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Mariene Ecologie

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SAMENVATTING

De gewone garnaal, *Crangon crangon* (Linnaeus 1758), is een van de meest voorkomende en belangrijkste soorten in estuaria en kustgebieden langs de Europese kust. De soort komt voor van de Witte Zee in het noorden tot de Zwarte Zee in het zuiden. Tot op heden is er geen genetische analyse naar de populatiestuctuur van *C. crangon* uitgevoerd. Deze studie beschrijft de resultaten van een genetische analyse naar de populatiestuctuur van *C. crangon* over het gehele verspreidingsgebied. Van meerdere garnalen per locatie werden de DNA sequenties bepaald, waarbij een drietal targets onderzocht zijn: [1] de zeer variabele Internal Transcribed Spacer (ITS); [2] het redelijk variabele mitochondriale Cytochroom Oxidase I (COI) gen; en [3] het conservatieve 18S rRNA gen. Ter controle werden ook DNA sequenties van *C. allmanni* en van *C. septemspinosa* bepaald. Sequenties van *C. septemspinosa* waren al in Genbank aanwezig en zijn ook meegenomen in de fylogenetische analyses. De 18S rRNA analyse werd gebruikt om in geval van twijfel na te gaan of werkelijk sprake was van de soort *C. crangon*. De sequenties van het ITS gebied waren te slecht voor een betrouwbare analyse van de populatiestructuur. Uit analyse van 418 bases van het COI gen bleek dat binnen *C. crangon* een tweetal (meta)populaties onderscheiden kunnen worden: een populatie langs de gehele Atlantische kust en één in de Middellandse Zee. Het is mogelijk dat er nog een derde populatie gevonden zal worden wanneer het onderzoek uitbreid wordt met *C. crangon* uit de Baltische Zee.

SUMMARY

The brown shrimp *Crangon crangon* (Linnaeus 1758) is one of the most abundant and important species in estuarine and coastal areas along the European coast. The range of distribution of *C. crangon* is from the White Sea in the north till the Black Sea in the south. At present no genetic analysis of the population structure of *C. crangon* is available. This study describes the results of a genetic analysis of the population structure of *C. crangon* over its distributional range. DNA sequences were determined from a number of shrimps per location to analyse the following three targets: [1] the highly variable Internal Transcribed Spacer (ITS) region; [2] the relatively variable mitochondrial Cytochrome Oxidase I (COI) gene; and [3] the conservative 18S rRNA gene. As a reference, the DNA sequences of *C. allmanni* were also measured. Sequences of *C. septemspinosa* were imported from Genbank and incorporated in the phylogenetic analyses. The 18S rRNA analysis was used to verify the species identity of *C. crangon* in case of uncertaincy. The quality of the sequences of the ITS region was insufficient for a reliable genetic analysis. Analysis of 418 bases of the COI gene indicated the presence of two (meta)populations: one along the Atlantic coast and another one along the Mediterranean coast. More populations may be revealed in the future when we have extended the genetic analyses to the Baltic region.

1. INTRODUCTION

The brown shrimp *Crangon crangon* (Linnaeus 1758) is one of the most abundant and important species on sandy and muddy substratum in especially estuarine and coastal areas along the coast of Europe (Tiews, 1970; 1978). Over almost its entire geographical range of distribution, this species is very abundant and hence must form an important component of the ecosystem, for instance as predators on bivalve spat (Van der Veer et al., 1998). Although the brown shrimp has been studied over the years, surprisingly little facts are known with respect to the population structure of the species and the available information is contradictory. In a morphometric analysis in British waters, it was concluded that in a relatively small area already six distinct populations could be identified (Henderson et al., 1990), whereby the boundaries between the populations corresponded with major plankton assemblages in British waters. A more extensive study by means of gel electrophoresis, indicated geographically four regional groups: North Sea and Baltic, N-Atlantic, Portugal and Adriatic (Bulnheim & Schwenzer, 1993). This study was done at protein level with iso-enzymes and the sampling grid was not dense. Non of these two studies covered the whole range of distribution of the species and also due to recent developments in DNA sequencing techniques, a more precise analysis at the DNA level with a higher resolution, has become possible.

