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Development of sensitive and specific real-time PCR systems for the detection of crustaceans in food

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Abstract

Crustaceans are known allergens for a remarkable number of people. For the detection of traces of crustaceans in food, a specific and sensitive real-time PCR method was developed. An approximately 205 bp long fragment of the mitochondrial 16S rRNA gene was chosen as molecular target region for the detection systems. The DNA sequence of this fragment was determined from 13 species belonging to different families and checked for homologies. Based on these data, primer-probe-systems were developed for the economically relevant decapods within the class of *Malacostraca* belonging to the families *Penaeidae, Palinuroidea, Astacoidea, Nephropoidea, Cancridae* and *Caridea*. The specificity of the primer-probe-systems was checked for inclusivity using DNA extracted from 17 different crustaceans. Exclusivity tests were carried out by analysing DNA samples derived from 21 mammals, six birds, 13 fishes, two molluscs and two insect species. Except for two systems, the molecular detection systems were optimized to be highly specific for the crustaceans. False positive signal was produced by DNA extracted from the hoverfly (*Psilota rubra)* in the system targeting the family *Astacoidea* and the common green lacewing (*Chrysoperla carnea)* in the systems targeting the family *Astacoidea* and *Cancroidea*. The $LOD_{95\%}$ was close to the theoretical value of 2.96 copies per reaction. The sensitivity of the real-time PCR systems was determined by using dilution series of crustacean DNA in rainbow trout DNA as animal matrix, and by artificial contamination of fish sticks and by artificial contamination of cassava chips with crustacean meat. The sensitivity ranging from 10 ppm to 0.01 ppm is considered being appropriate for food analysis.

Key Words *molecular detection, real-time PCR, crustaceae, food allergen, Penaeidae, Palinuroidea, Astacoidea, Nephropoidea, Cancridae, Caridea*

Abbreviations rRNA, ribosomal RNA; PCR, polymerase chain reaction; FAM, 6-carboxyfluorescein; BBQ, Black Berry Quencher

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Introduction

Crustaceans and products derived from crustaceans are significant food allergens. The protein tropomyosin serves as the main allergen. Due to structural homologies of proteins of the house dust mite, some people sensitized with mite tropomyosin could have an allergic reaction after eating crustacean seafood as result of cross reaction. In affected persons, crustaceans may trigger symptoms ranging from the mild oral allergy syndrome to a life-threatening systemic anaphylaxis. It is estimated that fish and crustacean allergy affects approximately 1 % of the general population with higher frequencies in regions where large amounts of crustaceans are consumed [1, 2, 3].

As there is no specific treatment for food allergies, strict avoidance of food allergens is the only way to prevent serious health consequences. For consumer protection, several countries, including member states of the European Union, require the labeling of ingredients that can trigger allergic or intolerance reactions from Crusteaceans [4, 5, 6]. This results in the need for specific and sensitive detection methods of crustaceans in food.

In principle, immunological and molecular methods are available for the detection of crustaceans in food [7, 8, 9, 10, 11, 12, 13]. ELISA systems are designed to target the allergenic protein tropomyosin directly. The limit of detection (LOD) of some systems is 1 ppm crustacean meat in food [10]. Due to the high level of homologies between tropomyosin from different taxonomic groups, cross reactivity is observed between tropomyosin from crustaceans and from other species belonging to insects and molluscs [8, 9, 10]. It is not possible to distinguish between tropomyosin from different sources by using the immunological tests which are commercially available. Despite the clinical relevance of the presence of the allergen tropomyosin in food, it could lead to misinterpretation. This could have legal consequences as the labelling requirements are clearly linked to the Subphylum *Crustacea*.

Molecular methods allow for specific species identification [11, 12, 13, 14]. The allergenic protein cannot be detected directly by application of highly sensitive and specific PCR methods, but detection of crustacean species will indicate the presence of potentially allergenic proteins. In addition to this, specific species identification enables the verification of the product composition as given on labels; and so the fulfilment of given legal requirements can be verified.

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At present, real-time PCR systems using specific probes are increasingly being applied in food analysis for ingredients with known allergenic potential [15, 16, 17, 18, 19]. Real-time PCR systems that were developed for the detection of crustaceans were recently published [13, 20, 21]. The main advantage of real-time PCR systems is the possibility of using specific probes within the reaction. This results in a simultaneous amplification of the target sequence and the sequence specific verification of the PCR product. This combination of detection and verification as closed tube reaction also reduces the potential contamination risk which could possibly lead to false positive results. Real-time PCR systems with probes also fulfil the requirements according to international standards in molecular food analysis which require molecular verification PCR results [22].

In this work, we describe the development and in-house validation of real-time PCR systems with TaqMan® probes for the detection of the crustacean species.

Materials and methods

Crustaceaen, insect and food samples

The crustacean samples (table 1) and the food products (table 2) were purchased at local supermarkets or at the wholesales fish market in Hamburg, Germany. The crayfish species *Orconectes limosus* was caught in a pond in Saxony-Anhalt, Germany. For exclusivity tests, insects were collected inside buildings in Germany, and DNA from birds and mammals were taken from the institute's collection of reference materials. The species of each sample was determined by morphological criteria and by DNA sequencing.

Table 1

Table 2

DNA extraction

DNA extraction was performed by using the CTAB (cetyltrimethylammonium bromide) method as described earlier [17, 23, 24]. Briefly, 200 mg of raw crustacean meat was digested in 1 mL CTAB extraction buffer (20 g/L CTAB, 1.4 mol/L NaCl, 0.1 mol/L Tris (tris(hydroxymethyl)aminomethan)-HCl, 0.02 mol/L Na_2EDTA, pH 8.0) and 20 µL Proteinase K solution (c = 20 mg/L). The samples were incubated at 65 °C under permanent

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agitation overnight. After centrifugation at 13 000 *g* for 10 min, the supernatant was transferred into a new vial, 750 µL chloroform was added, it was vigorously shaken and then centrifuged again at 13 000 *g* for 5 min. The upper phase was transferred into a new vial, its volume was determined and mixed with two volumes of CTAB precipitation buffer (5 g/L CTAB, 40 mmol/L NaCl). After incubation for 60 min at room temperature without agitation, the samples were centrifuged for 15 min at 13 000 *g*, the supernatant was discarded and the pellet was resuspended in 350 µL of a 350 mmol/L NaCl solution. 350 µL chloroform was added, the samples were vigorously shaken and then centrifuged for 10 min at 13 000 *g*. The upper phase was mixed with 0.6 volumes of isopropanol for nucleic acid precipitation and after 20 min incubation at room temperature the samples were centrifuged 10 min at 13 000 *g*. The supernatant was discarded; the pellet was washed with 500 µL ethanol solution (70 % ethanol) and resolved in 100 µL 0.1 x TE buffer (1 mmol/L Tris–HCl, 0.1 mmol/L Na_2EDTA, pH 8.0). The concentration of the extracted DNA was determined photometrically (Gene Quant II, Pharmacia Biotech, Cambridge, England).

