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Abteilung für Lebensmittelmikrobiologie

(Leiter: Univ.-Prof. Dr. med. vet. Dipl. ECVPH Martin Wagner)

Development of an analytical DNA metabarcoding method for species differentiation in food (focus on seafood)

Entwicklung eines DNA Metabaorcoding Systems zur Speziesidentifizierung in Lebensmitteln mit dem Fokus auf Meeresfrüchte

INAUGURAL-DISSERTATION

zur Erlangung der Würde eines

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vorgelegt von

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Wenn Du das Unmögliche ausgeschlossen hast, dann ist das, was übrigbleibt, die Wahrheit, wie unwahrscheinlich sie auch ist.

– Sherlock Homes

Danksagung

Es ist nun an der Zeit allen Menschen meinen DANK auszusprechen, die mir in dieser besonderen Zeit zur Seite gestanden haben. Vielen DANK für eure Hilfe, konstruktive Kritik, Ideen, Verständnis, Unterstützung, Lebensfreude, Essensversorgung, seelische Betreuung und körperliche Bewegung. Habt vielen DANK - ihr habt mir diese Zeit verschönert und bereichert! Ohne jeden Einzelnen wäre diese Arbeit nicht zu dem geworden was sie nun ist. Da ihr so viele Menschen in verschiedenen Positionen und Institutionen seid, die mir geholfen haben, möchte ich an der Stelle jedem Einzelnen von Herzen danken.

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© Kristina Gense

Geheime Spezies in Lebensmitteln und wie sie zu finden sind

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1. Introduction

1.1. Seafood in the human diet

The popularity of seafood has been rising steadily over the last three decades. The composition of essential macronutrients and micronutrients and the experience of eating an affordable delicacy are just a few of the reasons [1–8]. In German-speaking countries, seafood includes both crustaceans (e.g., crabs, prawns, lobsters) and molluscs (e.g., squid, octopus, mussels), in contrast to English-speaking countries where fish, marine mammalians and seaborne plants are also included. Seafood includes a wide spectrum of different marine species. Figure 1 shows a tiny overview of the commercial species eaten in Austria.

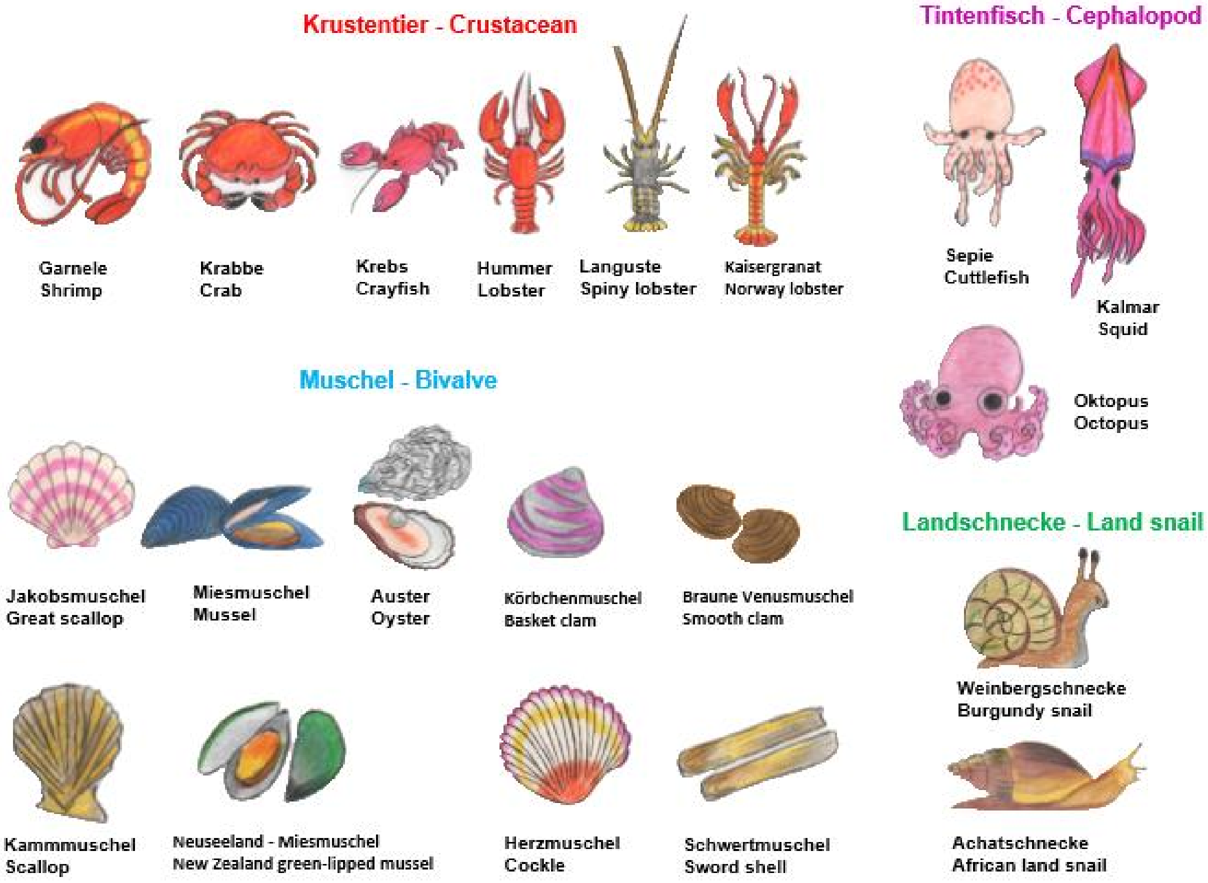

Figure 1 Variety of most eatable seafood in Austria. © Kristina Gense

Crustaceans and molluscs are divided into numerous genera comprising a high number of species with a global worldwide distribution [6,9]. The most important phylogenetic relationships of the edible seafood are shown in Figure 2 for crustaceans and Figure 3 for molluscs. Of course, the reality is even more complex and much more diverse, but even in this short overview the complexity and diversity of the species is visible.