In this project we aim to extend the study by Bulnheim & Schwenzer (1993) with a more refined method, by analysing specific targets on the DNA of *C. crangon* from different areas, to identify genetic differences within the species. Samples were collected over the entire geographical range of distribution of the species: from north, in Norwegian waters, to south in the Mediterranean and from east in the entrance of the Baltic, to west along the Irish coast.

2. MATERIAL AND METHODS

2.1. DATA COLLECTION

Samples were collected over the whole geographical range of distribution of the species (Fig. 1). In addition samples from a number of other *Crangon* species were obtained (Table 1).

All shrimps were checked individually for species identification according to Smaldon (1979). Of each sample about 50 shrimps were selected randomly (both males and females) and individually stored on 96% ethanol at 4°C. Subsequently, of each shrimp a muscle sample was collected by lifting the shield above the third pleopod and removing a little piece of the muscle tissue. This muscle sample was put in a 1.5 ml sterile collection tube and stored at -80°C for further genetic analyses. Five shrimps per location were used to obtain the DNA sequences. The remaining 45 shrimps were stored for PCR-RFLP.

2.2. GENETIC ANALYSIS

At the start of this study (September 2003) there were no nucleotide sequences of *C. crangon* available in public databases and hence no literature could be found describing molecular genetics. From personal communication with David W. Weetman (University of Hull, U.K.) we understood that after 2 years of trying his group was still unsuccessful in finding microsatellites for identifying population structure in *C. crangon*. Therefore, we excluded the approach of microsatellites in our study. Instead, three specific DNA targets were chosen for sequencing analysis: [1] the internal transcribed spacer regions ITS1 and ITS2; [2] the cytochrome oxidase I gene; and [3] the 18S rRNA gene.

The internal transcribed spacer (ITS) regions are uncoding regions with unknown functions that are located between the 5.8S and 18S ribosomal RNA genes. Within the class Crustacea the ITS1 region has been already analyzed of some fresh water crayfishes (Harris & Crandall, 2000) and of the ostracode *Darwinula stevensoni* (Gandolfi et al., 2001).

The mitochondrial Cytochrome Oxidase I (COI) gene is relatively variable and can contain sequence variation within populations. This gene has already been analyzed for some crustaceans: European populations of the green crab, *Carcinus maenas* (Rosman & Palumbi, 2004) north-east Atlantic and Mediterranean populations of the Norwegian lobster, *Nephrops aqueticus* (Stamatis et al., 2004); Slovenian populations of the water louse *Asellus aqueticus* (Verovnik et al., 2004); and north American populations of the waterflea *Daphnia obtusa* (Penton et al., 2004). In all studies mentioned above the universal primers LCO and HCO were used (Table 2) to amplify a region of 710bp of the mitochondrial Cytochrome Oxidase subunit (Folmer et al., 1992). From personal communication with Charles Franssen (University of Leiden) we learned that primers CO1F and CO1R (Table 2), worked relatively well for shrimp species. The 18S rRNA gene is conservative at species or higher taxonomic level and can be used to verify species identification. In a control study, we analysed this gene of *C. crangon* from the Mokbaai (Texel, The Netherlands) and of individuals from the Mediterranean (Etang de Vaccarès, France).

2.3. DNA EXTRACTION

DNA extraction was done with the GenElute™Mammalian Genomic DNA Miniprep Kit (Sigma®) according the Mammalian Tissue protocol (partB), provided by the manufacturer. The elution step was done with 100µl instead of 200µl elution buffer. DNA extraction was confirmed by standard submarine gel elecrophoresis,

using 1,0% agarose (Seakem® LE)/1XTBE). All gels during this genetic analysis were run in 1X TBE (10xTBE: 107.81g TRIS; 55.03g boric acid; 7.44g EDTA completed till 1L with MilliQ water; pH must be 8.3) for 45 min. at 75V. Subsequently, the gels were stained in an ethidium bromide solution (500 ml TBE buffer+25 µl ethidium bromide), and destained (500 ml TBE buffer), both during 20 min., and inspected under UV illumination using a Fluor-S Multi Imager (Bio-Rad).