Preparation of DNA mixtures and spiked samples

The limit of detection was assessed in two ways: mixtures of DNA and mixtures of food. Mixtures of DNA were prepared as weight per weight based on the $OD_{260\ nm}$ of the nucleic acids extracted. DNA of one species from each crustacean family was selected and mixed with fish DNA (rainbow trout - *Oncorhynchus mykiss*). Crustacean DNA was tenfold diluted using 0.1 TE buffer. 1.5 µL of this solution was mixed with 20 µL of rainbow trout DNA, resulting in a dilution series ranging from 100,000 ppm to 0.01 ppm crustacean DNA in rainbow trout DNA. In a second series, homogenized crustacean meat was added to ground fish sticks as the model for animal food and to finely ground cassava chips as the model for vegetarian food at contamination levels from 100,000 ppm to 0.01 ppm crustacean meat as weight per weight food material.

Conventional PCR and DNA sequencing

A PCR product of the 16S rRNA gene was generated from available crustacean species (Table 1) using the primers Brez f (5′- TTA TAG GGT CTT ATC GTC C -3′) and Brez r (5′- TAA AGT CTR GCC TGC CCA -3′) [11]. The reaction conditions were as follows: 2.5 µL 10 × PCR buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 2.5 µM of each primer, 0.125 µL HotStar Taq Polymerase (Qiagen, Hilden, Germany), 1 µL template in a reaction volume of 25 µL. Reactions were carried out on thermal cycler GeneAmp® PCR System 9700 (Applied

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Biosystems, Darmstadt, Germany) applying the following programme: initial denaturation 15 min at 95 °C, 40 cycles of 30 s at 94 °C, 30 s at 55 °C, 30 s at 72 °C, and a final elongation step of 7 min at 72 °C.

The PCR products were purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and sequenced using the Big Dye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany). The analysis was carried out on an automated DNA sequencer (ABI PRISM ™ 310 Genetic Analyzer, Applied Biosystems, Darmstadt, Germany). The resulting sequences were compared with sequences in Genbank using the computer program BLAST 2.0 [25].

Development of crustacean specific primer probe systems

The genetic sequences of the mitochondrial DNA of five crustacean species were retrieved from the NCBI GenBank [26] and checked for homologous sequences using the computer program ClustalW [27]. Based on these sequences and the data generated, primer-probe systems were developed using the software Primer-Express® 2.0 (Applied Biosystems, Darmstadt, Germany). Seven primer probe systems (see figure 1) for detection of the crustacean families were generated. All oligonucleotides were synthesized by TIB MOLBIOL (Berlin, Germany).

Real-time PCR

PCR conditions

Real-time PCRs were carried out in a LightCycler®480 Real-Time PCR Instrument (Roche, Mannheim, Germany). The optimized reaction conditions were as follows: 1 µL 10 × PCR buffer, 1.5 mM $MgCl_2$ for all primer probe systems but 2.0 mM $MgCl_2$ for the system 1 (*Astacoidea)*, 200 µM of each dNTP, 300 nM resp. 600 nM of each primer (see Table 3), 0.05 µL Hot Star Taq Polymerase (Qiagen, Hilden, Germany), 1 µL template and water up to 10 µL. The real time PCR protocol consisted of an initial step of 95 °C for 15 min, followed by 45 cycles with 15 s at 95 °C for and 1 min the annealing / elongation temperature as shown in table 3. The real-time PCR protocol was transferred to a BioRad CFX96 instrument (BioRad, Munich Germany) applying the same reaction conditions.

Determination of specificity and sensitivity

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The primer probe systems were checked for inclusivity by using the crustacean species shown in Table 1. Exclusivity tests were carried out with DNA preparations from species listed in table 4.

Reactions of crustacean DNA in rainbow trout DNA were carried out in duplicate by applying 5 µL DNA extract in a total amount of 25 µL reaction mix with and 1 µL DNA extract in 10 µL reaction mix. The spiked food samples were extracted in duplicates using 1 µL DNA extract in 10 µL reaction mix. These experiments were carried out using a Roche LC480. The $LOD_{95\%}$ was determined using 20, 10, 5, 2, 1, and 0.1 copy of crustacean DNA in background DNA (herring sperm DNA, 20 ng / µL). The target copy number was determined by droplet digital PCR in a BioRad QX200 instrument (BioRad, Munich, Grmany) and diluted background DNA (herring sperm DNA 20 ng / µL). These experiments were run on a BioRad CFX96 real-time PCR system.

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Optimization of the real time PCR

The optimization of the real time PCR was carried out by modifying the magnesium chloride concentration and the annealing temperature. To optimize the magnesium chloride concentration, the TaqMan®Universal real-time PCR Mastermix (5 mM $MgCl_2$, Applied Biosystems, Darmstadt, Germany) was replaced by using separate reagents (HotStar™ Taq polymerase, Qiagen, Hilden, Germany). The concentration was reduced to 4.0, 3.0, 2.5, 2.0 and 1.5 mM magnesium chloride. The annealing temperature was increased from 60 °C to 61 °C, 62 °C, 64 °C and 65 °C.

After determining the optimal magnesium chloride concentration and annealing temperature, all possible combinations of primer probe systems were checked for specificity as duplex real-time PCR. DNA from the species listed above was used as test material. Finally, the real-time PCR was transferred to a BioRad CFX96 instrument using the same reaction conditions.

Results

Specificity and sensitivity

The primer-probe combinations as conducted according to the reaction conditions described are specific to *Crustaceae*, except some insects. During method development, it became evident that false positive results occurred at most duplex real-time PCR combinations, e.g. with DNA of *Pomacea canaliculata, Psilota rubra, Sus scrofa*, *Ovis aries*, *Oryctolagus cuniculus, Macropus rufus, Macropus giganteus* (data not shown). No cross reactions were

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observed for the combination canc1-fw/ canc2-rev and Car1-fw/ Car5-rev only. Consequently, all systems but System 2 (*Cancridae* and *Caridea*) were carried out as single reactions. Assay specificities are summarized in table 4. *Psilota rubra* produces false positive results with system 1 (*Astacoidea*) and *Chrysoperla carnea* produces false positive results with both systems using probe Asta1 TMP; i.e. *Astacoidea* and *Cancridae* assay. Probe Asta-TMP and the primers have significant sequence homologies with these two insect species.

