Appendix

A

Table 11: PCR settings used to extract DNA from tissue samples. Adopted and modified from Maguire et al. (2014).

Materials	Vol / well (μL)	Concentration / well
Primer HCO2198	0.5	0.2 μM
Primer LCO1490	0.5	0.2 μM
AmpliTaq polymerase	5.0	1.0 U/μL
dNTPs	2.5	0.2 mM
$MgCl_2$		
10X Buffer (Amplitaq)	5.0	2 x
BSA		
H_20	6.5	
DNA-template	5.0	
	Total volume 25.0	

Table 12: PCR temperature settings used to extract DNA from tissue samples. Adopted and modified from Maguire et al. (2014).

	Cycles	Process	$°C$	Time
Phase 1	1	Initial denaturation	95	2 min
Phase 2	35	Denaturation	95	30 s
		Annealing	47	45 s
		Extension	72	1 min
Phase 3	1	Final extension	72	10 min
		Store	10	until removed

Table 13: Sequences acquired from Genbank® (March 15th, 2015), representing *Astacus leptodactylus*, *Astacus astacus* and *Pacifastacus leniusculus*, and used in genetic relationship analysis.

Species	Countries	Location	Genbank no.	Reference
A. leptodactylus	Turkey,	Lake Egirdir	KC311416	(Keskin and Atar, 2013)
A. leptodactylus	Croatia	Dobra	KF181928, KF181929	(Maguire et al., 2014)
A. leptodactylus	Croatia	Mrenica	KF181932, KF181937	(Maguire et al., 2014)
A. leptodactylus	Croatia	Odra	KF181933	(Maguire et al., 2014)
A. leptodactylus	Croatia	Una	KF181930, KF181931	(Maguire et al., 2014)
A. leptodactylus	Bulgaria	N/A	KF181943, KF181944	(Maguire et al., 2014)
A. leptodactylus	Poland	Gaj lake	AF525228, AF525229	(Maguire et al., 2014)
A. leptodactylus	Turkey	N/A	JQ623972	(Maguire et al., 2014)
A. leptodactylus	Russia	Siberia, Tyumen	KF181941, KF181942	(Maguire et al., 2014)
A. leptodactylus	Armenia	N/A	KF181938-KF181940	(Maguire et al., 2014)
A. leptodactylus	Armenia	N/A	KF181945-KF181954	(Maguire et al., 2014)
A. leptodactylus	Turkey	Altinyazi Dam Lake	JQ421465-JQ421468	(Akhan et al., 2014)
A. leptodactylus	Turkey	Kadikoy Dam Lake	JQ421465, JQ421466	(Akhan et al., 2014)
A. leptodactylus	Turkey	Kadikoy Dam Lake	JQ421468-JQ421474	(Akhan et al., 2014)
A. leptodactylus	Turkey	Karpuzlu Dam Lake	JQ421465, JQ421468	(Akhan et al., 2014)
A. leptodactylus	Karpuzlu Dam Lake	N/A	JQ421469, JQ421471	(Akhan et al., 2014)
A. leptodactylus	Karpuzlu Dam Lake	N/A	JQ421474	(Akhan et al., 2014)
A. leptodactylus	Turkey	Lake Iznik	JQ421506-JQ421509	(Akhan et al., 2014)
A. leptodactylus	Turkey	Lake Kucukcekmece	JQ421465, JQ421466	(Akhan et al., 2014)
A. leptodactylus	Lake Kucukcekmece	N/A	JQ421468, JQ421469	(Akhan et al., 2014)
A. leptodactylus	Turkey	Lake Apolyont	JQ421482, JQ421510	(Akhan et al., 2014)
A. leptodactylus	Turkey	Lake Manyas	JQ421496, JQ421500	(Akhan et al., 2014)
A. leptodactylus	Turkey	Lake Hamam	JQ421514, JQ421517	(Akhan et al., 2014)
A. leptodactylus	Turkey	Pabucdere,	JQ421518	(Akhan et al., 2014)
A. leptodactylus	Turkey	Terkos Dam Lake	JQ421511-JQ421513	(Akhan et al., 2014)
A. leptodactylus	Turkey	Lake Golmarmara	JQ421482, JQ421483	(Akhan et al., 2014)
A. leptodactylus	Turkey	Lake Egirdir	JQ421477-JQ421481	(Akhan et al., 2014)
A. leptodactylus	Lake Egirdir	N/A	JQ421501-JQ421505	(Akhan et al., 2014)
A. leptodactylus	Turkey	Lake Balik	JQ421487-JQ421492	(Akhan et al., 2014)
A. leptodactylus	Turkey	Lake Sera	JQ421488, JQ421489	(Akhan et al., 2014)
A. leptodactylus	Lake Sera	N/A	JQ421493-JQ421495	(Akhan et al., 2014)
A. leptodactylus	Turkey	Keban Dam Lake	JQ421482-JQ421486	(Akhan et al., 2014)
A. leptodactylus	Turkey	Lake Cildir	JQ421475, JQ421476	(Akhan et al., 2014)
A. leptodactylus	Turkey	Velika	JQ421519, JQ421520	(Akhan et al., 2014)
A. leptodactylus	Turkey	Lake Egirdir	KC789374-KC789393	(Keskin and Atar, 2013)
A. astacus	Croatia	N/A	GU727619	(Jadan et al., 2010)
A. astacus	Austria, Bulgaria,	N/A	JN254659-JN254681	(Filipová et al., 2011)
A. astacus	Croatia, Germany,	N/A	JN254659-JN254681	(Filipová et al., 2011)
A. astacus	Hungary, Romania,	N/A	JN254659-JN254681	(Filipová et al., 2011)
A. astacus	Czech Republic,	N/A	JN254659-JN254681	(Filipová et al., 2011)
A. astacus	Belgium, Poland	N/A	JN254659-JN254681	(Filipová et al., 2011)
P. leniusculus	Czech republic	Jedlova, Litomysl	JF437995	(Filipová et al., 2011)
P. leniusculus	Hungary	Gyongyos, Koszeg	JF437996	(Filipová et al., 2011)
P. leniusculus	Great Britain	Teil Burn, Five	JF437997	(Filipová et al., 2011)
P. leniusculus	Oregon	Upper Williamson River	JF437998	(Filipová et al., 2011)
P. leniusculus	California	Lower Klamath River	JF437999	(Filipová et al., 2011)
P. leniusculus	Oregon	Suislaw river	JF438000	(Filipová et al., 2011)
P. leniusculus	Poland	N/A	AF525226-AF525227	(Soroka et al., 2002)

Table 14: qPCR temperature settings used for analysis of extracted eDNA from filtered water

	Cycles	Process	°C	Time
Phase 1	1	Initial denaturation	50	5 min
			95	10 min
Phase 2	50	Denaturation	95	30 s
		Annealing	47	45 s
Phase 3	1	Final extension	72	10 min