2.4. POLYMERASE CHAIN REACTION (PCR)

PCR amplification (Saiki et al., 1988) was performed in a Perkin Elmer Gene Amp PCR System 2400 (Applied Biosystems). PCR of the nearly complete 18S rRNA gene was done according to Medlin et al. (1988).

PCR of Cytochrome Oxidase I (COI) gene. First a PCR was done according to Folmer et al. (1994) with LCO1490 and HCO2198 primers (Table 2), which work for a large range of invertebrates. However, with these primers, we were not able to obtain a PCR product. Therefore, two other primers were used: CO1F and CO1R (Table 2) according to Franssen (pers. comm.). With these primers a good PCR product could be obtained by using the following reactionmix: 1.25U Bio-Therm™DNA Polymerase (GeneCraft); 1x PCR buffer (GeneCraft; containing 16mM $(NH_4)_2SO_4$, 67 mM Tris-HCl pH 8.8, 1.5mM $MgCl_2$ and 0,01% Tween 20); 0.25 mM each dNTP; 0.5µM of each primer; 2.5mM additional $MgCl_2$, 1cl of DNA template in a final volume of 50µl. PCR program consisted of 40 cycles with annealing at 47°C (Table 3). Two samples, *C. crangon* from Etang de Vaccarès and *C. allmanni* from the North Sea, needed some adjustments to obtain a good PCR product. The adjustments were: doubling the amount of primers (1µM) and DNA Polymerase (2.5U); more buffer (1.3x); less $MgCl_2$ (1.5µl); more DNA template (5µl) and a lower annealing temperature of 42°C.

PCR of Internal Transcribed Spacer (ITS) region. Following White et al. (1990) PCRs with primerpair ITS1-ITS2 and ITS1-ITS4 were done. Primer sequences are listed in Table 2. The PCR with ITS1 and ITS4 primers did not provide a good result, but the combination of ITS1 and ITS2 primers worked. The PCR mix contained: 2.5U BioTherm™DNA Polymerase (GeneCraft); 1.0xbuffer(GeneCraft); 0.25mM of each dNTP; 1μM ITS1; 1μM ITS2; 5μl of the DNA template in a final volume of 50μl. Of the Portugese, Scotish, and French samples, 8 μl of DNA template was added. PCR program consisted of 39 cycles with annealing at 50°C (Table 3).

The amplifications were confirmed by standard submarine gel elecrophoresis. If there was only one fragment, the purification was done directly out of the PCR product with QIAquick columns. If more fragments were present (as occurred with the samples of *C. crangon* from Etang de Vaccarès and with the samples of *C. allmanni*), the complete PCR product was loaded on a new 1,5% gel (Seakem LE agarose/1xTBE buffer) and the fragment of interest was cut out of the gel and then purified with the QIAquick gel extraction kit. Purifications were done according to the QIAquick Spin Handbook (03/2001). For quantification, 4 μl of the purified product, was loaded on a 1,5% gel along with 4 concentrations of the PMMS (BioRad) ruler (concentrations 1:1/1:2/1:4/1:8).

2.5. SEQUENCING

Cycle sequence reactions were run with 5-20ng DNA of the COI fragment or 10-40ng DNA of the ITS fragment). The cycle sequence reaction was done in two directions using the Bigdye Terminator cycle sequence kit according to the BigdyeTerminator v1.1 protocol. The cycle sequence products were purified according the 75% isopropanol protocol (ABI Prism Bigdyeterminator Cycle Sequencing Ready Reaction kits, Origional and verion 2.0, p4-9). The end product

was dissolved in 12µl TSR and analysed (ABI prism™ 310 Genetic Analyzer) in ABI cups.