Data on sensitivity are summarized in table 5. Sensitive detection of crustaceans is possible in plant and animal matrix with the primer-probe systems developed. The $LOD_{95\%}$ as determined is close to the theoretical value of 2.96 copies [28]. These data including confidence intervals guarantee a reliable molecular detection of the target sequences. A slightly better sensitivity than the theoretical value is attributed to measurement uncertainty target copy number determination by droplet digital PCR and to the inherent statistical error of the $LOD_{95\%}$ determination.

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Application of then multiplex real-time PCR on crustacean food products

DNA from different crustacean food products was extracted as described and analysed with the optimized primer probe systems. With the multiplex PCR system 2 (*Cancridae* and *Caridea*) for the crab cream, Cq values of 25.1 and 25.2 were measured. The analysis of the crab cream was also positive close to the limit of detection with the system 1 (*Astacoidea)* and system 4 (*Penaeidae* a) resulting in a Cq value of 40.0. All other systems were negative.

The analysis of lobster soup paste showed Cq values of 23.7 and 23.9 with system 2 (*Cancridae* and *Caridea*) and Cq values of 35.3 and 35.4 with the system 4 (*Penaeidae* a). All other systems did not show any amplification. For the different shrimp chips, six of the seven developed primer probe systems showed Cq values between 25.9 and 40.0. Only system 6 (*Palinuroidea*) was negative.

Discussion

The goal was to develop an universal real-time PCR system that detected contamination of food with all economically relevant crustacean species. To achieve this, one species of each relevant family was selected as the model organism and checked for homologous genetic sequences. Surprisingly, only a few areas showed enough homologies, and sequences of the 16S rRNA gene were chosen based on a PCR product generated with the primer system of Brzezinski [11]. In between this region, primers and a hydrolysis probe were placed. A similar

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concept was applied by Herrero et al. [21]. For high sensitivity in processed food, we used a shorter amplicon length. Several combinations were checked for multiplexing to reduce the analytical costs. The application of multiplex PCR is only possible for the *Cancridae* and the *Caridea* system. All other multiplex real-time PCR combinations produced false positive results for some of the non-crustacean species checked. Despite very careful oligonucleotide design, sequence homologies with non-target species could not be avoided in the 16S rRNA gene. The same genetic region has also been used by Herrero et al. [21] by applying one single LNA hydrolysis probe to the primer pair proposed by Brzezinski [11]. In contrast to Herrero et al. [21], a basic differentiation into taxonomic groups is possible by using the strategy described in this work. This can be achieved because most systems need to be run as separate reactions. In addition to previously published real-time PCR assays [13, 20, 21], we also used a variety of land animals including insects for specificity tests. Some species did show cross reactions when multiplexing was applied, therefore most reactions were carried out as single reactions. We are very aware that false positive test results could lead to unjustified actions, therefore efforts were undertaken to find out specific reaction conditions. Optimization strategies for sensitivity and specificity included optimization of the $MgCl_2$ concentration and annealing temperature. The series started using pre-manufactured mastermixes which resulted in an insufficient specificity. Specific reaction conditions require a concentration of 1.5 mM of $MgCl_2$, except the system for amplification of *Astacoidea* which required 2.0 mM $MgCl_2$ to reach sufficient sensitivity. Compared to pre-manufactured mastermix formulations, this concentration is relatively low.

In a 2nd series, the annealing / elongation temperature was adjusted resulting in 65 °C annealing temperature for *Paluridae* (Pal1-fw/Pal2-rev) resp. 61 °C for the other real-time PCR systems. These studies were run on a Roche LightCycler® LC480 and successfully transferred onto a BioRad CFX96. Both instruments use an algorithm for temperature calculation in the tube. The use of other real-time PCR systems with different temperature calculation algorithms could need an adjustment of the temperature time programme. In contrast to the literature [20, 21], we used insects for exclusivity tests in addition to food. Cross reaction could be excluded for most species by reducing the $MgCl_2$ concentration and increasing the annealing temperature, *Psilota rubra* and *Chrysoperla carnea* however are positive in systems using probe Asta-TMP due to sequence homologies. These cross reactions would be of minor practical implication because an intended presence in food of this species is unlikely. It needs to be emphasized that stringent PCR conditions shall be kept.

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Determination of the LOD of the methods was done using three different approaches, DNA mixtures and food mixtures. Whereas the DNA mixtures demonstrate the LOD of the method using background DNA as copy numbers and is more of theoretical nature, the more practical 2nd approach was directed to food. For DNA mixtures, DNA of rainbow trout (*Oncorhynchus mykiss*) was used. We used fish DNA since crustaceans could occur as bycatch in industrial fishing and the same processing facilities could be used. Method validation included the determination of the $LOD_{95\%}$ as described by Uhlig et al [28] using 12 replicates of each 20, 10, 5, 10, 2, 1, and 0.1 copies per reaction. Our results are close to the theoretical $LOD_{95\%}$ of 2.96 copies. The two series of experiments show a very good sensitivity of the real-time PCR assays, independently from the real-time PCR instrument used.

Mixtures of crustacean meat into food were used for the practical contamination experiments. Fish sticks were used as example for food of animal origin and cassava chips were used as example for food of non-animal origin. Both foodstuffs were used as examples because they could be produced in factories or on production lines where the presence crustaceans is likely. This can lead to cross-contaminations when preventive measures are limited. Heating experiments were not carried out. Results obtained previously using the same genetic element but a longer PCR product showed that PCR systems based on the 16S rRNA gene are not seriously affected by food processing [11, 21]. The sensitivity of any DNA based detection system is influenced by acid treatment and autoclaving and depends on DNA integrity. When the systems will be applied by the food industry, the applicability of these methods to highly processed food should be assessed on case by case basis.

The LOD of all of the systems is of 10 ppm and below. This is considered appropriately taking into consideration the dose eliciting reactions in 10% of the allergic population (ED_{10}) is approximately 10 mg shrimp protein for subjective symptoms and 2.5 g for objective symptoms cooked shrimp [29]. The most sensitive individuals reacted at 2.5 mg shrimp protein (11 mg whole shrimp) with mild objective symptoms [30]. Recently, a reference dose of 10 mg protein was proposed [31]. This corresponds 50 mg of fresh weight, given a protein content of approximately 20 %. The sensitivity of 10 ppm and below of crustacean meat will guarantee sufficient food safety for allergic consumers.

Meanwhile, the system has been used in routine diagnostics for six years using different real-time PCR instruments (Roche LC 480 and BioRad CFX96) and this shows the robustness of the real-time PCR systems.

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In conclusion, the probe based real-time PCR systems allow for sensitive and specific detection of crustacean in food. To ensure a high level of specificity, stringent reaction conditions, especially the concentration of $MgCl_2$ and annealing temperature, need to be kept.