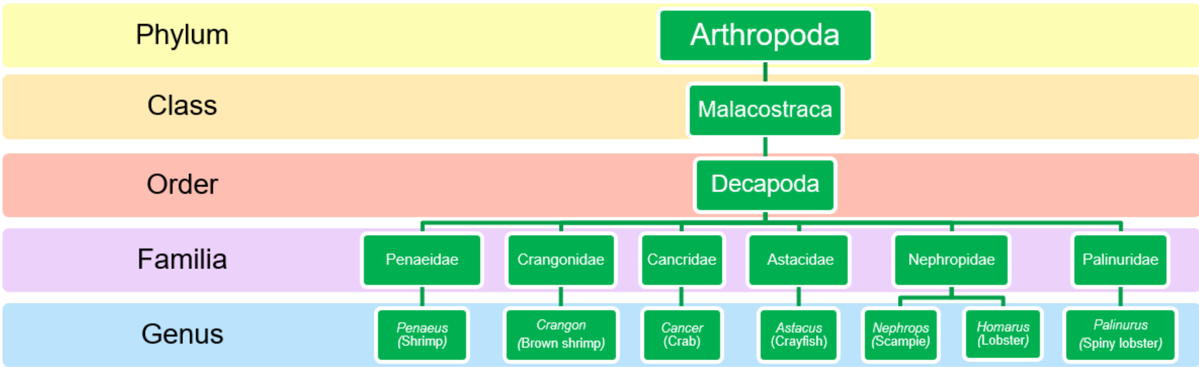

Figure 2 Brief overview of the most important crustacean phylogenetic relationship.

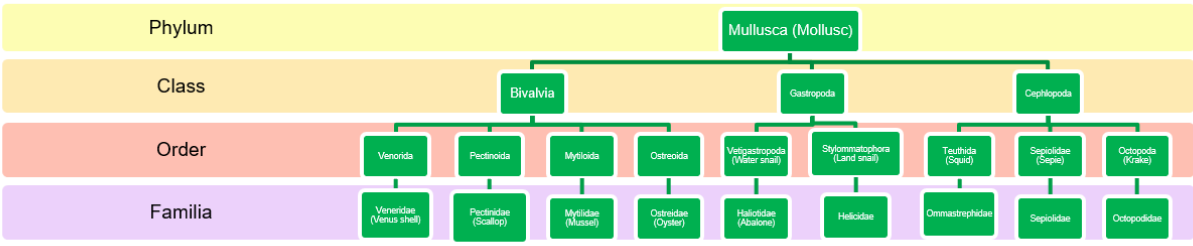

Figure 3 Brief overview of the most important mollusc phylogenetic relationship.

Worldwide, 6.1 million tonnes of crustaceans were caught and 10.5 million tonnes were cultivated in 2019. In the same year, 6.4 and 17.6 million tonnes of molluscs were caught and were cultivated, respectively. In total, 107.6 billion US $ were made with the marketing of seafood (crustaceans and molluscs) 2019, compared to 8.1 billion US $ 30 years ago [10]. Figure 4 and Figure 5 compare the volume and value composition of the main trade types of agricultural production in the European Union (EU) between the years 2008 and 2017 [11]. This significant increase highlights the importance and popularity of seafood in human nutrition.