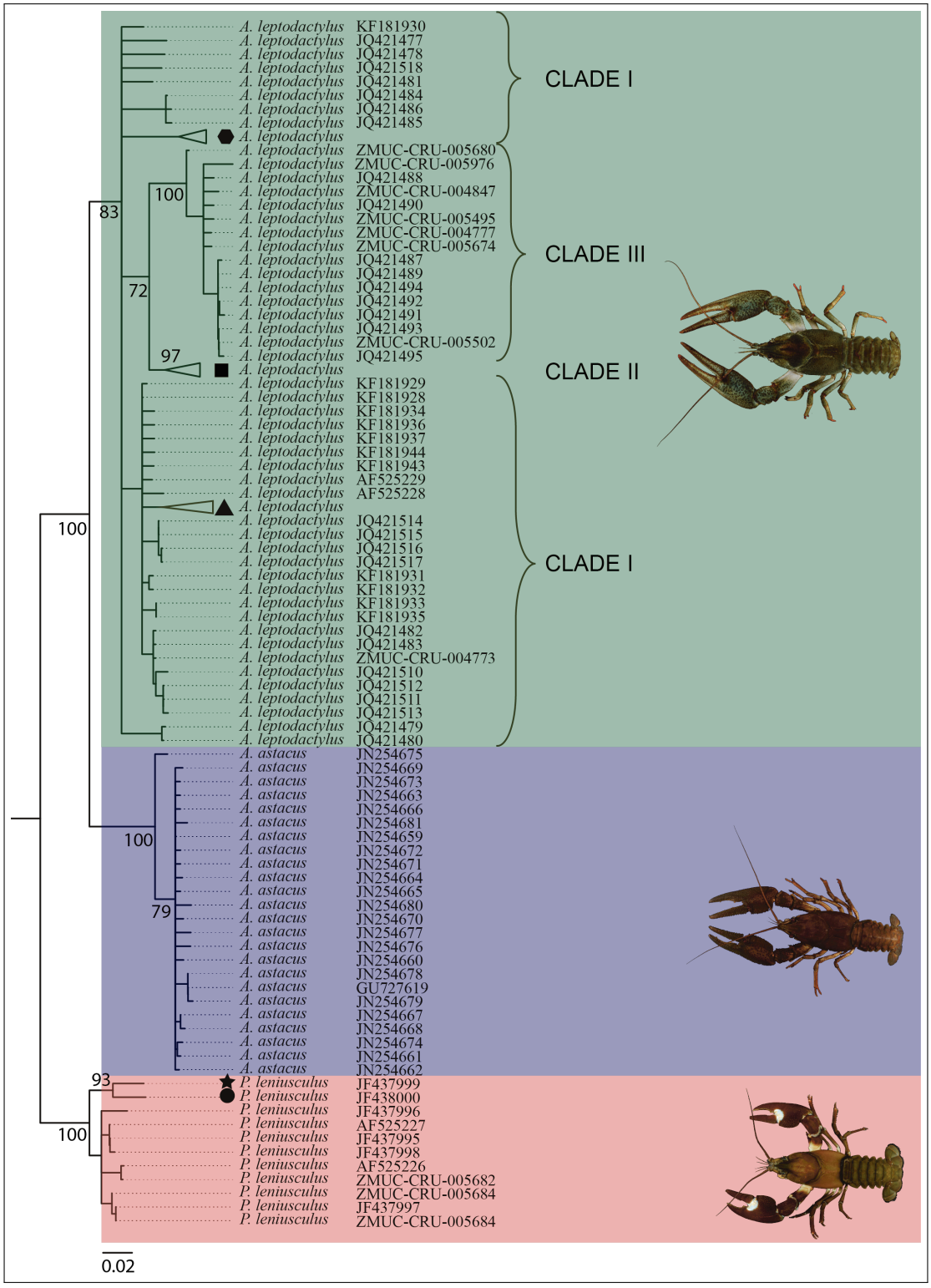


Figure 9: A neighbour joining tree showing mtDNA COI relationship between *A. leptodactylus*, *A. astacus* and *P. leniusculus*. For each branch, species and accession numbers/museums ID are shown. Three clades have been collapsed: the hexagon is accession numbers JQ421496-JQ421509 within clade I , the square represents the entire clade II and the triangle represents accession numbers KC311416, KC789374-KC789393 within clade 1. The star and circle represent *P. leniusculus* subspecies *klamathensis* and *trowbridgii*, respectively. *P. leniusculus* was used as outgroup and from 1000 bootstrap pseudoreplicates, relevant bootstrap values above 60 % are shown. ZMUC-CRU specimens were collected for the present study. Photos: W. B. Larsen and S. Agersnap.

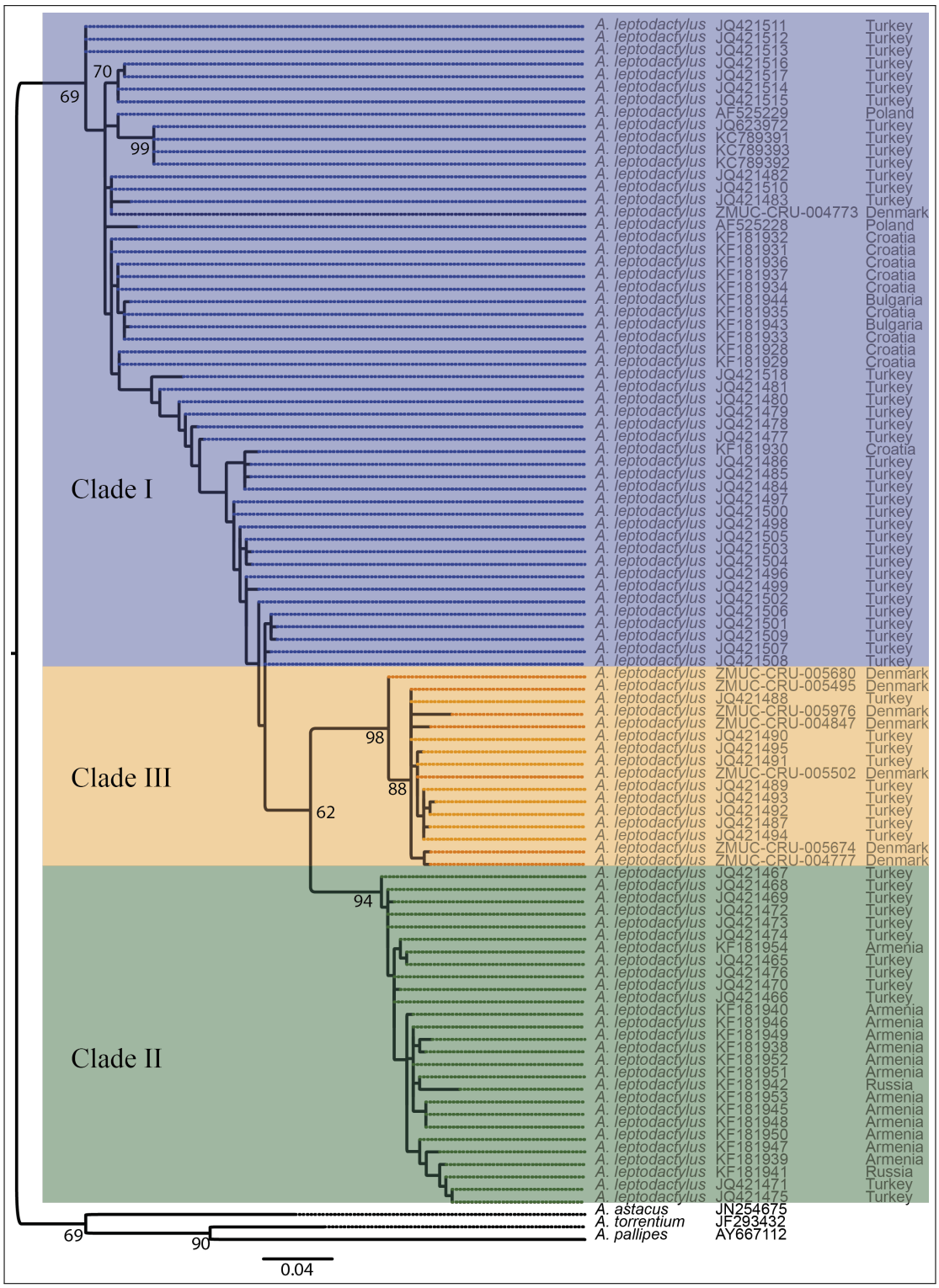


Figure 10: A Maximum likelihood tree showing the mtDNA COI relationship between the *A. leptodactylus* species complex. It is divided into three clades, matching the clade numbering presented by Akhan et al. (2014) and for each branch species, accession numbers/museums ID and country of origin is presented. *Astacus astacus*, *Austropotamobius torrentium* and *Austropotamobius pallipes* was used as outgroup. From 1000 bootstrap pseudoreplicates, relevant bootstrap values above 60 % are displayed. ZMUC-CRU specimens were collected for the present study.

B

QIAGEN® DNeasy® Blood & Tissue – **Quick-Start** Protocol

1. Tissue: Cut tissue into small pieces, and place in a 1.5 ml microcentrifuge tube. Add 180 µl Buffer ATL. Add 20 µl proteinase K, mix by vortexing, and incubate at 56°C until completely lysed. Vortex occasionally during incubation. Vortex 15 s directly before proceeding to step 2.

2. Add 200 µl Buffer AL. Mix thoroughly by vortexing. Incubate blood samples at 56°C for 10 min.
2a. Add 200 µl Buffer AL. Mix thoroughly by vortexing. Incubate blood samples at 56°C over night.