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Conflict of Interest

The authors declare that they have no conflict of interest.

References

1. Wüthrich B, Jäger L (2002) Nahrungsmittelallergien und –intoleranzen, Urban & Fischer München, 2. Auflage
2. Alonso E, Zavala B, Escoda S (2006) Mantis Shrimp Allergy. Clinical Immunologie 16:394-396
3. EFSA (2006) Opinion of the Scientific Panel on Dietetic Products, Nutrition and Allergies on a request from the Commission related to the evaluation of mollusks for labelling purposes. The EFSA Journal 327: 1-25
4. Paschke A (2008) Proteine als Lebensmittelallergene. Nachrichten aus der Chemie 56: 1005-1009
5. Anonymus (2004): Food Allergen Labeling and Consumer Protection Act of 2004. Public Law 108:282, 118, Stat. 905
6. Regulation (EU) No 1169/2011of the European Parliament and of the Council of 25 October 2011 on the revision of food information to consumers, amending Regulations (EC) No 1924/2006 and (EC) No 1925/2006 of the European Parliament and of the Council, and repealing Commission Directive 87/250/EEC, Council Directive 90/496/EEC, Commission Directive 1999/10/EC, Directive 2000/13/EC of the European Parliament and of the Council, Commission Directives 2002/67/EC and 2008/5/EC and Commission Regulation (EC) No 608/2004
7. Rehbein H (2001) Identification of Shrimp Species by Protein- and DNA-based Analytical Methods. Annales Societatis Scientiarum Faeroensis Supplementum 28:195-205
8. Fuller HR, Goodwin PR, Morris GE (2006) An enzyme-linked immunosorbent assay (ELISA) for the major crustacean allergen, tropomyosin in food. Food and Agricultural Immunology 17: 43-52
9. Seiki K, Oda H, Yoshioka H, Sakai S, Urisu A, Akiyama H, Ohno Y (2007) A Reliable and Sensitive Immunoassay for the Determination of Crustacean Protein in Processed Foods. Journal of Agricultural and Food Chemistry 55: 9345-9350
10. Werner MT, Fæste CK, Egaas E (2007) Quantitative Sandwich ELISA for the Determination of Tropomyosin from Crustaceans in Foods. Journal of Agricultural and Food Chemistry 55: 8025-8032
11. Brzezinski JL (2005) Detection of Crustacean DNA and Species Identification Using a PCR-Restriction Fragment Length Polymorphism Method. Journal of Food Protection 68:1866-1873
12. Unterberger C, Luber F, Demmel A, Grünwald K, Huber I, Engel K-H, Busch, U (2014) Simultaneous detection of allergenic fish, cephalopods and shellfish in food by multiplex ligation-dependent probe amplification. Eur Food Res Technol 239:559-566
13. Cao J, Yu B, Ma L, Zheng Q, Zhao X, Xu J (2011) Detection of Shrimp-Derived Components in Food by Real-Time Fluorescent PCR. J Food Protect. 74:1776-1781

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14. Wolf C, Burgener M, Hübner P, Lüthy J (2000) PCR-RFLP Analysis of Mitochondrial DNA: Differentiation of Fish Species. Lebensm Wiss Technol. 33:144-150
15. Brežná B, Hudecová L, Kuchta T (2006) A novel real-time polymerase chain reaction (PCR) method for the detection of walnuts in food. Eur Food Res Technol 223:373-377
16. Hupfer Ch, Waiblinger HU, Busch U (2007) Development and validation of a real-time PCR detection method for celery in food. Eur Food Res Technol 225:329-335
17. Zeltner D, Glomb MA, Maede D (2009) Real-time PCR systems for the detection of the gluten-containing cereals wheat, spelt, kamut, rye, barley and oat. Eur Food Res Technol 228:321-330
18. Köppel R, Dvorak V, Zimmerli F, Breitenmoser A, Eugster A, Waiblinger HU (2010) Two tetraplex real-time PCR for the detection and quantification of DNA from eight allergens in food. Eur Food Res Technol 230 367-374
19. Tetzlaff C, Mäde D (2016) Development of a real-time PCR system for the detection of the potential allergen fish in food. Eur Food Res Technol doi:10.1007/s00217-016-2799-5
20. Eischeid A, Kim BH, Kasko SM (2012) Two Quantitative Real-Time PCR Assays for the Detection of Penaeid Shrimp and Blue Crab, Crustacean Shellfish Allergens. J Agric Food Chem 61 (24), pp 5669–5674 DOI: 10.1021/jf3031524. Publication Date (Web): November 28, 2012
21. Herrero B, Vieites JM, Espineira M (2012) Fast real-time PCR for the detection of crustacean allergen in foods. J Agric Food Chem 60:1893−1897
22. Anonymous (2005) Microbiology of food and animal feeding stuffs - Polymerase chain reaction (PCR) for the detection of food-borne pathogens - General requirements and definitions. International Standard ISO 22174:2005. International Organization for Standardization, Geneva, Switzerland
23. Rogers SO, Bendich AJ (1988) Extraction of DNA from plant tissues. Plant Molecular Biology Manual A6: 1-10
24. Anonymous (2005) Foodstuffs -- Methods of analysis for the detection of genetically modified organisms and derived products - Nucleic acid extraction. International Standard ISO 21571:2005. International Organization for Standardization, Geneva, Switzerland
25. Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DL (1997) Nucl Acids Res 25:3389-3402
26. National Center for Biotechnology Information, U.S. National Library of Medicine. http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucleotide (accessed on January 25, 2017)
27. The European Bioinformatics Institute. Multiple Sequence Alignment. http://www.ebi.ac.uk/Tools/clustalw2/. New version: http://www.ebi.ac.uk/Tools/msa/clustalo/ (accessed on January 25, 2017)
28. Uhlig S, Frost K, Colson B, Simon K, Mäde D, Reiting R, Gowik P, Grohmann L (2015) Validation of qualitative PCR methods on the basis of mathematical-statistical modelling of the probability of detection. Accred Qual Assur 20:75-83
29. Ballmer-Weber BK, Fernandez-Rivas M, Beyer K, Defernez M, Sperrin M, Mackie AR, Salt LJ, Hourihane JO'B, Asero R, Belohlavkova S, Kowalski M, de Blay F, Papadopoulos NG, Clausen M, Knulst AC, Roberts G, Popov T, Sprikkelman AB, Dubakiene R, Vieths S, van Ree R, Crevel R, Mills ENC (2015) How much is too much? Threshold dose distributions for 5 food allergens. J Allergy Clin Immunol 135:964-971
30. Nordlee JA, Remington BC, Ballmer-Weber BK, Lehrer SB, Baumert JL, Taylor SL (2013) Threshold Dose for Shrimp: A Risk Characterization Based On Objective Reactions in Clinical Studies. J Allergy Clin Immunol 131: AB88
31. Taylor SL, Baumert JL, Kruizinga AG, Remington BC, Crevel RWR, Brooke-Taylor S, Allen KJ, Houben G (2014) Establishment of Reference Doses for residues of allergenic foods: Report of the VITAL Expert Panel. Food Chem Toxicol 63:9-17

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Development of sensitive and specific real-time PCR systems for the detection of crustaceans in food

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Figures and Tables

Table 1: Common and scientific names, family and the origin of the crustacean samples used for method development. All samples were taken as whole animal (samples from the fish market and caught animal) or degutted animal without exoskeleton (bought frozen in local supermarkets). The species was verified by DNA sequencing.

