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A novel screening approach based on six real-time PCR systems for the detection of crustacean species in food

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ABSTRACT

Analysis of crustacean species plays a role in authenticity issues as well as allergen detection. A real-time PCR-based screening assay was developed for the detection of crustaceans in food. In order to cover most relevant species in one analytical step, PCR systems were newly developed for the detection of shrimps (Penaeidae), lobster (*Homarus* sp.), Common shrimp (*Crangon crangon*), river prawns (*Macrobrachium* sp.) and Chinese mitten crab (*Eriocheir sinensis*). In addition a published system targeting Northern prawn (*Pandalus borealis*) was selected. All PCR-systems are based on mitochondrial 16S rRNA gene sequences and were optimized to be run with a standard program at a universal annealing temperature of 60 °C. Validation experiments confirmed a sensitivity of the PCR systems of 0.01–0.1 genome copies or 0.04–2.5 pg DNA, respectively. Specificity was demonstrated with 25,000 copies of pure genomic DNA from about thirty plant and animal species relevant in food and the performance in food matrix was evaluated. Finally primer and probes were pre-spotted steadily on 96-well PCR plates and the practical applicability of the assay was proven with selected food products. The assay enables detection of multiple species of market relevance.

1. Introduction

Crustaceans such as shrimps, prawns or lobsters may elicit allergic reactions up to severe anaphylactic shock syndrome after oral intake. Hence, to protect sensitized persons, foods containing crustaceans as an ingredient have to be labeled in the European Union (Regulation (EU) No 1169/2011). On the other hand, food fraud and authenticity problems are of increasing interest and relevance in Europe as well as at a global scale.

In order to control correct labeling of seafood allergens, sensitive detection methods for crustaceans are needed. PCR (polymerase chain reaction) is the method of choice if reliable species-specific DNA detection is required. DNA is considerably stable and present in nearly any organic matter. Moreover, PCR methods are easy to standardize and the approach is in accordance with the European law (Regulation (EU) No 1169/2011) which demands the detection of the allergenic organism but not necessarily the allergenic protein.

However, crustaceans (about 51,000 species) constitute the biggest subphylum of the arthropod phylum (WoRMS, 2016). This imposes a big challenge on food control laboratories since the huge biodiversity makes it impossible to set up one single PCR system that detects all relevant species with the same sensitivity.

An amount of 6.5 M (million) metric tons (t) of crustaceans which can be ascribed to 32 families have been fished worldwide in 2014 (FishstatJ, 2016). Ten out of these wild-caught crustacean families exhibited economical important fishing quotas of >100,000 tons. Again eight of these mostly fished ten families belonged to one order, the Decapoda, which thus amount up to 60% (~4 M t) of all fished crustacean species. Whether tiny krill-like shrimps like *Acetes japonica* (Segestidae) which are used in great amounts for Akiamipaste production or big sized prawns like the Giant tiger prawn (*Penaeus monodon*; Penaeidae), shrimps and prawns are of highest ranking with approximately 2 tons, followed by marine crabs like e. g. the Gazami crab (*Portunus trituberculatus*; Portunidae) which is a popular food in Japan.

In addition, in 2014, further 6.9 M t crustaceans were produced in aquaculture for local markets and export. In fact, the aqua farmed white leg shrimp *Penaeus vannamei* (Syn. *Litopenaeus vannamei*), is the most frequently consumed crustacean species worldwide with a production of 3.7 M tons. *Penaeus monodon*,

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P. japonicus and *P. chinensis* are of commercial significance as well. Altogether nearly half of all crustaceans on the global seafood market, about 6 M tons, belonged to shrimps of the Penaeidae family. Next to these the Chinese mitten crab (*Eriocheir sinensis*; Varunidae), swim crabs of the Genus *Portunus* and freshwater prawns like *Macrobrachium rosenbergii, M. nipponense* (Palaemonidae) and *Procambarus clarkii* (Cambaridae), are produced in noteworthy amounts (>100,000 t/year).

Apart from the global market situation also local consumption habits should be considered if developing appropriate detection systems. Whereas in the northern hemisphere, e.g. *Pandalus borealis* (Northern prawn), *Crangon crangon* (North Sea crab) or lobster (Homaridae) are high-valued, the Chinese mitten crab, mangrove crabs (*Scylla serrata*) or the aforementioned shrimppaste species are appreciated and consumed in indo-pacific and eastern Asia.

In this study, to compensate for the variable performance of PCR methods targeting different crustaceans, we assembled group- and species-optimized PCR systems to build up a small-sized screening array in 96-well format. The work focuses exemplary on decapods of economic relevance on the northern European market including members of Penaeidae, Varunidae, Homaridae and Palaemonidae families. PCR systems were either newly developed based on a sequence of the mitochondrial 16S RNA gene or derived from a published source in one case. Group-as well as single species specific PCR-systems were combined by pre-spotting and conservation of the respective primer and probes on a PCR microtiter plate with trehalose as protecting agent. All systems were validated and adapted for a uniform standard annealing temperature at 60 °C. Finally the practical applicability was tested with selected food products from the market.