If the sequences were double, the initial PCR product was loaded on a 3% Nusieve gel and run for 210 min. at 50V. If there were two fragments visible, the one of interest was cut out of the gel and was processed in the same way as the other samples.

2.6. PHYLOGENETIC ANALYSIS

Forward and reverse sequences were edited manually with the software Sequencing Analyses (Applied Biosystems) and assembled into consensus sequences using the program AutoAssembler (Applied Biosystems).

In the phylogeny programme ARB (Ludwig et al., 2004) a new database was constructed from the consensus sequences of our samples, selected sequences of Malacostraca and a sequence of *Drosophila yacuba* obtained from GenBank. Nucleotide sequences of the cytochrome oxidase gene were first translated into amino acid sequences using the invertebrate mitochondrial code. The sequence of *Drosophila yacuba* was used as reference for the translation. Amino acid sequences were aligned using the Clustalw Protein Alignment module incorporated in the ARB programme. The amino acid alignment was used to align the nucleotide sequences.

Aligned nucleotide sequences were analyzed using Neighbour Joining and Maximum Likelihood (FastDnaML) methods implemented in the ARB programme, with the shortest sequence as filter (418 bases for COI, 1603 bases for 18S rRNA and 248 bases for ITS).

3. RESULTS

The DNA extraction check on agarose gel (Fig. 2) indicated sufficient high molecular DNA (>10 kb) for a succesfull PCR. For most of the samples, PCR of the cytochrome oxidase gene resulted in a good PCR product of about 500 bp (Fig. 3). However, some samples did not generate products (Fig. 3, lowest row) and PCR conditions for these samples were adjusted (Table 3). PCR of the ITS region (Fig. 4, upper row) provided products of around 1250bp, which is bigger than expected based on White et al. (1990), although a fragment of this size might occur because of known size polymorphism in the ITS region (Harris et al., 2000) For some of the locations, PCR conditions had to be optimized (Table 3).

DNA sequences were incorporated in phylogenetic trees for COI (Fig. 5) and for 18S rRNA (Fig. 6). The ITS sequences were not good enough for constructing a phylogenetic tree and they need to be re-analysed. The Cytochrome Oxidase I analysis (Fig. 5) showed that *C. allmanni* and *C. septemspinosa*, were genetically different from *C. crangon*. It also became clear that the 'Mediterranean' *C. crangon* sample (Etang de Vaccarès) was genetically different from the *C. crangon* samples from the other locations.

With the Cytochrome Oxidase primers no significant difference was found within the Atlantic region between *C. crangon* from The Netherlands, Schotland, Norway and Portugal. The PCR product of the COI fragment was around 500bp and the phylogenetic analysis was restricted to 418 bases of this fragment, which means that with a sequence difference of 10%, 42 bases out of 418 bases are different.

Based on the genetic analysis with COI primers, *C. crangon* from the Mediterranean Sea could be distinguished from the 'Atlantic *C. crangon* group' consisting of samples of The Netherlands, Portugal, Schotland and Norway. Based on the same

analysis, it was also possible to distinguish the different *Crangon* species: *C. septemspinosa*, *C. allmanni* and *C. crangon*.

The 18S analysis (Fig. 6) showed that there was no significant difference between the *C. crangon* from Etang de Vaccarès (France) and the *C. crangon* from the Mokbaai (The Netherlands), which means that the sample from Etang de Vaccarès (France) did not contain another *Crangon* species.