3. Add 200 µl ethanol (96–100%). Mix thoroughly by vortexing.

4. Pipet the mixture into a DNeasy Mini spin column placed in a 2 ml collection tube. Centrifuge at ≥ 6000 x g (8000 rpm) for 1 min. Discard the flow-through and collection tube.

5. Place the spin column in a new 2 ml collection tube. Add 500 µl Buffer AW1. Centrifuge for 1 min at ≥6000 x g. Discard the flow-through and collection tube.

6. Place the spin column in a new 2 ml collection tube, add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000 x g (14,000 rpm). Discard the flow-through and collection tube.
6a. Place the spin column in a new 2 ml collection tube, add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000 x g (14,000 rpm). Discard the flow-through and centrifuge again for 3 min at 20,000 x g (14,000 rpm). Discard the flow through and the collection tube.

7. Transfer the spin column to a new 1.5 ml or 2 ml microcentrifuge tube.

8. Elute the DNA by adding 200 µl Buffer AE to the center of the spin column membrane. Incubate for 1 min at room temperature (15–25°C). Centrifuge for 1 min at ≥6000 x g.

Figure 11: Blood and tissue protocol with modifications, 2a. replaces 2. and 6a. replaces 6..

Protocol: Extraction from sterivex filter:
Procedure (for directly frozen sterivex filters – i.e. no alcohol added)

1. *Day 1:* Mix (720 µL ATL + 80 µL Proteinase K / sterivex-filter)
2. Take filter from freezer. Wipe off with 5% bleach solution. Remove inlet cap from top end.
3. Add mix (800 uL / filter) directly into the sterivex-filter. Reseal the top of the sterivex-filter
4. Incubate at 56°C for overnight (12-24h) in a rotating sample holder.
5. *Day 2:* Remove liquid from the filter with a syringe (Use a 3 mL soft ject syringe with lock tip. Do NOT reuse syringes – to avoid cross contamination of samples)
6. Use the 3 mL syringe to transfer liquid to a 2 mL Eppendorf tube.
7. Mix equal amount sample, AL-buffer and ice-cold Ethanol
8. Vortex vigorously
9. Transfer 750 µL of the liquid to a spin column
10. Centrifuge at ≤ 6000 x g (8000 rpm) for 1 min.
11. Discard flow-through
12. Repeat this (step 9) until all liquid is spin through the filter
13. Place the DNeasy Mini spin column in a new 2 ml collection tube
14. Add 500 µL Buffer AW1
15. Centrifuge for 1 min at ≤6000 x g (8000 rpm).
16. Discard flow-through and collection tube.
17. Place the DNeasy Mini spin column in a new 2 ml collection tube
18. Add 500 µL Buffer AW2
19. Centrifuge for 3 min at 20,000 x g (14,000 rpm)
20. Discard flow-through
21. Blot collection tubes on a clean paper towel to remove residual AW2 buffer. Blot on unused areas of the paper towel to minimize risk of cross contamination between samples.
22. Centrifuge for 1 min at 20,000 x g (14,000 rpm) to dry the DNeasy membrane.
23. Place the DNeasy Mini spin column in a clean premarked and cap free LoBind 1.5 mL Eppendorf tube.
24. Place on a heating block 70°C for 1 min, to evaporate residual ethanol.
25. Add 100 µL preheated (70°C) 1x TE buffer directly onto the centre of DNeasy membrane. (*End up will only have ~ 70-80 µL DNA extract)
26. Incubate at room temperature for 7min
27. Centrifuge for 1 min at 6000 x g (8000 rpm) to elute.
28. Re-elute and repeat steps 26. And 27
29. Transfer elute to a new premarked LoBind 1.5 mL Eppendorf tube. Parafilm the tubes.
30. Store at -20°C for at least 24h before further use.

Figure 12: Extraction protocol developed by Micaela A. Helström, Philip F. Thomsen and Steen W. Knudsen, Center for Evolutionary Genomics, Copenhagen University. The protocol is still under modifications by Micaela A. Helström, and is not to be shared.

5 Appendix

Felt-guide til krebs i Danmark

Sune Agersnap og William Brenner Larsen

Statens Naturhistoriske Museum
Københavns Universitet
Universitetsparken 15
København Ø.

Dette dokument er en guide til identifikation af krebsearter kendt fra Danmark, beregnet til brug i felten.

Grafisk design, tekst og billeder af William Brenner Larsen og Sune Agersnap.
Forside: fra venstre: han af flodkrebs, han af galizisk sumpkrebs, han af signalkrebs.

Introduktion

Krebs har stor betydning for de omgivelser de lever i og betragtes ofte som nøglearter i ferskvand. Derfor er det vigtigt for forvaltningen af ferske vande at kende til deres udbredelse. I Danmark mangler vi viden om udbredelsen af krebs og den eksisterende viden er hovedsageligt baseret på mindre undersøgelser med andet fokus.

Kendskabet til krebs i Danmark er generelt dårligt, sammenlignet med, f. eks. vores naboland, Sverige, og ikke mange er i stand til at adskille arterne fra hinanden. I Danmark er der registreret tre arter af krebs; den hjemmehørende flodkrebs *Astacus astacus* (Linnaeus, 1758) og de to ikke hjemmehørende arter; galizisk sumpkrebs *Astacus leptodactylus* (Eschscholtz, 1823 artskompleks) og signalkrebs *Pacifastacus leniusculus* (Dana, 1852).

Begge ikke hjemmehørende arter er kommet til gennem menneskelige aktiviteter tilbage i 1960'erne og har nu veletablerede bestande i Danmark. De er af Naturstyrelsen listet som invasive arter, da de er kendt for at kunne fortrænge den rødlistede flodkrebs.
Det er anerkendt at udryddelse af de invasive krebsearter er nært umuligt. Det er dog muligt at begrænse videre spredning, ved at informere om problemerne ved introduktioner af fremmede krebsearter, da spredningen i naturen ofte sker gennem menneskelige aktiviteter.

En simpel felt-guide til identifikation af de danske krebsearter, er et vigtigt værktøj til at øge kendskabet til arterne og dermed sikre bedre kvalitet af fremtidige registreringer, hvilket vil forbedre udgangspunktet for videre forvaltning af krebs i Danmark.

Denne guide er baseret på morfologiske undersøgelser af individer fra Danmark (fra Agersnap og Larsen, in prep.) og repræsenterer derfor bedst muligt de danske populationer af krebs.

Udbredelsen af krebs er angivet som tilstedeværelse, registreret efter 1960, i 10 x 10 km UTM-kvadrater og er baseret på registreringer verificeret af forfatterne.