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Species	Scientific name	Crustacean family	Origin
Red swamp crayfish	*Procambarus clarkii*	*Astacoidea*	Germany (Saxony, supermarket)
Stone crayfish	*Austropotamobius torrentium*	*Astacoidea*	Germany (Hamburg, fish market)
American crayfish	*Orconectes limosus*	*Astacoidea*	Caught in Saxony-Anhalt, Germany
Edible crab	*Cancer pagurus*	*Cancridae*	Germany (Hamburg, fish market)
American lobster	*Homarus americanus*	*Nephropoidea*	Germany (Hamburg, fish market)
Norway lobster	*Nephrops norvegicus*	*Nephropoidea*	Germany (Hamburg, fish market)
Pacific white shrimp	*Litopenaeus vannamei*	*Penaeidae*	Germany (Saxony, supermarket)
Black tiger shrimp	*Penaeus monodon*	*Penaeidae*	Germany (Saxony-Anhalt, supermarket)
Pink Shrimp	*Metapenaeus affinis*	*Penaeidae*	Germany (Saxony-Anhalt, supermarket)
Northern shrimp	*Pandalus borealis*	*Caridea*	Germany (Saxony, supermarket)
North sea shrimp	*Crangon crangon*	*Caridea*	Germany (Saxony,

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			supermarket)
Giant freshwater prawn	*Macrobrachium rosenbergii*	*Caridea*	Germany (Saxony-Anhalt, supermarket)
Caribbean spiny lobster	*Panulirus argus*	*Palinuroidea*	Germany (Hamburg, fish market)

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Table 2: Food products used to determine the sensitivity and applicability of the methods.

Food	**Producer**	**Number of samples**
Fish sticks	Germany (Saxony-Anhalt, supermarket)	1
Cassava (manioc) chips	Germany (Saxony-Anhalt, supermarket)	1
Lobster soup paste	Germany (Saxony-Anhalt, supermarket)	1
Crab cream	Germany (Saxony-Anhalt, supermarket)	1
Shrimp chips	Germany (Saxony-Anhalt, supermarket)	8

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Table 3: Seven different primer probe systems for the detection of crustaceans by real time PCR. Two systems were developed for *Penaeidae* to cover all sequence variants. It is necessary to run system 5 (*Penaeidae b*) and system 6 (*Palinuroidea*) at an annealing temperature of 65 °C for higher specificity. 6FAM – 6-carboxyfluorescein, BBQ – BlackBerry® Quencher, YAK - Yakima Yellow®.

Real-time PCR system and Crustacean family (Species)	Annealing temperature	Oligo-nucleotide name	Oligonucleotide sequence (5´-3´)	Length of the PCR-product in bp	Final concentration in PCR (nM)
System 1 Astacoidea	*61 °C*	asta1-fw asta2-rev Asta1 TMP	AGM TTA TTT CTT GTY CAA CCA TTC A AAG GCC GCG GTA TTA TGA C 6FAM-ACT AAT GAT TAT GCT ACC TT -BBQ	90	300 300 100
System 2 Cancridae and *Caridea*	*61 °C*	canc1-fw canc2-rev Asta1 TMP (Cancridae)	CAA CCA TTC ATA CGA GTT TCT AAT T AA GA AAA GAG CCG CGG TAT TTT GA 6FAM- ACT AAT GAT TAT GCT ACC TT -BBQ	77	300 300 100
		Car1-fw Car5-rev Neph1 TMP (Caridea)	CCA TTC ATT CCA GCC TCC AAT AAG GGC CGC GGT AAT TTG YAK-TAT GCT ACC TTC GCA CGG T -BBQ	73	600 600 100
System 3 Nephropoidea	*61 °C*	PenNeph1-fw Neph2-rev Neph1 TMP	GTC CAA CCA TTC ATA CRA GCC T AAA GGG CCG CGG TAT TTT A YAK-TAT GCT ACC TTC GCA CGG T -BBQ	80	300 300 100
System 4 Penaeidae a	*61 °C*	PenNeph1-fw	GTC CAA CCA TTC ATA CRA GCC T	81	300

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		Pen4-rev Neph1 TMP	TAA AGG GCC GCG GTA TTT TG YAK-TAT GCT ACC TTC GCA CGG T -BBQ		300 100
System 5 Penaeidae b	*65 °C*	PenNeph1-fw Pen3-rev Neph1 TMP	GTC CAA CCA TTC ATA CRA GCC T TAA AGG GCC GCG GTA TAC TG YAK-TAT GCT ACC TTC GCA CGG T -BBQ	81	300 300 100
System 6 Palinuroidea	*65 °C*	Pal1-fw Pal2-rev Pal1 TMP	AGC CGT TTA TAC CAG TCC TCA ATT GGC CGC GGT AGC ATG AC 6FAM-ACA AAT GAT TAT GCT ACC TT-BBQ	72	300 300 100

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Table 4: Results of the exclusivity tests of crustacean specific real-time PCR systems. DNA was extracted from muscle tissue, except for all insects and the common earth worm which were totally subjected to DNA extraction.