4. DISCUSSION

This study showed that two different sub-populations of *C. crangon* could be identified by sequence analyses of the Cytochrome Oxidase I gene: an Atlantic population with samples from Norway, Scotland, Portugal and The Netherlands, and a Mediterranean population with a sample from France (Etang de Vaccarès). It was also possible with this analysis to distinguish between different *Crangon* species. Based on the analysis of Bulnheim & Schwenzer (1993) and the distance between the most extreme locations, Norway and Portugal, we expected to find sub-populations along the Atlantic coast, but no such structure was found yet. In the future more shrimps per location should be analysed and also more locations should be incorporeted in the analyses. It is possible that the cytochrome oxidase gene is too conservative and therefore not specific enough to distinguish between populations along the European coast. The ITS region, which is more variable could perhaps distinguish between the sub-population along the Atlantic coast, but we were not yet able to retrieve high quality sequences of this region. One of the reasons may be the occurrence of microsatellites (repeating sequences) in the variable ITS region: DNA polymerase can make a "hickup" due to such a repeating part. It is also possible that ITS primer binding was not optimal. Finally, in some cases, damage of DNA due to UV radiation during processing of the PCR products may have reduced the quality of the sequences.

At present we still lack enough samples from the Baltic and the Adriatic Sea. Due to the relative isolation of these basins from the Atlantic, it is possible that these areas also represent subpopulations from a genetic point of view in a similar way as the Mediterranean. However, more material is needed to confirm this hypothesis.

This study does not confirm the morphometric analysis in British waters, whereby over a relatively small area already six distinct populations could be identified (Henderson et al., 1990). These differences might be the consequence of phenotypic plasticity for instance caused by irreversible non-genetic adaptation during early life (Kinne, 1962; Van der Veer et al., 2000). However more data are required to support this suggestion. It might therefore be of interest to expand the present genetic analysis with a study on the morphometric variability among the various samples.

Another approach remains the study of microsatellites in shrimps. Whereas pilot studies on tri-repeats by collegues were not succesfull it is possible that looking for di-repeats may lead to a next step in the genetic analysis on the *C. crangon*. From birds it is known (Baker pers. comm.) that di-repeats occur more frequently than tri-repeats and tetra-repeats.