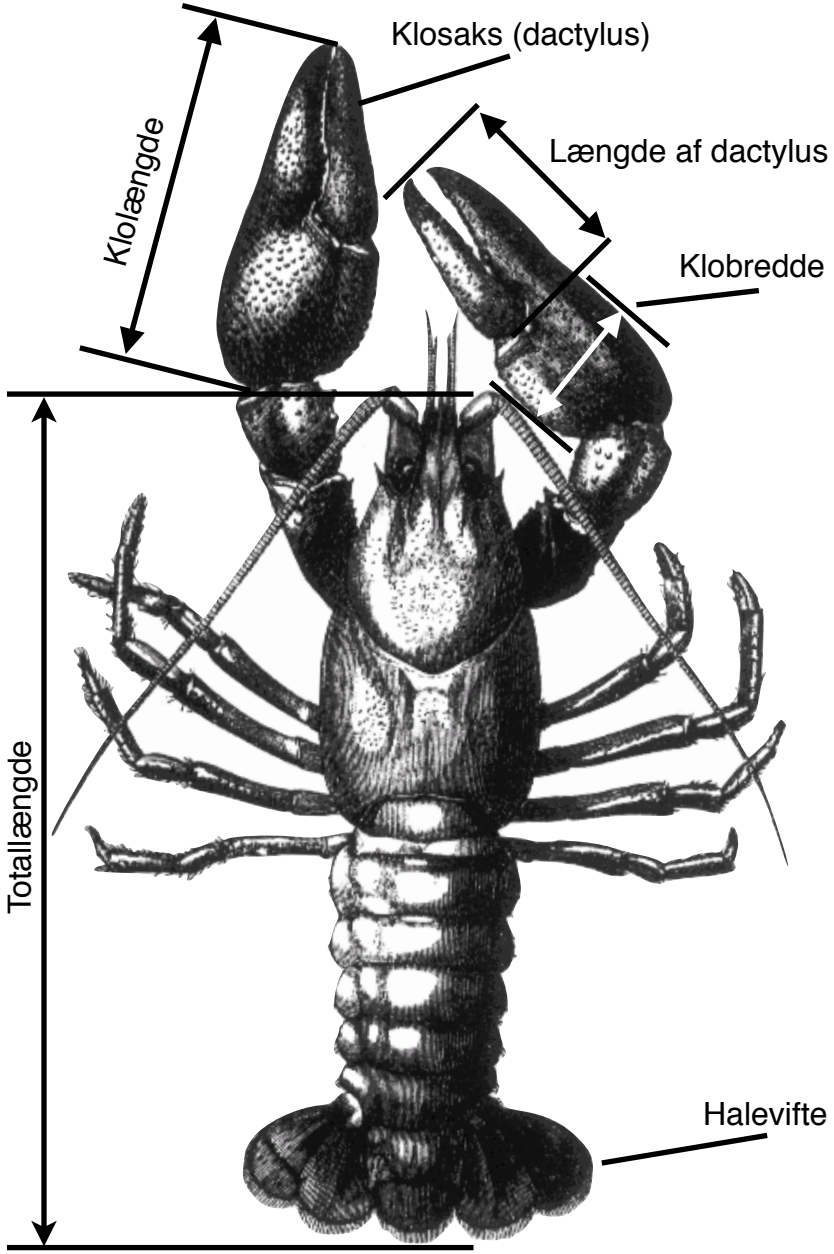
Brug af guiden

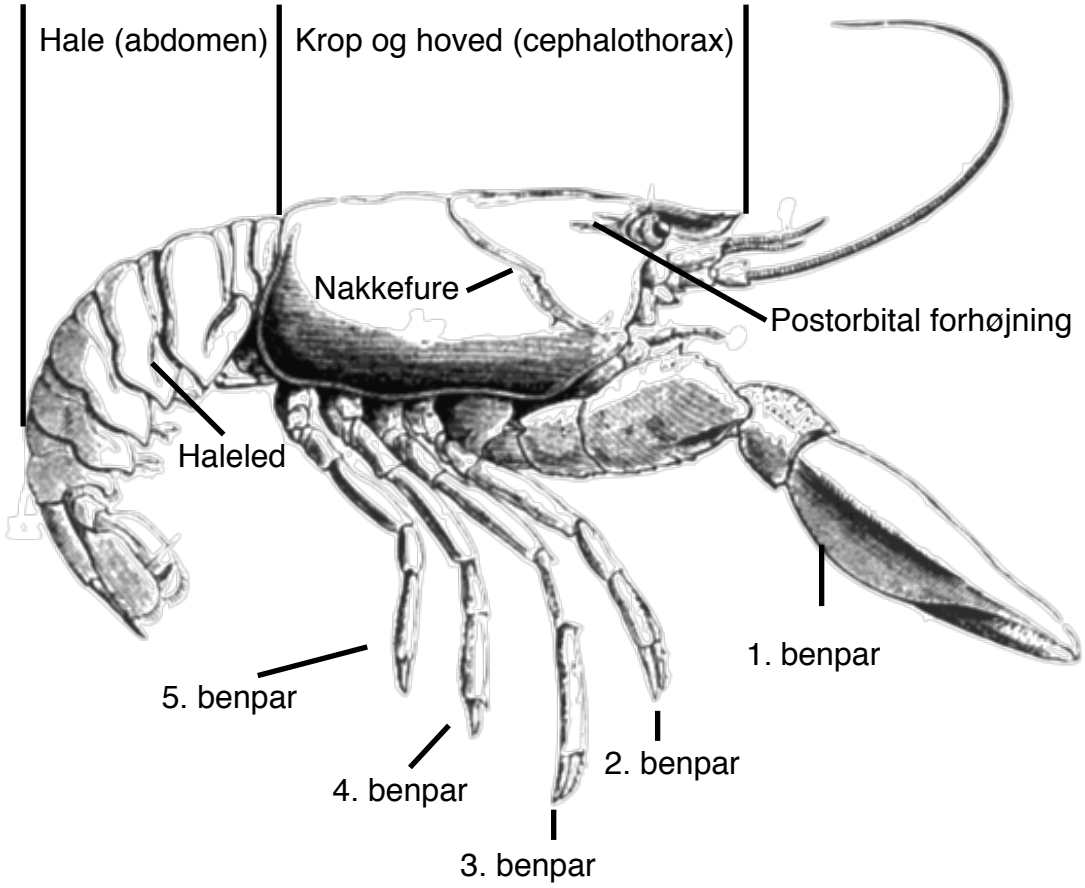
Denne guide fungerer som en billedidentifikationsnøgle og indeholder billeder af alle relevante karakterer. Der er lagt vægt på karakterer som kan anvendes til identifikation i felten, uden brug af særligt udstyr.

Karakterer er gældende for både hanner og hunner, med mindre andet er anført.
Farver kan variere meget mellem individer, og er generelt dårlige kendetegn. Det anbefales at anvende andre karakterer før farver ved bestemmelse af arter.

Krebsen

- Vigtige termer





Han og hun

- Forskelle til kønsbetemmelse

♂ ♀

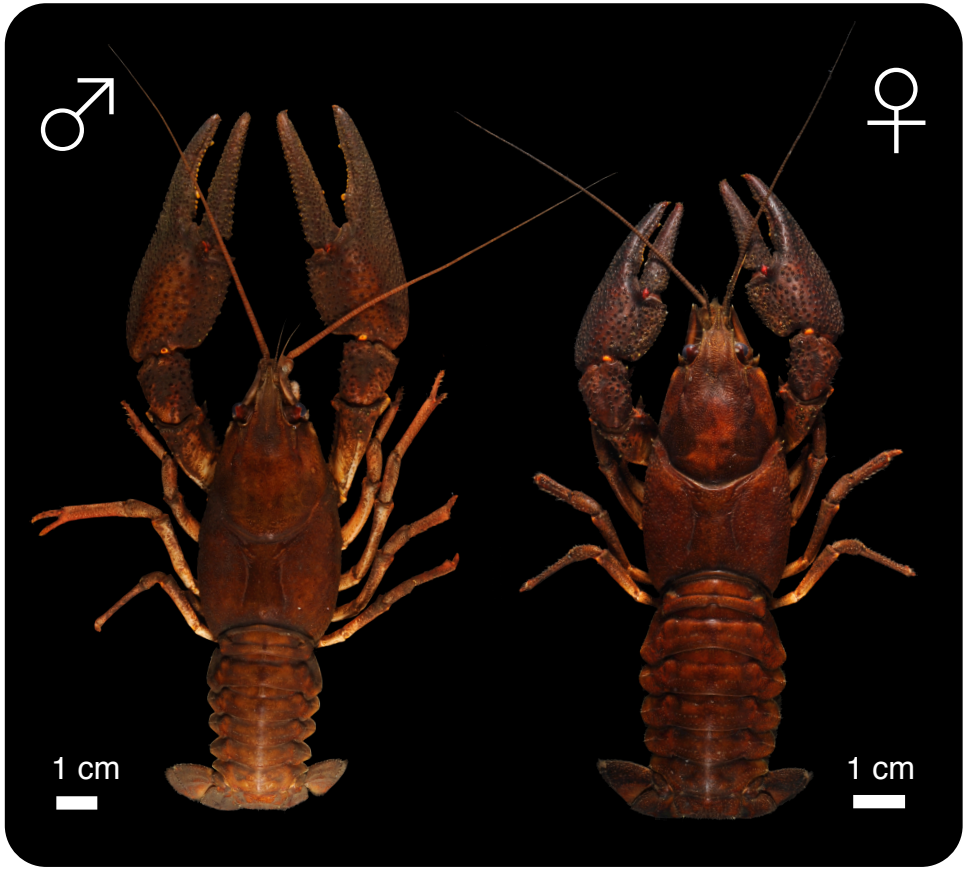
Hanner er kendetegnet ved en smallere hale end hunner.

Hunner er kendetegnet ved den brede hale, som bruges til at bære æg.