Species name (scientific name)	*System 1 Astacoidea*	*System 2 a Cancridae*	*System 2 b Caridea*	*System 3 Nephropoidea*	*System 4 Penaeidae a*	*System 5 Penaeidae b*	*System 6 Palinuroidea*
Cattle (*Bos taurus*),	-	-	-	-	-	-	-
Domestic pig (*Sus scrofa*)	-	-	-	-	-	-	-
Wild boar (*Sus scrofa*)	-	-	-	-	-	-	-
Sheep (*Ovis aries*)	-	-	-	-	-	-	-
Goat (*Capra hircus*)	-	-	-	-	-	-	-
Horse (*Equus caballus*)	-	-	-	-	-	-	-
Roe deer (*Capreolus capreolus*)	-	-	-	-	-	-	-
Red deer (*Cervus elaphus*)	-	-	-	-	-	-	-
Bison (*Bison bison*)	-	-	-	-	-	-	-
Elk (*Alces alces*)	-	-	-	-	-	-	-
Badger (*Meles meles*)	-	-	-	-	-	-	-
Rabbit (*Oryctolagus cuniculus*)	-	-	-	-	-	-	-
European hare (*Lepus europaeus*)	-	-	-	-	-	-	-
Domestic cat (*Felis catus*)	-	-	-	-	-	-	-
Domestic dog (*Canis familiaris*)	-	-	-	-	-	-	-
Rat (*Rattus norvegicus*)	-	-	-	-	-	-	-
Mouse (*Mus musculus*)	-	-	-	-	-	-	-
Coypu (*Myocastor coypus*)	-	-	-	-	-	-	-

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Giraffe (*Giraffa camelopardalis*)	-	-	-	-	-	-	-
Red giant kangaroo (*Macropus rufus*)	-	-	-	-	-	-	-
Grey giant kangaroo (*Macropus giganteus*)	-	-	-	-	-	-	-
Chicken (*Gallus domesticus*)	-	-	-	-	-	-	-
Turkey (*Meleagris gallopavo*)	-	-	-	-	-	-	-
Duck (*Anas sparsa*)	-	-	-	-	-	-	-
Goose (*Anser cygnoides*)	-	-	-	-	-	-	-
Ostrich (*Struhio camelus*)	-	-	-	-	-	-	-
Quail (*Coturnix coturnix*)	-	-	-	-	-	-	-
Plaice (*Pleuronectus platessa*)	-	-	-	-	-	-	-
Sole (*Solea solea*)	-	-	-	-	-	-	-
Flounder (*Platichthys flesus*)	-	-	-	-	-	-	-
Cod (*Gadus morrhua*)	-	-	-	-	-	-	-
Lemon sole (*Microstomus kitt*)	-	-	-	-	-	-	-
Rainbow trout (*Oncorhynchus mykiss*)	-	-	-	-	-	-	-
Brook trout (*Salmo trutta*)	-	-	-	-	-	-	-
Haddock (*Melanogrammus aeglefinus*)	-	-	-	-	-	-	-
Mackerel (*Scomber scombrus*)	-	-	-	-	-	-	-
Coalfish (*Pollachius virens*)	-	-	-	-	-	-	-
Sprat (*Sprattus sprattus*)	-	-	-	-	-	-	-
Herring (*Clupea harengus*)	-	-	-	-	-	-	-

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Red pandora (*Pagellus bellottii*)	-	-	-	-	-	-	-
Channeled applesnail (*Pomacea canaliculata*)	-	-	-	-	-	-	-
Blue mussel (*Mytilus edulis*)	-	-	-	-	-	-	-
Common earthworm (*Lumbricus terrestris*)	-	-	-	-	-	-	-
Bloody-nosed beetle (*Timarcha tenebricosa*)	-	-	-	-	-	-	-
Cereal leaf beetle (*Oulema melanopus*)	-	-	-	-	-	-	-
Ground beetle (*Amara similata*)	-	-	-	-	-	-	-
Red wood ant (*Formica rufa)*	-	-	-	-	-	-	-
Blue bottle fly (*Calliphora vicina)*	-	-	-	-	-	-	-
Blue bottle fly *Calliphora vomitoria*	-	-	-	-	-	-	-
Hoverfly (*Psilota rubra*)	+	-	-	-	-	-	-
Housefly (*Musca domestica*)	-	-	-	-	-	-	-
Common green lacewing (*Chrysoperla carnea)*	+	+	-	-	-	-	-

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Table 5: Performance characteristics of the real-time PCR system for the different crustacean families. The $LOD_{95\%}$ was assessed using pure target DNA using a BioRad CFX96 real-time PCR instrument, DNA mixtures and food mixtures were analysed using a Roche LC480 machine.

Crustacean family	$LOD_{95\%}$ including 95 % confidence interval (CI)	Detection limit in rainbow trout DNA	Detection limit in fish sticks	Detection limit in cassava chips
System 1 *(Astacoidea)* (crayfishes)	3.546 [2.249, 5.601]	1 ppm	1 ppm	10 ppm
System 2 *(Cancridae)* (crab)	2.656 [1.654, 4.273]	0.1 ppm	0.1 ppm	0.01 ppm
System 2 *(Caridea)* (shrimp)	3.261 [2.055, 5.172]	1 ppm	1 ppm	1 ppm
System 3 *(Nephropoidea)* (lobster)	2.492 [1.544, 4.031]	0.01 ppm	0.1 ppm	0.01 ppm
System 4 *(Penaeidae a)* (shrimp)	3.044 [1.908, 4.853]	1-10 ppm	1-10 ppm	1-10 ppm
System 5 *(Penaeidae b)* (shrimp)	4.720 [3.043, 7.340]	1-10 ppm	1-10 ppm	1-10 ppm
System 6 *Palinuroidea* (spiny lobster)	2.987 [1.878, 4.751]	0.1	1 ppm	0.01 ppm

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Figure 1: Sequence alignment of the 16S rRNA region which was chosen as molecular target from crustaceans. Oligonucleotides are underlined with primer sequences in italics and probe sequences in bold. Asterisks in the bottom line indicate homologous sequences.