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FIGURES

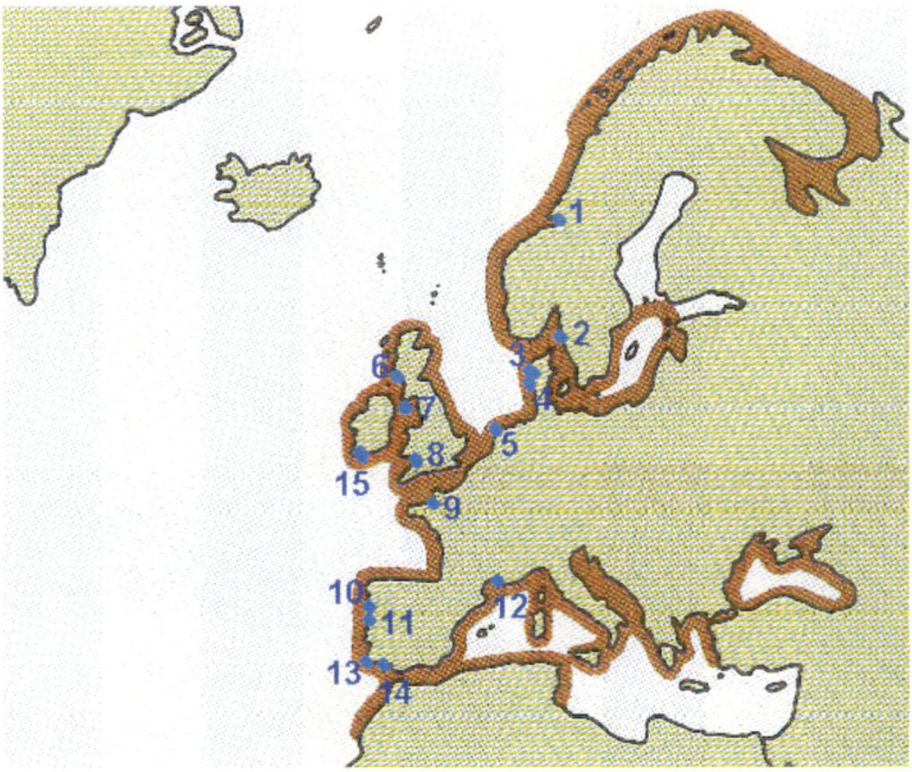

Figure 1: The distribution of the *C. crangon* (Muus & Nielsen, 1999), together with the various sampling stations in blue: 1=Sletvik, Norway; 2=Fiskebäckskil, Sweden; 3+4=Denmark; 5=Texel, The Netherlands; 6=Oban, Schotland; 7=Port Erin Bay, Isle of Man; 8=Hinkley Point, England; 9=Mt. St. Michel, France; 10=Minho river, Portugal; 11=Lima river, Portugal; 12= Étang de Vaccarès, France; 13= Algarve, Portugal; 14= Guidalquivir, Spain; 15=Cork, Ireland

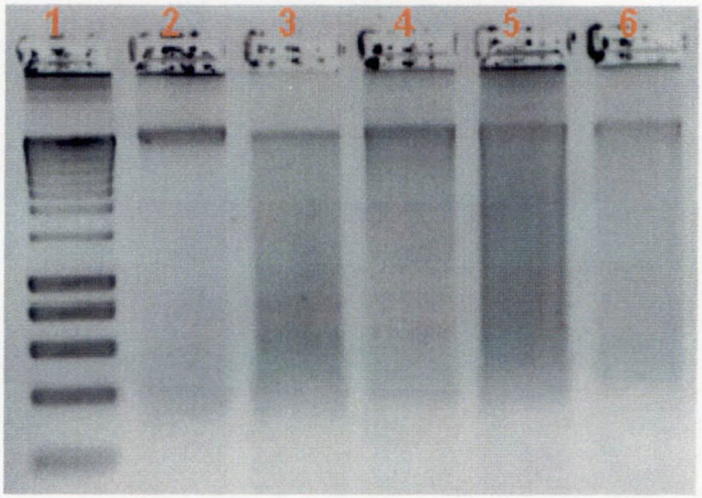

Figure 2: DNA extraction from *C. crangon* from the Mokbaai (Texel).
1=smart ladder (10000-200bp); 2-5= five different samples from the Mokbaai (Texel)

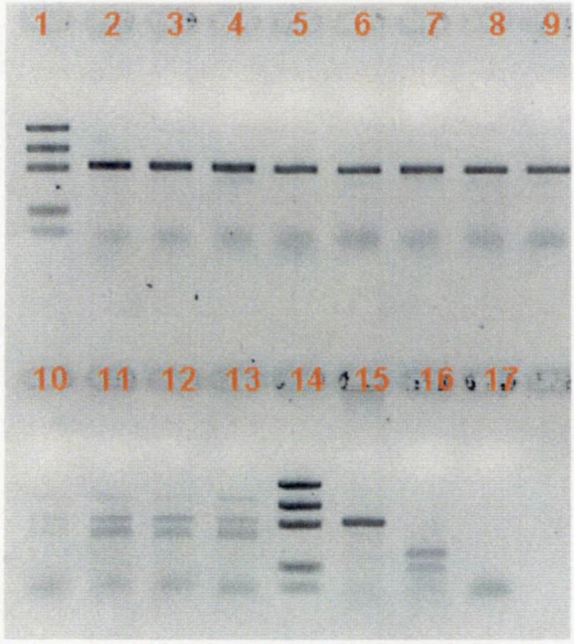

Figure 3: PCR with CO1F and CO1R primers from three different locations.
1+14=pmms ladder (1000-100bp); 2-5=Norway; 6-9=Portugal; 10-13=France; 15+16= different positive controles (*C. crangon and Crassostrea gigas*); 17=negative controle.