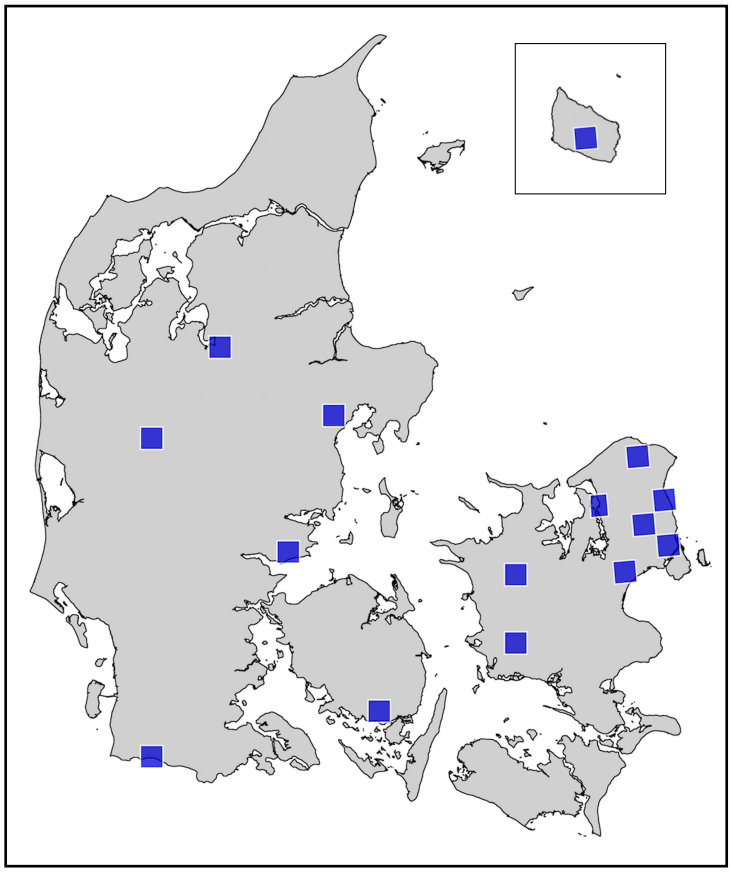
Hanner kendes på de tydelige kønsorganer på undersiden mellem hale og krop, og kønsåbningerne på femte benpar.

Hunner er kendetegnet ved manglen på sædledere og kønsåbningerne på tredje benpar.

Flodkrebs *Astacus astacus* (Linneaus, 1758)

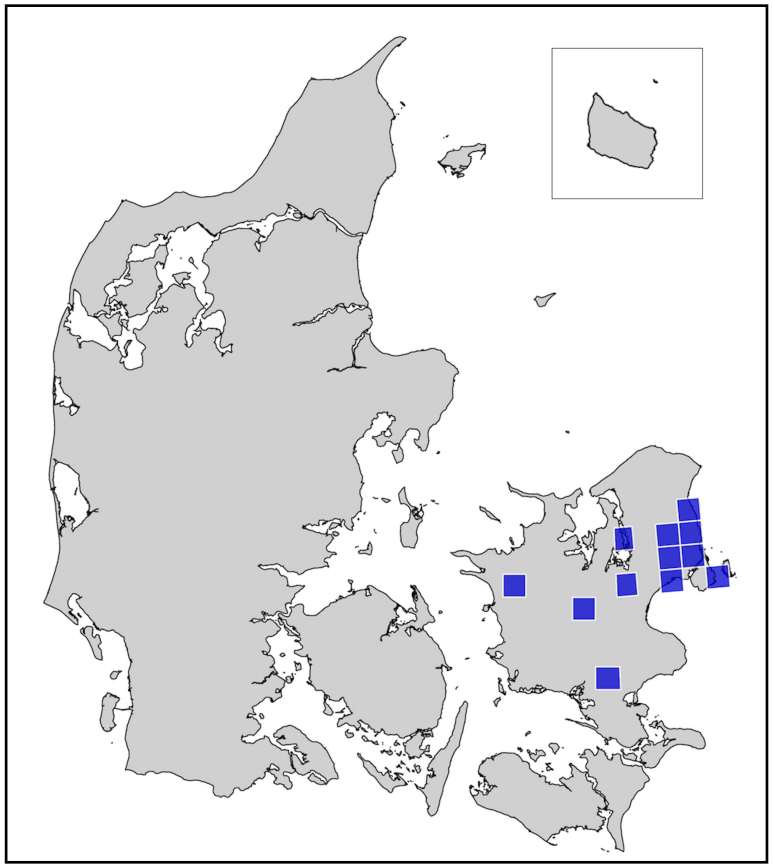


Størrelse:
Op til 18 cm (totallængde). I Danmark er den registreret op til 15 cm.
Oprindelsesområde:
Europa.
Levesteder:
Søer, mindre vandhuller og vandløb.
Status i Danmark:
Rødlistet.



Udbredelse af flodkrebs

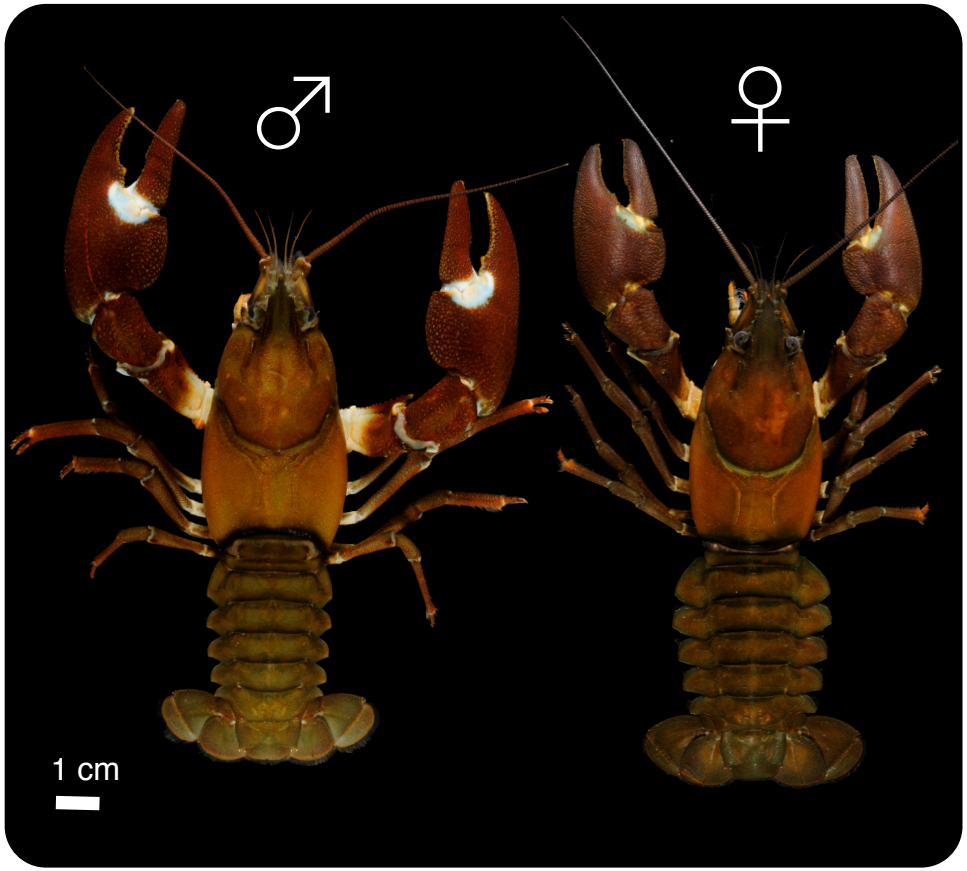
Galizisk sumpkrebs *Astacus leptodactylus (Esch., 1823)*