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Procambarus clarkii         GACAGATTATTTCTTGTTCAACCATTCATTCTAGCCTTCAATTAAAAAACTAATGATTATGCTACCTTTGCACGGTCATAATACCGCGGCCTTTTAG
Orconectes limosus          GACAGATTATTTCTTGTCCAACCATTCATTCTAGCCTTCAATTAAAAAACTAATGATTATGCTACCTTTGCACGGTCATAATACCGCGGCCTTTTAG
Austropotamobius torrentium GACAGCTTATTTCTTGTCCAACCATTCATACCAGCCTCCAATTAAAAGACTAATGATTATGCTACCTTAGCACGGTCATAATACCGCGGCCTTTTAG
Cancer pagurus              GACAGCTTTTCTTTTGTTCAACCATTCATACGAGTTTCTAATTAAGAAACTAATGATTATGCTACCTTTGCACGGTCAAAATACCGCGGCTCTTTAA
Penaeus monodon             GACAGATTACTTTTTGTCCAACCATTCATACAAGCCTTCAATTAAAAGACTAATGATTATGCTACCTTCGCACGGTCAGTATACCGCGGCCCTTTAA
Litopenaeus vannamei        GACAGTTTGCTTTTTGTCCAACCATTCATACGAGCCTCCAATTAAAAGACTAATGATTATGCTACCTTCGCACGGTCAGTATACCGCGGCCCTTTAA
Metapenaeus affinis         GACAGCTTATTTTTTGTCCAACCATTCATACAAGCCTCCAATTAAAAGACTAATGATTATGCTACCTTCGCACGGTCAAAATACCGCGGCCCTTTAA
Nephrops norvegicus         GACAACTTACTTCTTGTCCAACCATTCATACAAGCCTCCAATTAAGAGACTAGTGACTATGCTACCTTCGCACGGTTAAAATACCGCGGCCCTTTAG
Homarus americanus          GACAGTTTGCTTCTTGTCCAACCATTCATACAAGCCTCCAATTAAGAGACTAATGACTATGCTACCTTCGCACGGTTAAAATACCGCGGCCCTTTAG
Pandalus borealis           GACAGCTTCCCCCTTGTCCAACCATTCATTCCAGCCTCCAATTAAAAGACTACTGATTATGCTACCTTCGCACGGTCAAATTACCGCGGCCCTTAAA
Crangon crangon             GACAGCTTCCCCCTTGTCCGACCCTTCATTCCAGCCTCCAATTAARAGGCTACTGATTATGCTACCTTCGCACGGTCAAATTACCGCGGCCCTTAAA
Macrobrachium rosenbergii   GACAGTTTATCCCTCGTCCAACCATTCATTCAAGCCTCCAATTAAAAGACTACTGACTATGCTACCTTCGCACGGTCAAATTACCGCGGCCCTTTAA
Panulirus argus             GACAGACAATTTCTTGTGCAGCCGTTTATACCAGTCCTCAATTAAAGGACAAATGATTATGCTACCTTAGCACGGTCATGCTACCGCGGCCGTTAAA
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Table 3: Seven different primer probe systems for the detection of crustaceans by real time PCR. Two systems were developed for *Penaeidae* to cover all sequence variants. It is necessary to run system 5 (*Penaeidae b*) and system 6 (*Palinuroidea*) at an annealing temperature of 65 °C for higher specificity. 6FAM – 6-carboxyfluorescein, BBQ – BlackBerry® Quencher, YAK - Yakima Yellow®.

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System 2 Cancridae and *Caridea*	*61 °C*	canc1-fw canc2-rev Asta1 TMP (Cancridae)	CAA CCA TTC ATA CGA GTT TCT AAT T AA GA AAA GAG CCG CGG TAT TTT GA 6FAM- ACT AAT GAT TAT GCT ACC TT -BBQ	77	300 300 100
		Car1-fw Car5-rev Neph1 TMP (Caridea)	CCA TTC ATT CCA GCC TCC AAT AAG GGC CGC GGT AAT TTG YAK-TAT GCT ACC TTC GCA CGG T -BBQ	73	600 600 100
System 3 Nephropoidea	*61 °C*	PenNeph1-fw Neph2-rev Neph1 TMP	GTC CAA CCA TTC ATA CRA GCC T AAA GGG CCG CGG TAT TTT A YAK-TAT GCT ACC TTC GCA CGG T -BBQ	80	300 300 100
System 4 Penaeidae a	*61 °C*	PenNeph1-fw	GTC CAA CCA TTC ATA CRA GCC T	81	300

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		Pen4-rev	TAA AGG GCC GCG GTA TTT TG		300
		Neph1 TMP	YAK-TAT GCT ACC TTC GCA CGG T - BBQ		100
System 5 Penaeidae b	*65 °C*	PenNeph1-fw	GTC CAA CCA TTC ATA CRA GCC T	81	300
		Pen3-rev	TAA AGG GCC GCG GTA TAC TG		300
		Neph1 TMP	YAK-TAT GCT ACC TTC GCA CGG T - BBQ		100
System 6 Palinuroidea	*65 °C*	Pal1-fw	AGC CGT TTA TAC CAG TCC TCA ATT	72	300
		Pal2-rev	GGC CGC GGT AGC ATG AC		300
		Pal1 TMP	6FAM-ACA AAT GAT TAT GCT ACC TT-BBQ		100

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Table 4: Results of the exclusivity tests of crustacean specific real-time PCR systems. DNA was extracted from muscle tissue, except for all insects and the common earth worm which were totally subjected to DNA extraction.

Species name (scientific name)	*System 1 Astacoidea*	*System 2 a Cancridae*	*System 2 b Caridea*	*System 3 Nephropoidea*	*System 4 Penaeidae a*	*System 5 Penaeidae b*	*System 6 Palinuroidea*
Cattle (*Bos taurus*),	-	-	-	-	-	-	-
Domestic pig (*Sus scrofa*)	-	-	-	-	-	-	-
Wild boar (*Sus scrofa*)	-	-	-	-	-	-	-
Sheep (*Ovis aries*)	-	-	-	-	-	-	-
Goat (*Capra hircus*)	-	-	-	-	-	-	-
Horse (*Equus caballus*)	-	-	-	-	-	-	-
Roe deer (*Capreolus capreolus*)	-	-	-	-	-	-	-
Red deer (*Cervus elaphus*)	-	-	-	-	-	-	-
Bison (*Bison bison*)	-	-	-	-	-	-	-
Elk (*Alces alces*)	-	-	-	-	-	-	-
Badger (*Meles meles*)	-	-	-	-	-	-	-
Rabbit (*Oryctolagus cuniculus*)	-	-	-	-	-	-	-
European hare (*Lepus europaeus*)	-	-	-	-	-	-	-
Domestic cat (*Felis catus*)	-	-	-	-	-	-	-
Domestic dog (*Canis familiaris*)	-	-	-	-	-	-	-
Rat (*Rattus norvegicus*)	-	-	-	-	-	-	-
Mouse (*Mus musculus*)	-	-	-	-	-	-	-
Coypu (*Myocastor coypus*)	-	-	-	-	-	-	-

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Giraffe (*Giraffa camelopardalis*)	-	-	-	-	-	-	-
Red giant kangaroo (*Macropus rufus*)	-	-	-	-	-	-	-
Grey giant kangaroo (*Macropus giganteus*)	-	-	-	-	-	-	-
Chicken (*Gallus domesticus*)	-	-	-	-	-	-	-
Turkey (*Meleagris gallopavo*)	-	-	-	-	-	-	-
Duck (*Anas sparsa*)	-	-	-	-	-	-	-
Goose (*Anser cygnoides*)	-	-	-	-	-	-	-
Ostrich (*Struhio camelus*)	-	-	-	-	-	-	-
Quail (*Coturnix coturnix*)	-	-	-	-	-	-	-
Plaice (*Pleuronectus platessa*)	-	-	-	-	-	-	-
Sole (*Solea solea*)	-	-	-	-	-	-	-
Flounder (*Platichthys flesus*)	-	-	-	-	-	-	-
Cod (*Gadus morrhua*)	-	-	-	-	-	-	-
Lemon sole (*Microstomus kitt*)	-	-	-	-	-	-	-
Rainbow trout (*Oncorhynchus mykiss*)	-	-	-	-	-	-	-
Brook trout (*Salmo trutta*)	-	-	-	-	-	-	-
Haddock (*Melanogrammus aeglefinus*)	-	-	-	-	-	-	-
Mackerel (*Scomber scombrus*)	-	-	-	-	-	-	-
Coalfish (*Pollachius virens*)	-	-	-	-	-	-	-
Sprat (*Sprattus sprattus*)	-	-	-	-	-	-	-
Herring (*Clupea harengus*)	-	-	-	-	-	-	-