2. Material and methods

2.1. Samples

Market samples were purchased from local retailers in Berlin either fresh or deep-frozen in a raw or precooked state. In addition, defined reference fish and crustaceous specimen were obtained in 95% ethanol from the Max Rubner-Institut, Federal Research Institute of Nutrition and Food (MRI, Hamburg, Germany). *Eriocheir sinensis* was purchased from a local fishery at Strodehne, Brandenburg. Fish and crustacean specimen were identified by sequencing regions of the cytochrome *b* or 16S rRNA genes according to German National Food Act standard operations (BVL L 10.00–12; BVL L 12.01–3) and/or by determination based on morphological features (e.g. *Cancer pagurus*, *Eriocheir sinensis*). All species investigated in this study are listed in Table 1.

2.2. DNA extraction

For DNA isolation 200 mg of each sample were extracted using a slightly modified cetyltriammonium bromide (CTAB) protocol according to a method under the German Food Act (BVL L-23.01.22-1). To eliminate RNA contamination, crustacean DNA extracts were further digested with 20 µl RNase A per 1000 µl sample volume for 20 min at 65 °C under gentle agitation. After digestion, DNA was purified according to the following protocol: the final volume of DNA extract was adjusted to 200 µl by adding nuclease-free water and vortexed with 200 µl of phenol/Ready Red™ solution (1:1 v/v). After centrifugation for 2 min at 14,000 rpm (20 °C) the aqueous supernatant was pipetted into a clean 1.5 ml microcentrifuge tube. 150 µl of nuclease-free water were added again to the remaining organic phase, vortexed and centrifuged as described above. Both supernatants were unified. DNA was precipitated by adding isopropanol, with a volume of 80% of sample solution, and 3 M sodium acetate (pH 5.2), with a volume of 10% of sample solution. After incubation for 30 min at 4 °C, the mixture was centrifuged for 30 min at 14,000 rpm (4 °C). The supernatant was discarded and 500 µl of 70% ethanol was added. After centrifugation for 5 min at 14,000 rpm (4 °C), the liquid phase was discarded. The DNA pellet was dried using a speed vacuum concentrator and was then suspended in 30 µl nuclease-free water.

Table 1
Species used for method development and validation.

Crustaceans
Cancer pagurus (Edible crab)[a]
Crangon crangon (Common shrimp)[a]
Eriocheir sinensis (Chinese mitten crab)[a]
Homarus americanus (American lobster)[a]
Homarus gammarus (European lobster)[a]
Macrobrachium rosenbergii (Giant river prawn)[a]
Nephrops norvegicus (Norway lobster)[a]
Pandalus borealis (Northern shrimp)[b]
Penaeus brasiliensis (Redspotted shrimp)[a]
Penaeus japonica (Kuruma prawn)[a]
Penaeus monodon (Giant tiger prawn)[a,b]
Penaeus vannamei (Whiteleg shrimp)[a,b]
Procambarus clarkii (Red swamp crawfish)[a]
Varuna litterata (Peregrine crab)[a]
Xiphopenaeus kroyeri (Atlantic Seabob)[a]

Molluscs and Fish
Gadus morhua (Atlantic cod)[b]
Melanogrammus aeglefinus (Haddock)[b]
Merluccius productus (North Pacific hake)[b]
Mytilus edulis (Blue mussle)[a]
Octopus vulgaris (Common octopus)[a]
Ostrea edulis (European flat oyster)[a]
Pecten jacobaeus (Great Mediterranean scallop)[a]
Pleuronectes platessa (European plaice)[b]
Scomber scombrus (Atlantic mackerel)[b]
Theragra chalcogramma (Alaska pollock)[a,b]
Thunnus sp. *(Tuna)*[a]

Plants
Allium sativum (Garlic)
Allium cepa (Onion)
Anethum graveolens (Crown dill)
Capsicum annuum (Capsicum)
Glycine max (Soya)
Origanum majorana (Marjoram)
Oryza sativa (Rice)
Petroselinum crispum (Parsley)
Piper nigrum (Black pepper)
Sesamum indicum (Sesame)
Sinapis alba (White mustard)
Triticum sp. (Wheat)
Zea mays (Maize)

Mammalia
Bos taurus (Cattle)
Gallus domesticus (Chicken)
Melaegris gallopavo (Turkey)
Ovis aries (Sheep)
Sus scrofa demesticus (Swine)

Insects
Dermatophagoides farinae (House dust mite)
Musca domestica (Common house fly)

[a] Market sample.
[b] Max-Rubner Institute.