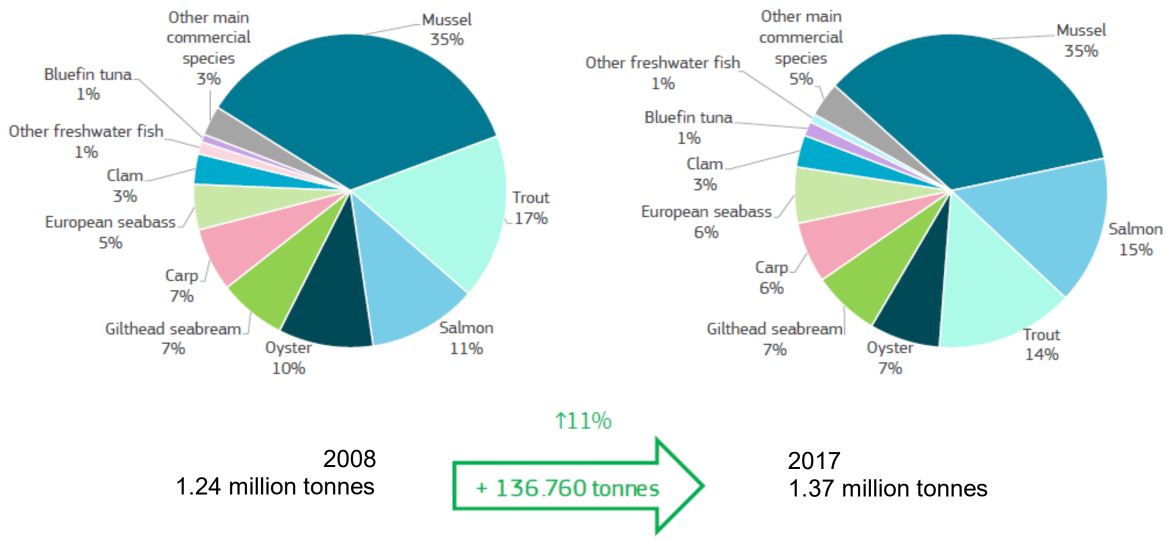

Figure 4 Composition of EU aquaculture production by main commercial species (in volume) [11], modified.

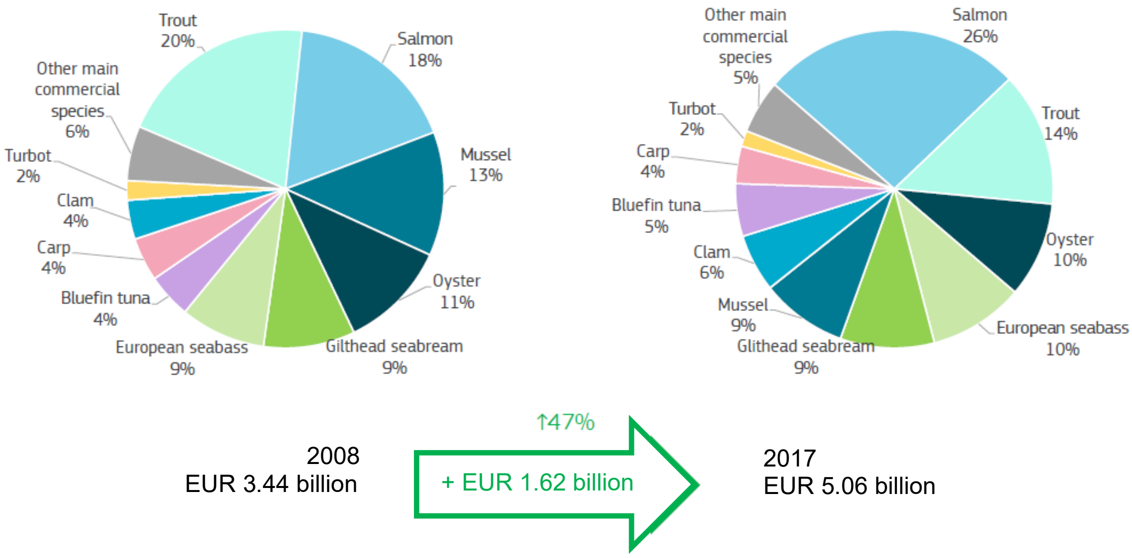

Figure 5 Composition of EU aquaculture production by main commercial species (in value) [11], modified.

1.2. Food fraud in seafood in the European Union

Like all foods with a high value, rarity and complicated supply chains, seafood is not safe from food adulteration and has a high risk of fraud and their products are often mislabelled [12–24]. Even the definition of authentic and adulterated food is sometimes under debate [25,26]. Authentic food is simple to explain by using the definition of Spink et al." Food is what it says it is" [26]. Adulteration is generally the misdeclaration of food with the goal of increasing the economic benefit [25,26]. Pardo et al. reviewed in 2016 that up to 27 % of the seafood is mislabelled worldwide [18,27]. Food adulteration includes, but is not limited to, (i) replacement (an/a (valuable) ingredient is replaced by one of a lower value), (ii) relabelled food or (iii) incorrectly labelled food. Incorrect labelling can result either from using different traditional local names which are used for the same species or from the use of identical denominations for different species, as well as errors in translation into other languages [24,28–31].

In the EU, international and national regulations are in place to ensure the legal trade of seafood and its products. Regulation EU 1169/2001 specifies prerequisites of food labelling and Regulation EU 1379/2013 implements measures for inappropriate market regulation in fishery and aquaculture industry [32,33]. These regulations stipulate that seafood has to be labelled with the commercial name (in the national language(s)) and its scientific name (in Latin), production method, and catching method or cultivation approach. The manufacturers are responsible for accuracy of information and have to guarantee the safety and authenticity of their traded seafood [19,33]. For consumers, labelling is important for individual and personal reasons, such as religion (e.g., the absence of animal-derived food is mandatory for some religions), lifestyle (seafood is deemed a luxury food in many modern societies), health concerns (mitigation of seafood associated allergies), and individual diet request (e.g. vegan, vegetarian and pescetarian diets) [34–36]. Furthermore correct labelling of seafood products is important for traceability issues, protection of endangered species and mitigation of illegal fishing practice [37].