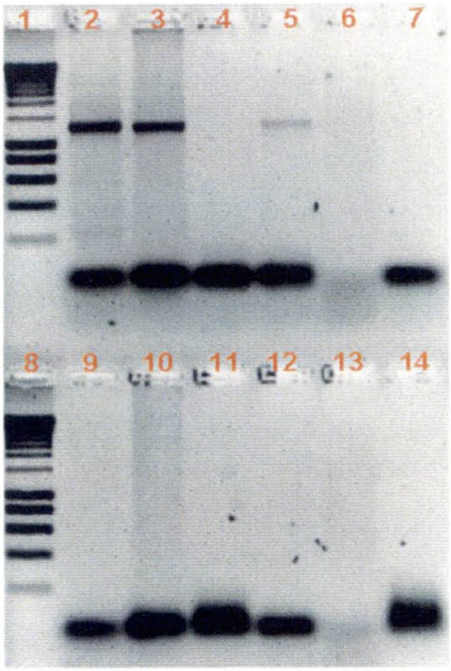

Figure 4: PCR with ITS1/ITS2 and ITS1/ITS4 from two different locations. 1+8=smart ladder (10000-200bp);2-3=Mokbaai (Texel) ITS1/ITS2; 4-5=Portugal ITS1/ITS2; 9-10=Mokbaai(Texel ITS1/ITS4); 11-12=Portugal ITS1/ITS4; 6+13=positive controle (*Venerupis senegalensis*); 7+14=negative controle

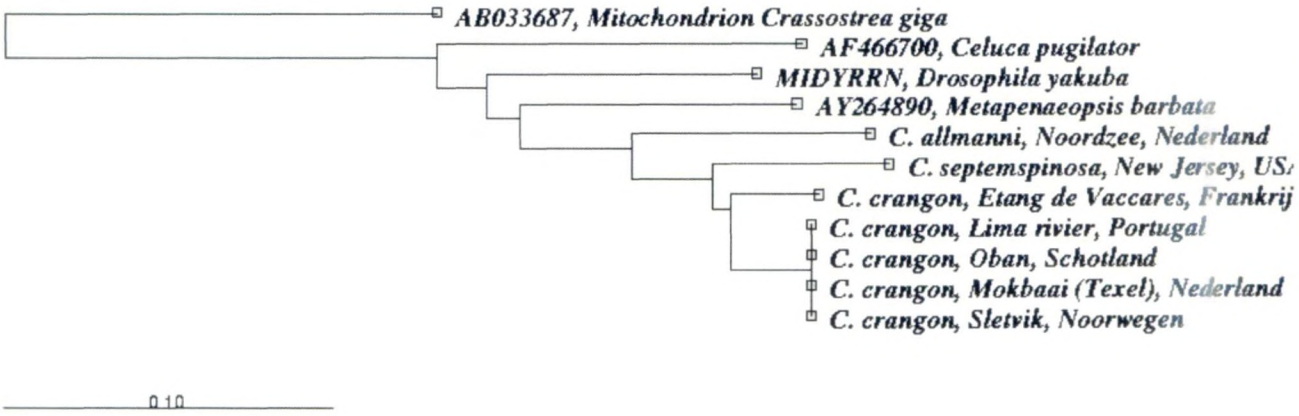

Figure 5: Phylogenetic tree based on COI analysis of the different *Crangon* species and of Crangon crangon from different locations. The scale indicates 10% sequence difference.