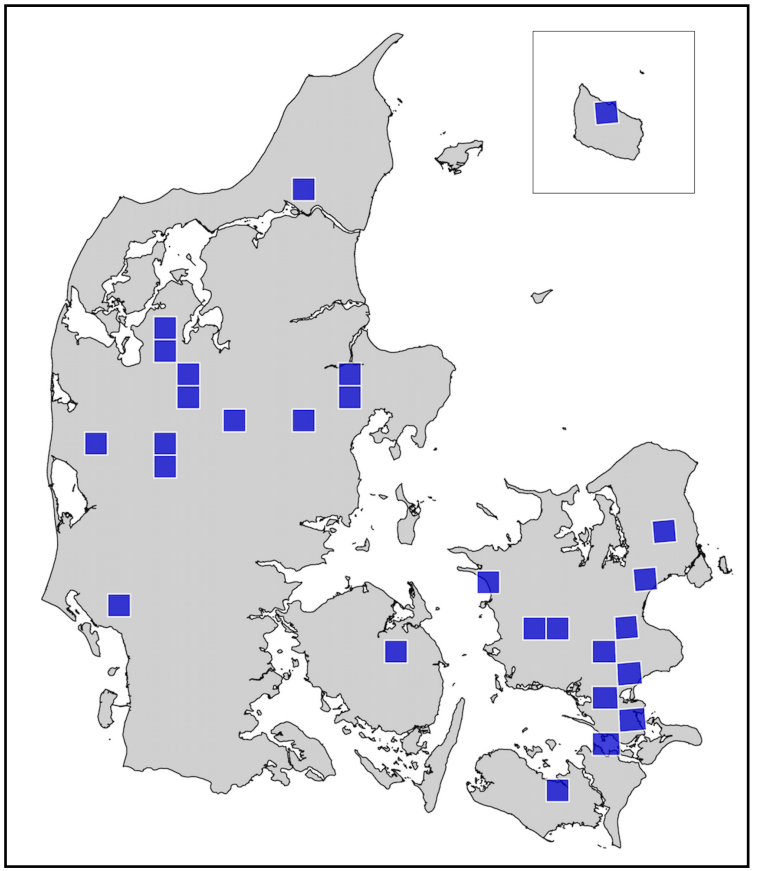


Udbredelse af galizisk sumpkrebs

Størrelse:
Op til 20 cm (totallængde). I Danmark er den registreret op til 16 cm.
Oprindelsesområde:
Sydøstlige Europa.
Levesteder:
Søer, mindre vandhuller og vandløb. Kan opholde sig i brakvand.
Status i Danmark:
Invasiv, sortlistet. Udsætningsforbud.

Signalkrebs *Pacifastacus leniusculus* (Dana, 1852)





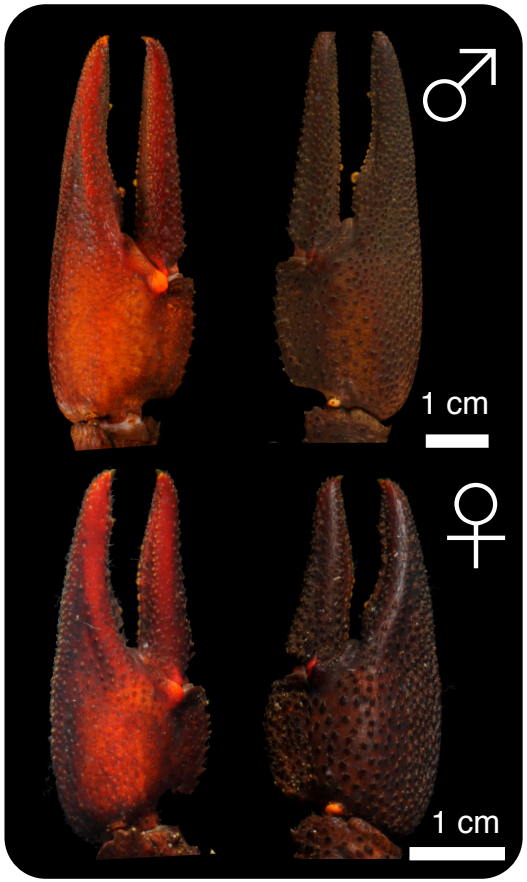
Udbredelse af signalkrebs

Størrelse:
Op til 16 cm (totallængde). I Danmark er den registreret op til 13,6 cm.
Oprindelsesområde:
Nordamerika.
Levesteder:
Søer, mindre vandhuller og vandløb. Kan opholde sig i brakvand.
Status i Danmark:
Invasiv, sortlistet. Udsætningsforbud.

Særlige kendetegn

- Klør

Flodkrebs



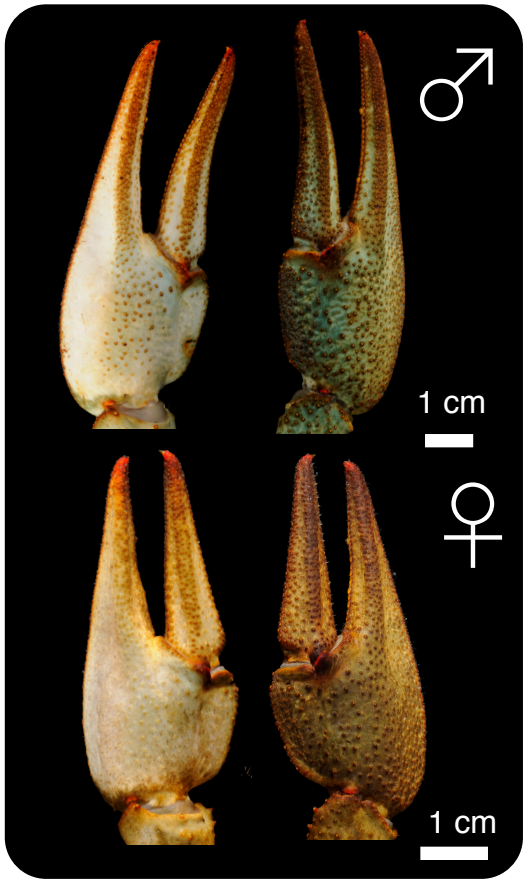
tv: underside, th: overside

Længde:
Han: 34-67% af totallængde.
Hun: 31-44% af totallængde.

Form:
Hunner bredere (35-45%) end hanner (34-41%) relativt til klolængde.

Farve:
Rødlig (særligt undersiden).

Galizisk sumpkrebs



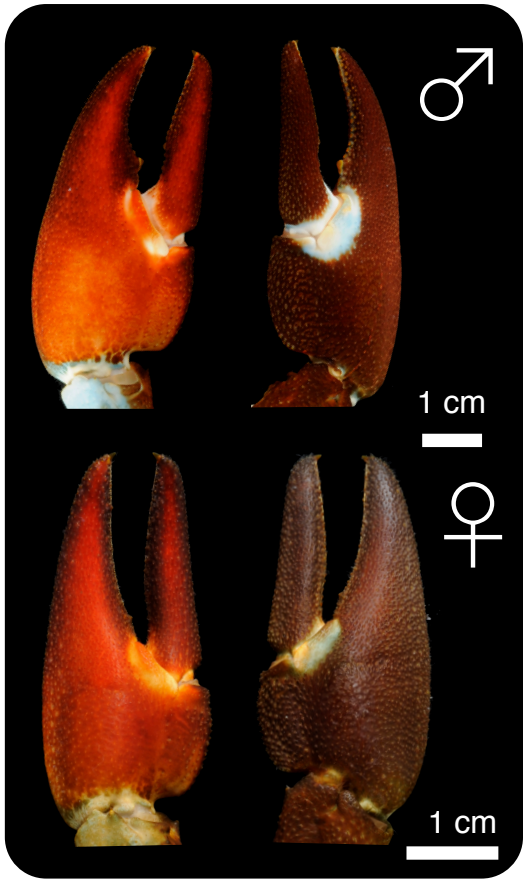
tv: underside, th: overside

Længde:
Han: 27-72% af totallængde.
Hun: 25-40% af totallængde.

Form:
Hunner bredere (31-50%) end hanner (27-44%) relativt til klolængde.

Farve:
Brun/gul. Undersiden ofte bleg.