According to the legal guidelines, each EU member state has a register of commercial species name (in the national language(s)) and its scientific name (in Latin) for each species permitted for consumption. The Codex Alimentarius Austriacus chapter B 35 (the guideline list for species designation in Austria) clarifies how to declare each species correctly (for example, in Germany this is the “Verzeichnis der Handelsbezeichnungen für Erzeugnisse der Fischerei und Aquakultur”). However, falsely declared seafood products have repeatedly been detected in Europe [14,38–41]. For example, in German-speaking countries scallops of the genus *Pecten* should only be labelled as “Jakobsmuschel” (“Jacobsmuschel”) but, species of other genera (especially *Placopecten* and *Mizuhopecten*) are also labelled as “Jakobsmuschel” [38,42,43]. Sometimes in seafood pasta, cheaper shrimps (especially *Litopenaeus vannamei* and *Penaeus monodon*) are prepared instead of the ten times more expensive scampi (*Nephrops* *norvegicus*) [31,44–48]. A last example of food adulteration is pig intestines that are labelled as squid rings [49].

1.3. Current identification methods for seafood

In order to check the authenticity of food, official laboratories have various analytical methods. The morphological characteristics, such as shell, colour, size, claws, antennas etc., give an indication of the species. However, after shell removal or mechanical processing, the classification by morphology is hampered or quite impossible [18,39,50,51]. In the field of species identification methods have been proposed based on deoxyribonucleic acid (DNA) analysis, matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) or mass spectrometry based proteomics [43,52]. However, the protein-based methods are not suitable for processed foods, as the proteins can be destroyed during the food treatment process [53]. The DNA based methods are more suitable even in highly processed food [54–59]. For species differentiation it is possible to use both, the nuclear DNA and the mitochondrial DNA (mt DNA), which exists in a number of copies in one cell [60,61]. Different DNA regions were found in the mt DNA for species discrimination like cytochrome c oxidase subunit I (COI), cytochrome *b (*cyt *b),* 12S ribosomal DNA (12S rDNA), and 16S ribosomal DNA (16S rDNA) [12,51,62–66]. In various studies, the 16S rDNA has been shown to be useful for seafood species differentiation, polymerase chain reaction (PCR) using specific probes (real-time PCR) or intercalating dyes (high resolution melt (HRM) analysis) are widely distributed in the analytical laboratories [12,15,38,46,51,53,65–79]. The principles are very similar in the PCR-based methods, an overview is given in Figure 6. It is about the amplification of specific DNA target regions. For detection and identification, different DNA target region lengths (species-specific PCR), light signals by using probes (real time PCR) or melting of the PCR products (HRM analysis) are used. The application can be used with for raw or processed single or mixed species products (not with HRM analysis). The advantages are a rather fast technique and cost effective with respective to equipment. Additionally, in the case of real time PCR, it is highly sensitive, quantitative and the primers can be multiplexed. The disadvantage of PCR based methods is that separate primers are necessary for each species target and the potential of cross reactivity. Moreover, real-time PCR probes can be very expensive. HRM analysis does not enable to distinguish between species with a low genetic difference, it cannot be applied to mixed species products and only be used with real-time PCR machines that have HRM functions [15,50,51,66,70–73,75,79–83].

Another method of analysis is hybridisation-based assays using DNA microarrays (DNA chips, biochips). This technique is based on attaching an oligonucleotide probe to a chip of a microarray surface. The previously amplified DNA target out of the sample is then attached to these probes. A positive signal is generated by a clustered binding. This method is relatively new and has just a few applications in the species identification of seafood [83–85]. It is applicable to raw and processed as well as single and mixed multi species food products. The disadvantages are high costs, complex development and need of several post-PCR steps [83].