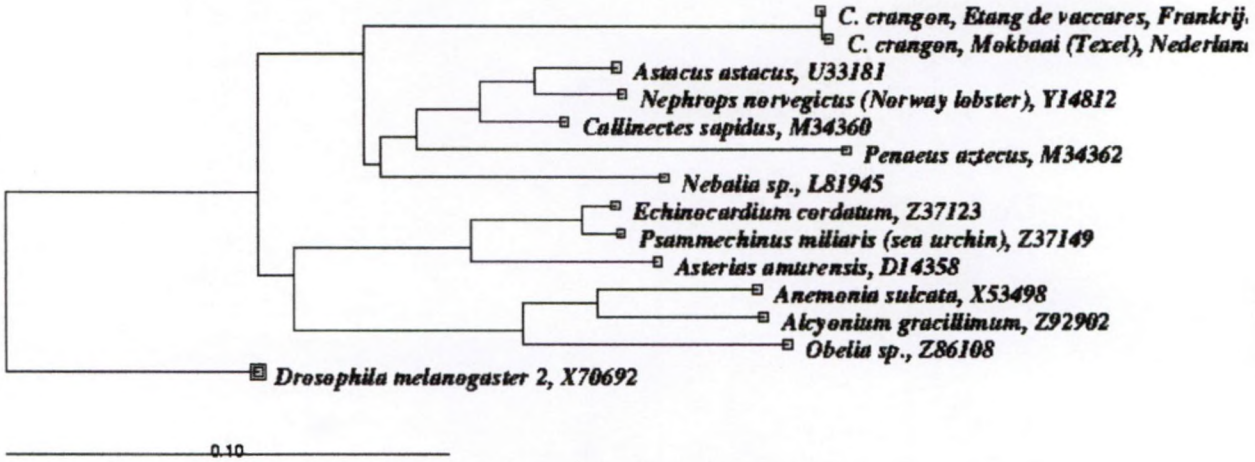

Figure 6: Phylogenetic tree of the 18S analysis from the *C. crangon* from the Mokbaai (Texel) and the *C. crangon* from Etang de Vaccarès.

Table 1: Sampling characteristics of the various *Crangon* species collected, together with the codes used.

Code	Species	Location	Month	Source
Ne2	*C. allmanni* (Kinahan 1857)	North Sea (53°30' 4°30')	February 2004	Sjollema (Royal NIOZ)
Nj1	*C. septemspinosa* (Say 1818)	New Jersey, Tuckerton	November 2003	Able, (Rutgers University)
Ne1	*C. crangon* (Linnaeus 1758)	The Netherlands, Wadden Sea Mokbaai	September 2003	Witte (Royal NIOZ)
Po1	*C. crangon* (Linnaeus 1758)	Portugal, Lima River	September 2003	Campos (Royal NIOZ)
No1	*C. crangon* (Linnaeus 1758)	Norway, Sletvik	October 2003	Karlson (Trontheim Biological Station)
Sc1	*C. crangon* (Linnaeus 1758)	Schotland, Oban	October 2003	Gibson (DML)
Fr2	*C. crangon* (Linnaeus 1758)	France , Étang de Vaccarès	January 2004	Crivelli (France)

Table 2.: Overview of the primers used for genetic analysis of *Crangon crangon*

Primers	Sequence (5'→3')	Reference
LCO1490	ggt caa caa atc ata aag ata ttg g	Folmer *et al.* 1992
HCO2198	taa act tca ggg tga cca aaa aat ca	Folmer *et al.* 1992
CO1F	tat tat tag aca aga atc tgg tta a	Franssen (personal communication)
CO1R	agg aaa tgt tga ggg aag aaa gta a	Franssen (personal communication)
ITS1	tcc gta ggt gaa cct gcg g	White *et al.* 1990
ITS2	gct gcg ttc ttc atc gat gc	White *et al.* 1990
ITS4	tcc tcc gct tat tga tat gc	White *et al.* 1990

Tabel 3: The PCR programs for the different primercombinations.

Primer Combinatie	CO1F CO1R	ITS1 ITS2
Denaturatie	94°C, 300 sec.	95°C, 300 sec.
Aantal cycli	40	39 *[2]
Denaturatie	94°C, 30 sec.	95°C, 60 sec.
Annealing	47°C, 30 sec. *[1]	50°C, 60 sec.
Extentie	72°C, 30 sec.	72°C, 90 sec.
Laatste extentie	72°C, 420 sec.	72°C, 600 sec.

*[1] *C. crangon* France and *C. allmanni* :annealingstemperature: 42°C

*[2] *C. crangon* The Netherlands: 35 cycles, *C. crangon* Norwey; *C. allmanni* and *C. septemspinosa*: 37cycles

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