Signalkrebs



tv: underside, th: overside

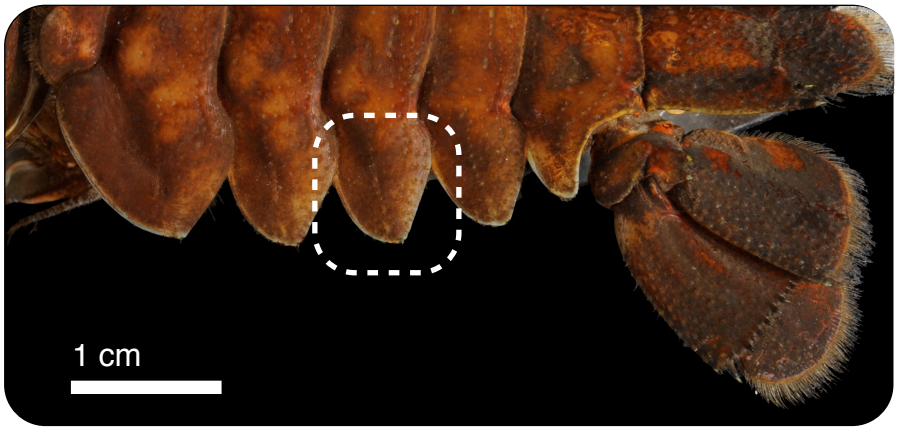
Længde:
Han: 27-52% af totallængde.
Hun: 25-39% af totallængde.

Form:
Kort og bred (35-49% af klolængde). Ingen kønsforkelle.

Farve:
Rødlig (særligt undersiden). Hvid eller blålig plet ved basis af klosaks (kan være utydelig).

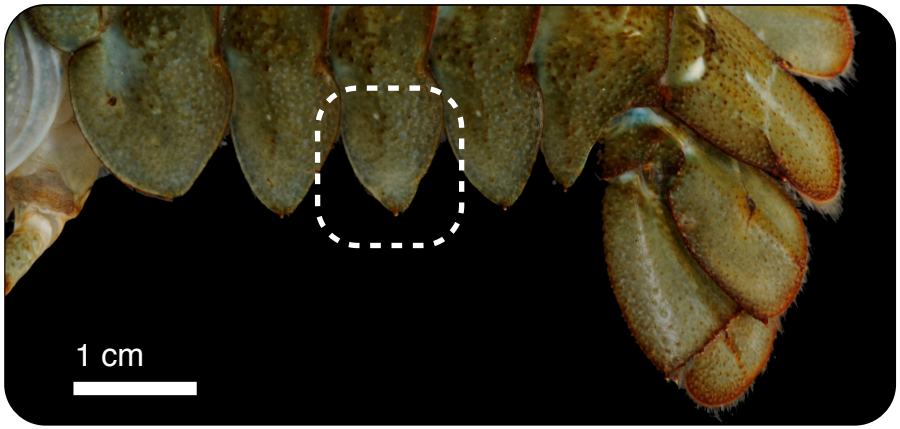
Særlige kendetegn

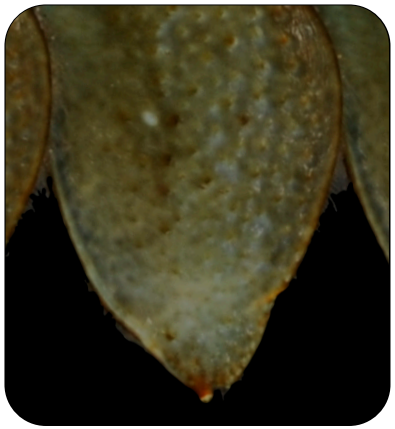
- Haleled set fra siden



Flodkrebs

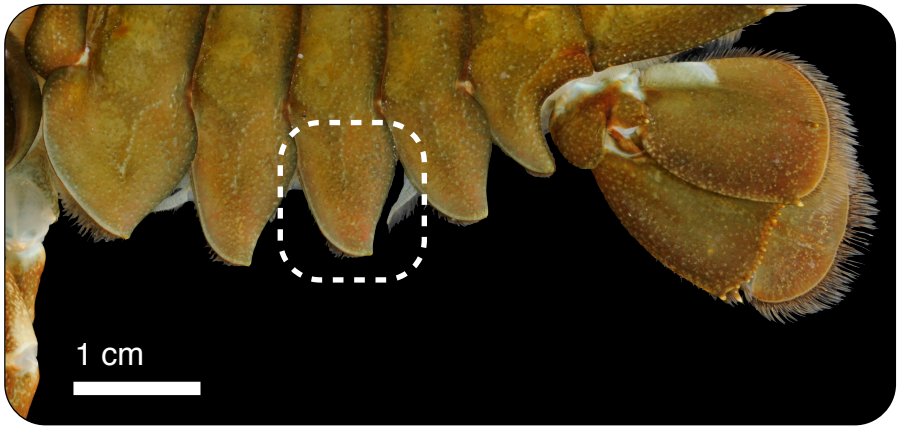
Haleled asymmetriske, afrundet fortil, med spidsen pegende mod haleviften.

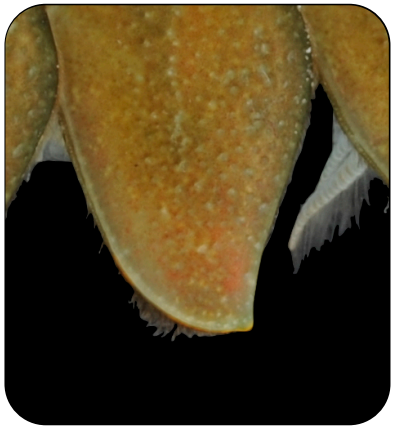




Galizisk sumpkrebs

Haleled symmetriske, afsluttet i tydelig, mærkbar pig.



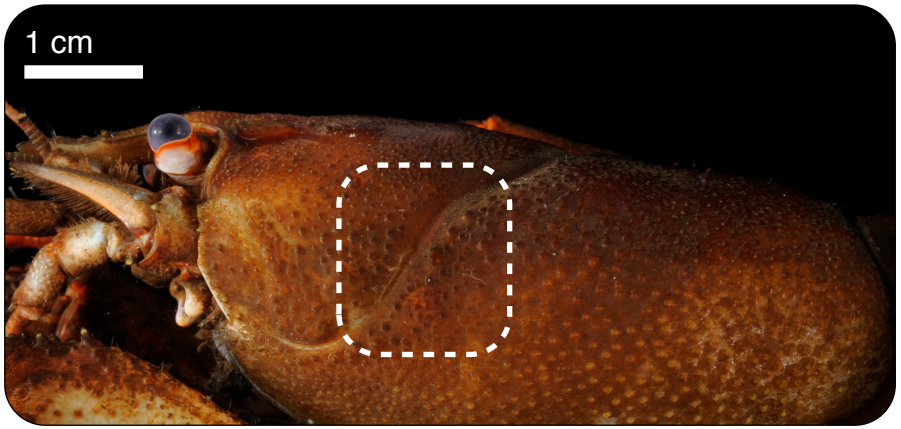


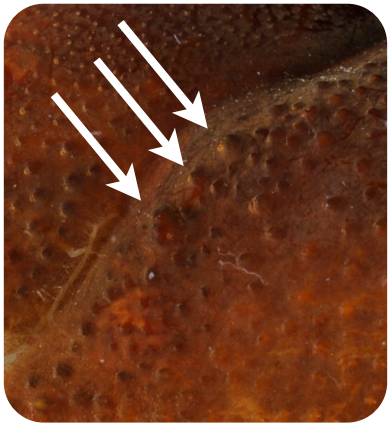
Signalkrebs

Haleled asymmetriske, afrundet fortil, med spidsen pegende mod haleviften.