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Red pandora (*Pagellus bellottii*)	-	-	-	-	-	-	-
Channeled applesnail (*Pomacea canaliculata*)	-	-	-	-	-	-	-
Blue mussel (*Mytilus edulis*)	-	-	-	-	-	-	-
Common earthworm (*Lumbricus terrestris*)	-	-	-	-	-	-	-
Bloody-nosed beetle (*Timarcha tenebricosa*)	-	-	-	-	-	-	-
Cereal leaf beetle (*Oulema melanopus*)	-	-	-	-	-	-	-
Ground beetle (*Amara similata*)	-	-	-	-	-	-	-
Red wood ant (*Formica rufa)*	-	-	-	-	-	-	-
Blue bottle fly (*Calliphora vicina)*	-	-	-	-	-	-	-
Blue bottle fly *Calliphora vomitoria*	-	-	-	-	-	-	-
Hoverfly (*Psilota rubra*)	+	-	-	-	-	-	-
Housefly (*Musca domestica*)	-	-	-	-	-	-	-
Common green lacewing (*Chrysoperla carnea)*	+	+	-	-	-	-	-

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Table 5: Performance characteristics of the real-time PCR system for the different crustacean families. The $LOD_{95\%}$ was assessed using pure target DNA using a BioRad CFX96 real-time PCR instrument, DNA mixtures and food mixtures were analysed using a Roche LC480 machine.

Crustacean family	$LOD_{95\%}$ including 95 % confidence interval (CI)	Detection limit in rainbow trout DNA	Detection limit in fish sticks	Detection limit in cassava chips
System 1 *(Astacoidea)* (crayfishes)	3.546 [2.249, 5.601]	1 ppm	1 ppm	10 ppm
System 2 *(Cancridae)* (crab)	2.656 [1.654, 4.273]	0.1 ppm	0.1 ppm	0.01 ppm
System 2 *(Caridea)* (shrimp)	3.261 [2.055, 5.172]	1 ppm	1 ppm	1 ppm
System 3 *(Nephropoidea)* (lobster)	2.492 [1.544, 4.031]	0.01 ppm	0.1 ppm	0.01 ppm
System 4 *(Penaeidae a)* (shrimp)	3.044 [1.908, 4.853]	1-10 ppm	1-10 ppm	1-10 ppm
System 5 *(Penaeidae b)* (shrimp)	4.720 [3.043, 7.340]	1-10 ppm	1-10 ppm	1-10 ppm
System 6 *Palinuroidea* (spiny lobster)	2.987 [1.878, 4.751]	0.1	1 ppm	0.01 ppm

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Figure 1: Sequence alignment of the 16S rRNA region which was chosen as molecular target from crustaceans. Oligonucleotides are underlined with primer sequences in italics and probe sequences in bold. Asterisks in the bottom line indicate homologous sequences.

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Procambarus clarkii         GACAGATTATTTCTTGTTCAACCATTCATTCTAGCCTTCAATTAAAAAACTAATGATTATGCTACCTTTGCACGGTCATAATACCGCGGCCTTTTAG
Orconectes limosus          GACAGATTATTTCTTGTCCAACCATTCATTCTAGCCTTCAATTAAAAAACTAATGATTATGCTACCTTTGCACGGTCATAATACCGCGGCCTTTTAG
Austropotamobius torrentium GACAGCTTATTTCTTGTCCAACCATTCATACCAGCCTCCAATTAAAAGACTAATGATTATGCTACCTTAGCACGGTCATAATACCGCGGCCTTTTAG
Cancer pagurus              GACAGCTTTTCTTTTGTTCAACCATTCATACGAGTTTCTAATTAAGAAACTAATGATTATGCTACCTTTGCACGGTCAAAATACCGCGGCTCTTTAA
Penaeus monodon             GACAGATTACTTTTTGTCCAACCATTCATACAAGCCTTCAATTAAAAGACTAATGATTATGCTACCTTCGCACGGTCAGTATACCGCGGCCCTTTAA
Litopenaeus vannamei        GACAGTTTGCTTTTTGTCCAACCATTCATACGAGCCTCCAATTAAAAGACTAATGATTATGCTACCTTCGCACGGTCAGTATACCGCGGCCCTTTAA
Metapenaeus affinis         GACAGCTTATTTTTTGTCCAACCATTCATACAAGCCTCCAATTAAAAGACTAATGATTATGCTACCTTCGCACGGTCAAAATACCGCGGCCCTTTAA
Nephrops norvegicus         GACAACTTACTTCTTGTCCAACCATTCATACAAGCCTCCAATTAAGAGACTAGTGACTATGCTACCTTCGCACGGTTAAAATACCGCGGCCCTTTAG
Homarus americanus          GACAGTTTGCTTCTTGTCCAACCATTCATACAAGCCTCCAATTAAGAGACTAATGACTATGCTACCTTCGCACGGTTAAAATACCGCGGCCCTTTAG
Pandalus borealis           GACAGCTTCCCCCTTGTCCAACCATTCATTCCAGCCTCCAATTAAAAGACTACTGATTATGCTACCTTCGCACGGTCAAATTACCGCGGCCCTTAAA
Crangon crangon             GACAGCTTCCCCCTTGTCCGACCCTTCATTCCAGCCTCCAATTAARAGGCTACTGATTATGCTACCTTCGCACGGTCAAATTACCGCGGCCCTTAAA
Macrobrachium rosenbergii   GACAGTTTATCCCTCGTCCAACCATTCATTCAAGCCTCCAATTAAAAGACTACTGACTATGCTACCTTCGCACGGTCAAATTACCGCGGCCCTTTAA
Panulirus argus             GACAGACAATTTCTTGTGCAGCCGTTTATACCAGTCCTCAATTAAAGGACAAATGATTATGCTACCTTAGCACGGTCATGCTACCGCGGCCGTTAAA
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