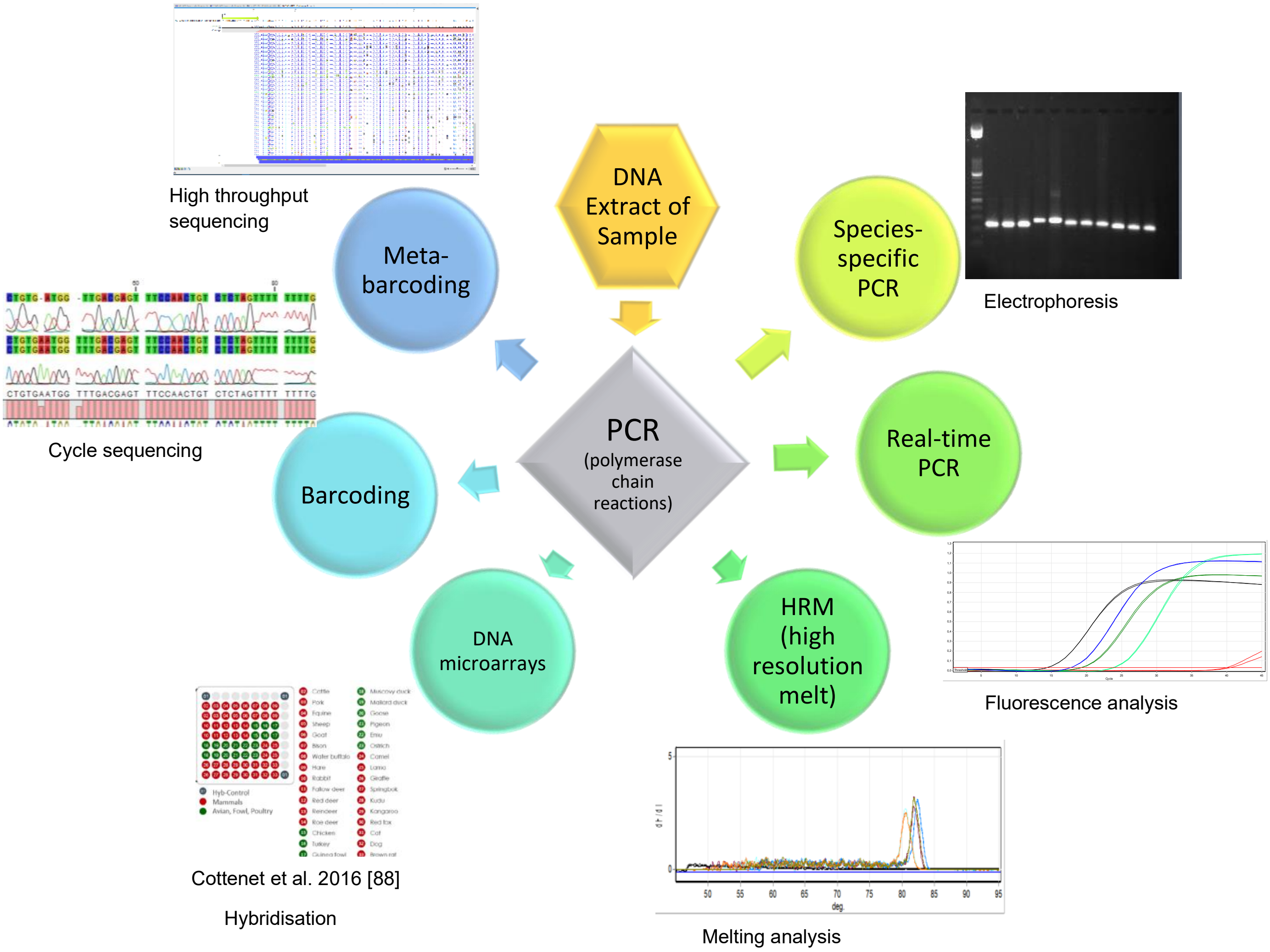

Figure 6 Current DNA-based methods of species identification.

1.4. Next generation methods

The DNA barcoding method is a very good alternative to detect a larger range of different DNA sequence fragments. Since the term “barcode” was established in 2003 by Hebert et al. the barcode has been used as a tool for the identification of species; similar to the commercial barcode on various products in the supermarket [60]. Currently, there are several barcoding systems available which use different DNA based biomarkers for species identification [12,17,41,86–90]. The DNA target length is a limiting factor because shorter barcodes have the benefit of being able to withstand higher processing conditions [14,51,55,57,62,63]. However, mini barcodes need to be composed of enough base pairs (bp), to allow an accurate species differentiation at the species level. Barcoding systems have been proposed for discrimination through Sanger sequencing using universal primers [39,86,87,91]. Barcoding systems use DNA regions conserved in a variety of species for the primer binding site to obtain the largest coverage among as many target species as possible (high coverage). The variable region in between should be diverse to be able to differentiate between individual seafood species after sequencing of PCR products (high discriminate power) [51,92]. An advantage is a large number of species identification with high content information of the DNA, meanwhile low cost per analysis and trusted species identifications. Unfortunately, this system has its own limitations as it enables species determination by sequencing of just one DNA target strand, but not multiple different DNA target strands simultaneously. Other disadvantages are a high labour intensive procedure, time intensity and the need for advanced instruments [40,41,51,83,86]

An extended version of the barcoding approach is the metabarcoding method which enables the identification of a wide number of different species in a single sample using next generation sequencing (NGS) [13,59,65,92]. Figure 7 shows the individual steps of metabarcoding. In the first step, out of the food sample of interest (A), the DNA is extracted (B). For this purpose, the laboratories established numerous protocols. The DNA library is produced afterwards (C). Different protocols are used depending on different sequencing machines. Basically, certain company adaptors and overhangs are added to the DNA target sequence. Afterwards, the sequencing itself takes place. Due to the high throughput, much data is generated (D). This data has to be analysed with special data analysis programmes and then compared with a database (E). This type of analysis is a qualitative analysis and shows only the species that have been identified (F). The advantage is the high information content of DNA (by high throughput sequencing) and trusted species identifications. The analysis of ultra-processed complex matrices (such as insect DNA in flour and bars, as well as mammalian species in sausages or different animal and plant species used in traditional Chinese medicine) has already been shown in previous studies [51,59,93,94]. At the moment the costs are high for the equipment, analysis and qualified personnel [51].