Særlige kendetegn

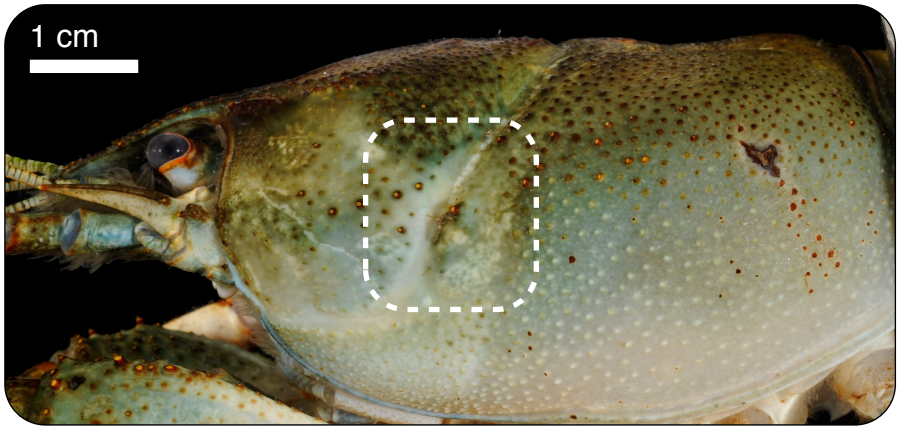
- Nakkefuren set fra siden

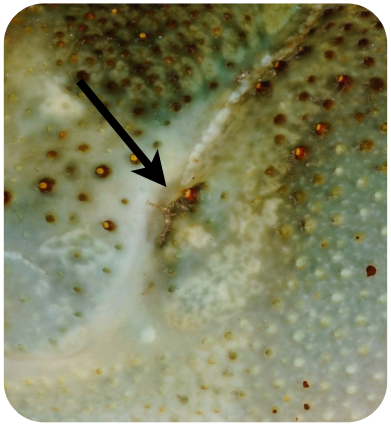




Flodkrebs

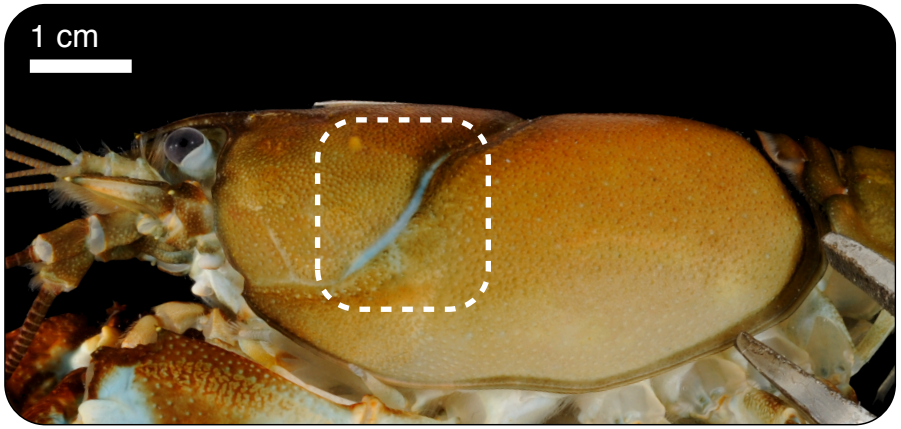
1-4 mærkbare pigge langs nakkefuren.





Galizisk sumpkrebs

1-3 mærkbare pigge langs nakkefuren.

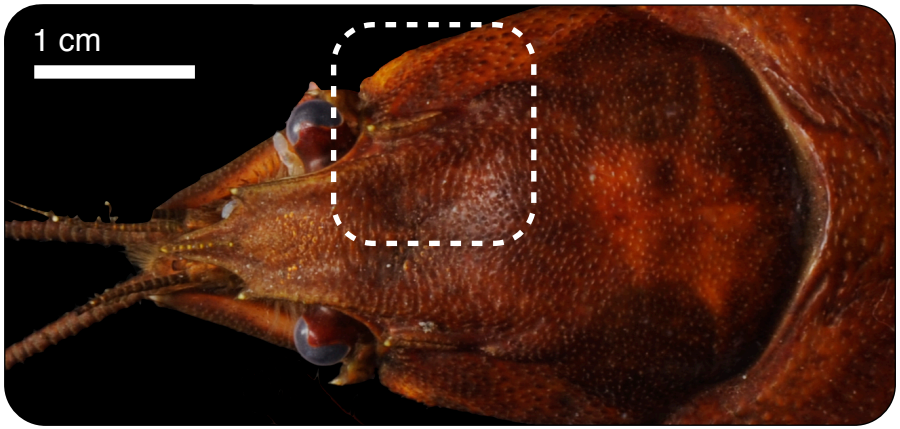


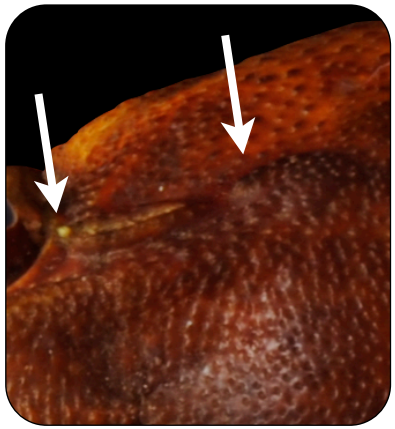
Signalkrebs

Ingen mærkbare pigge langs nakkefuren.

Særlige kendetegn

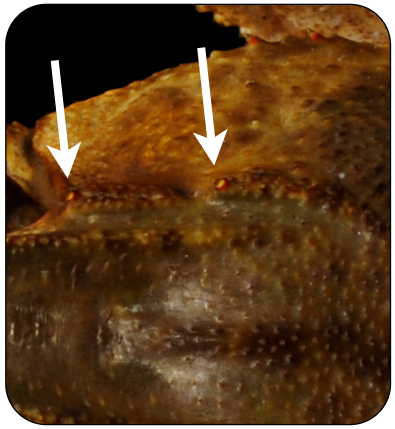
- Postorbitale forhøjninger set fra oven





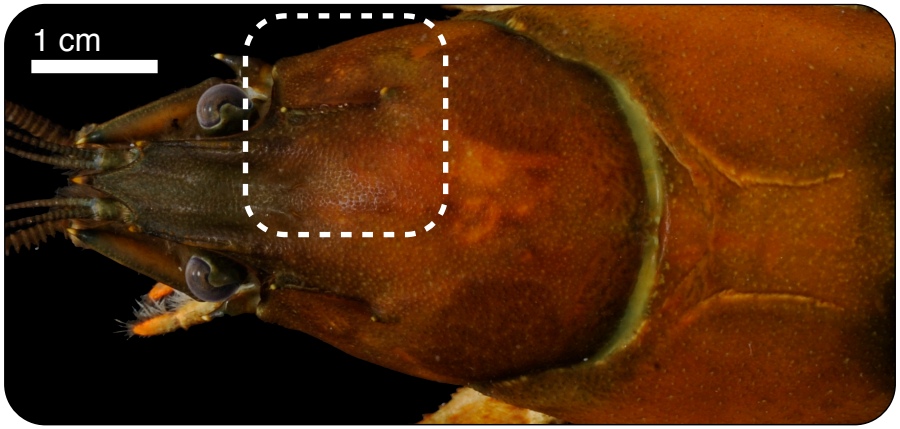
Flodkrebs

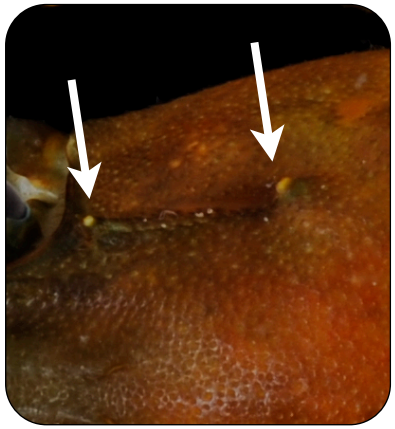
2 postorbitale forhøjninger. 1-2 med mærkbar pig. På billedet er vist et individ med mærkbar pig på forreste forhøjning og uden pig på bageste.



Galizisk sumpkrebs

2 postorbitale forhøjninger. Begge med mærkbar pig.





Signalkrebs

2 postorbital forhøjninger. 1-2 med mærkbar pig. På billedet er vist et individ med mærkbar pig på begge forhøjninger.

Relevante referencer

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Skov, C., K. Aarestrup, F. Sivebæk, S. Pedersen, T. Vrålstad, og S. Berg (2010). Non-indigenous signal crayfish *Pacifastacus leniusculus* are now common in Danish streams: preliminary status for national distribution and protective actions. Biologicl Invasions 13, 1269–1274.

Skov, C., F. Sivebæk, K. Aarestrup, T. Vrålstad, P. Hansen, og S. Berg (2009). Udbredelse og bekæmpelse af signalkrebs i Alling å. Pilotprojekt og anbefaling til fremtidige tiltag. Teknisk rapport, DTU Aqua, Sektion for Ferskvandsfiskeri. Tilgængelig her.

Souty-Grosset, C., D. M. Holdich, P. Y. Noel, J. D. Reynolds, og P. Haffner (2006). Atlas of Crayfish in Europe. Museum national d'Histoire naturelle, Paris, 187 p. (Patrimoines naturels, 64). ISBN: 978-2856535790.

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THE END