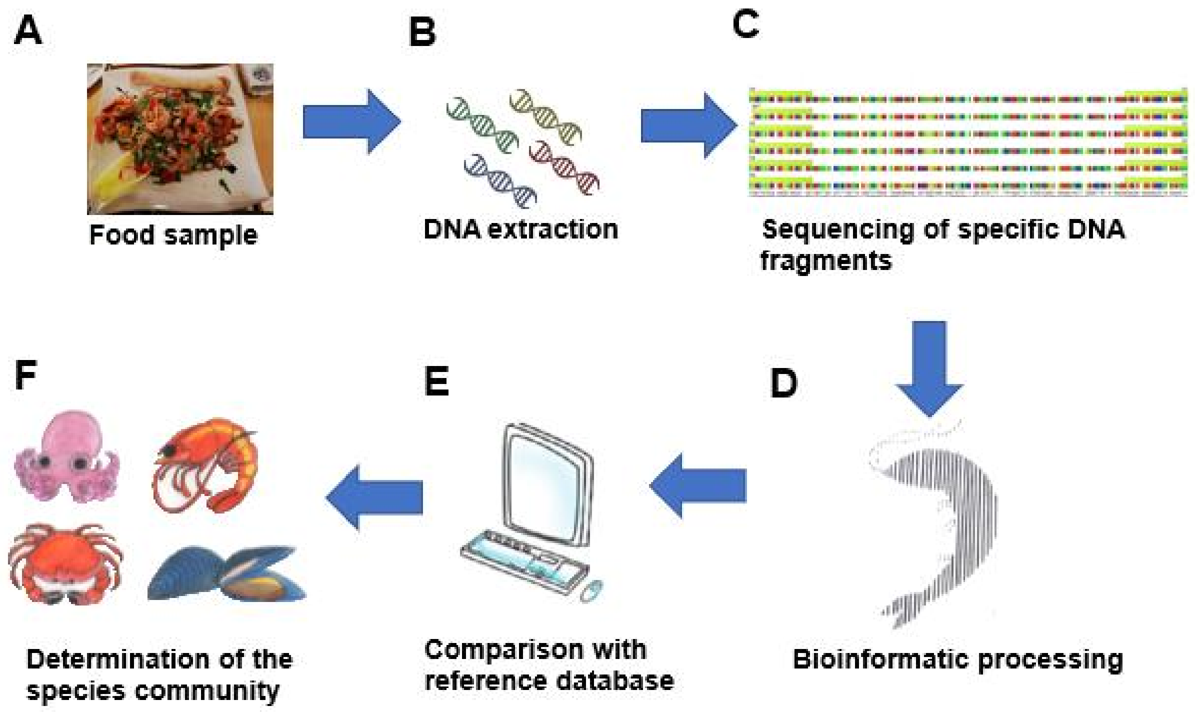

Figure 7 Operations of metabarcoding.

1.5. Aims

The aim of this study was to develop a DNA metabarcoding method that allows the identification of several commercial seafood in raw and processed conditions to accurately and rapidly detect food adulteration. The novelty consists of a non (specific to seafood) targeted analysis of a large number of seafood species simultaneously and the combination of several different single assays into one single method.

For this purpose, seafood was categorized according to their phylogenetic properties. This approach should facilitate the identification of primer sites appropriate for an efficient amplification of diverse species DNA. The following systems were developed on the Illumina platform: CruTin system (system for crustaceans and squids' identification), snail system (system for eatable land snails' identification), MOS system (system for three bivalve families: mussels (M), oysters (O) and scallops (S) identification), and Ven system (system for Venus clams' identification). Illumina MiSeq® and iSeq® platform sequencing (San Diego, California, USA) was selected for this project due to the low error rates compared to other sequencing platforms. The DNA metabarcoding method on the Illumina platform was applied to complex processed seafood products. In this dissertation, only the currently published work on bivalve triplex system was added, as the other three systems are still in progress and not fully evaluated. However, these systems have been summarised briefly and described in an own separate chapter. A patent has also been filed encompassing applied for all four seafood systems. The patent documents are not included in their totality but the most important pages for an overview. The complete manuscript of this invention report is available online.

2. Publication

foods

MDPI

Article

Development of a DNA Metabarcoding Method for the Identification of Bivalve Species in Seafood Products

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Abstract: The production of bivalve species has been increasing in the last decades. In spite of strict requirements for species declaration, incorrect labelling of bivalve products has repeatedly been detected. We present a DNA metabarcoding method allowing the identification of bivalve species belonging to the bivalve families Mytilidae (mussels), Pectinidae (scallops), and Ostreidae (oysters) in foodstuffs. The method, developed on Illumina instruments, targets a 150 bp fragment of mitochondrial 16S rDNA. We designed seven primers (three primers for mussel species, two primers for scallop species and a primer pair for oyster species) and combined them in a triplex PCR assay. In each of eleven reference samples, the bivalve species was identified correctly. In ten DNA extract mixtures, not only the main component (97.0–98.0%) but also the minor components (0.5–1.5%) were detected correctly, with only a few exceptions. The DNA metabarcoding method was found to be applicable to complex and processed foodstuffs, allowing the identification of bivalves in, e.g., marinated form, in sauces, in seafood mixes and even in instant noodle seafood. The method is highly suitable for food authentication in routine analysis, in particular in combination with a DNA metabarcoding method for mammalian and poultry species published recently.

Keywords: DNA metabarcoding; next generation sequencing; food authentication; bivalves; Mytilidae; Pectinidae; Ostreidae; species identification; mitochondrial 16S rDNA; seafood

1. Introduction

Bivalves, a class of molluscs, are distributed worldwide. Due to their high content of essential nutrients, their production has steadily been increased over the last three decades [1–5]. Mytilidae (mussels), Pectinidae (scallops), and Ostreidae (oysters) are the most important bivalve families for human consumption. Each of these bivalve families is divided into several genera comprising a high number of species [6]. In 2019, 1.03 million tons of mussels, scallops, and oysters were caught in nature and 10.25 million tons were cultivated in aquaculture, earning a profit of millions of US dollars [7].

In the EU, international and national regulations exist to ensure legal trade in seafood and seafood products. The EU directive 1379/2013 regulates market organization of fishery and aquaculture products, including correct declaration of seafood [8]. To comply with legal regulations, labels must include both the local trade name in the official language(s) and the correct scientific Latin name [8,9]. Correct labelling of seafood products is important for traceability issues, protection of endangered species, mitigation of illegal fishing, and for individual reasons of end consumers [10,11]. Regardless of clear and strict requirements for species declaration, incorrect labelling of bivalve products has repeatedly been detected in Europe [12–17]. In German and Swiss studies, more than half of the products declared to contain "Jakobsmuschel" (or "Jacobsmuschel") were labelled incorrectly [15,18,19]. Although the German name "Jakobsmuschel" (or "Jacobsmuschel") may only be used for scallop species belonging to the genus *Pecten*, species of other genera (particularly *Placopecten* and *Mizuhopecten*) were identified in these products.

For authentication of seafood products, laboratories may choose from a variety of methodologies. In the case of bivalves, morphological characteristics such as shell, color, and size may allow correct species classification. However, after shell removal or mechanical processing, classification by morphology may be hampered or even be impossible [16,20]. Recently, matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) has been shown to be suitable for accurate species identification of scallops [19]. However, since MALDI-TOF MS instruments are rather expensive and do not allow high-throughput analysis, this methodology is less applicable for routine analyses.

To date, DNA-based methods are considered most suitable for the identification of seafood species, even in highly processed food products [21–23]. Due to its high copy number and robustness, mitochondrial DNA (mtDNA) is frequently preferred over genomic DNA [24,25]. The mtDNA regions most commonly used for species identification are cytochrome c oxidase subunit I (COI), cytochrome b (cyt b), and 16S ribosomal DNA (16S rDNA) [15,26–33]. Compared to other seafood, e.g., fish, crustaceans, and cephalopods, (real-time) polymerase chain reaction (PCR) assays for bivalve species are limited in number [18,32,34–41]. The disadvantage of (real-time) PCR is that for each target species, a specific primer (probe) system is required [18,31,33,36,39–43].

A powerful alternative is DNA barcoding, aiming at detecting a broader range of species by using universal primer systems [22,26,34,44]. DNA barcodes commonly contain conserved regions at both ends, serving as binding sites for universal primers, and a variable part in between the primer binding sites, for differentiation between the species of interest [34,45]. DNA barcodes of approximately 600 base pairs (bp) in length have been found to be suitable for the analysis of highly processed food products [22,26,27,34,44,46–48]. In conventional DNA barcoding, PCR products obtained by amplifying the selected DNA barcode region are then subjected to Sanger sequencing [22,34,44,49,50]. However, sample throughput of Sanger sequencing is limited since samples are sequenced one by one. A much more efficient approach is to combine DNA barcoding with next-generation sequencing (NGS) technologies [22,26,34]. So-called DNA metabarcoding allows the identification of multiple species in multiple food samples in one and the same sequencing run [45,46,51–54]. The suitability of DNA metabarcoding for the analysis of ultra-processed food products has already been demonstrated, e.g., for the detection of mammals in sausages or insects in bars [47,48].

In this study, we present a DNA metabarcoding method allowing the differentiation between species from three bivalve families, Pectinidae, Ostreidae, and Mytilidae, in raw and processed food products to detect food adulteration. The method was developed on the Illumina MiSeq® (San Diego, CA, USA) and iSeq® (San Diego, CA, USA) platforms due to their low error rates compared to other NGS platforms [55].

2. Materials and Methods

2.1. Sample Collection and Storage

A total of 86 commercial food products were collected from regional supermarkets, fish markets, and delicacy shops in Austria from summer 2018 until winter 2020 (Supplementary Table S1). Samples were either fresh, deep-frozen, or in processed condition. Each sample was given a specific ID number, with the letter "O" referring to oysters, "S" to scallops, "M" to mussels, and "Mi" to mixed-species seafood. Samples were stored at −20 °C until DNA extraction.

Eleven out of the 86 samples ("reference samples"), comprising three mussel, six scallop, and two oyster species (see Table 1), were used for method development. Identity of bivalve species in these reference samples (samples M12, M13 and M27 for mussels; samples S42, S46, S47, S49, S50, and S55 for scallops; samples O2 and O3 for oysters; Supplementary Table S1) was verified by subjecting DNA extracts to Sanger sequencing (Microsynth, Balgach, Switzerland) and matching the sequences against the public databases provided by the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA). For Sanger sequencing, the forward and reverse primers listed in Table 2 were used.

Table 1. Bivalve species used for development of the DNA metabarcoding method.

Scientific Name	Commercial Name (German)	Commercial Name (English)
Mytilidae	Miesmuscheln	Mussels
Mytilus edulis	Gemeine Miesmuschel	Blue mussel
Mytilus galloprovincialis	Mittelmeer-Miesmuschel	Mediterranean mussel
Perna canaliculus	Neuseeland-Miesmuschel	New Zealand green-lipped mussel
Pectinidae	Kammmuscheln	Scallops
Placopecten magellanicus	Atlantischer Tiefseescallop	Atlantic deep-sea scallop
Mizuhopecten yessoensis	Japanische Kammmuschel	Yesso scallop
Pecten jacobaeus	Jakobsmuschel	Great scallop
Zygochlamys patagonica	Patagonische Kammmuschel	Patagonian scallop
Argopecten purpuratus	Purpur-Kammmuschel	Purple scallop
Aequipecten opercularis	Kleine Pilgermuschel	Queen scallop
Ostreidae	Austern	Oysters
Magallana gigas	Pazifische Felsenauster	Pacific oyster
Ostrea edulis	Europäische Auster	European flat oyster

2.2. DNA Extraction and Quantification

Raw material was cut into smaller pieces or homogenized. To 2.0 g of each sample, 10 mL of a hexadecyltrimethylammonium bromide (CTAB) buffer was added. After addition of 80 µL proteinase K, the mixture was incubated on an Intelli-Mixer™ RM2 (LTF Labortechnik, Wasserburg, Germany) overnight at 50 °C.

For DNA isolation, a commercial kit (Maxwell® 16 FFS Nucleic Acid Extraction System Custom-Kit, Promega, Madison, WI, USA) was used according to the manufacturer's instructions. DNA concentration was determined fluorometrically (Qubit® 2.0 fluorometer, Thermo Fisher Scientific, Waltham, MA, USA). For higher concentrations, the Qubit® dsDNA broad range assay kit (2 to 1000 ng) was used, and for lower concentrations, the Qubit® dsDNA high-sensitivity assay kit (0.2 to 100 ng) was used. DNA purity was assessed from the ratio of the absorbance at 260 and 280 nm (QIAxpert spectrophotometer, software version 2.2.0.21, Qiagen, Hilden, Germany). DNA extracts were stored at −20 °C until further use.

2.3. DNA Extract Mixtures

Ternary DNA extract mixtures were prepared by mixing DNA extracts (DNA concentration 5 ng/μL) from *Pecten* spp., *Magallana gigas* and *Mytilus galloprovincialis*, representing the three bivalve families Pectinidae, Ostreidae, and Mytilidae, respectively. Individual DNA extracts were mixed in a ratio of 98.0:1.5:0.5 (*v*/*v*/*v*).

In addition, DNA extract mixtures consisting of DNA from species belonging to one bivalve family were prepared. In these mixtures, DNA from one species was present as the main component, DNA from the other species as minor components (1.0% each). Since only two oyster species were available, the DNA extract mixture representing the bivalve family Ostreidae contained the closely related scallop (*Placopecten magellanicus*) as a major component (98.0%) and DNA from the two oyster species as minor components (1.0% each).

In addition to mixtures consisting of DNA from bivalve species only, a DNA extract mixture containing another mollusc species was prepared. DNA extract from a squid species (*Sepiella inermis*) was chosen as the main component (97.0%) and DNA from the bivalve species *Placopecten magellanicus*, *Ostrea edulis* and *Perna canaliculus* was present as minor components (1.0% each).

2.4. Reference Sequences

A 150 bp fragment of the mitochondrial 16S rDNA gene was used as a DNA barcode. Reference sequences for commonly consumed bivalve species and some exotic seafood species, that are permitted for consumption in Austria ("Codex Alimentarius Austriacus" chapter B35, [56]), were downloaded from the NCBI databases (Supplementary Table S2) by using CLC Genomics Workbench software (version 10.1.1, Qiagen, Hilden, Germany). If available, complete reference sequences from the RefSeq database were preferentially downloaded due to their reliability. In case complete reference sequences were not available, all DNA sequences of the mitochondrial 16S rDNA available for one and the same species, submitted by individual scientists, were aligned and checked for similarity and unidentified nucleotides. Subsequently, the DNA sequence with the highest quality (e.g., without unknown nucleotides, full-length of the DNA barcode) was chosen as a reference sequence.

2.5. Primer Systems

Primers were designed manually on a multiple DNA sequence alignment of the mitochondrial 16S rDNA of approximately 90 bivalve species using the CLC Genomics Workbench software (version 10.1.1, Qiagen, Hilden, Germany). The designed primers were checked for their physical and structural properties (e.g., formation of dimers, secondary structure, annealing temperature) using Oligo Calc, the OligoAnalyzer Tool provided by Integrated DNA Technologies (IDT, Coralville, IA, USA) and the online product descriptions from TIB Molbiol (Berlin, Germany). The primers, listed in Table 2, were synthesized by TIB Molbiol. Table 2 also shows the Illumina overhang adapter sequences which were linked to the target-specific primers.

All in-house-designed primers were tested in real-time PCR with DNA extracted from the eleven reference samples. During optimization, the following PCR conditions/parameters were kept constant and applied as published previously: DNA input amount of 12.5 ng, 'ready-to-use' HotStarTaq Master Mix Kit, annealing temperature (62 °C), 25 cycles [47]. Only one variable, the addition of magnesium chloride solution, was modified (addition of 1.5 or 3 mM $MgCl_2$). Real-time PCR reactions were carried out using a fluorescent intercalating dye (EvaGreen® (20x in water)) in strip tubes or in 96-well plates, depending on the thermocycler used, the Rotor-Gene Q (Qiagen, Hilden, Germany) or the LightCycler® 480 System (Roche, Penzberg, Germany), respectively. The total volume of the PCR reactions was 25 μL, consisting of 22.5 μL reaction mix and 2.5 μL of template DNA (diluted DNA samples (5 ng/μL)) or water as negative control. In the reaction mix, the HotStarTaq Master Mix Kit (Qiagen, Hilden, Germany) was used at a final concentration of 1x and the final concentration of primers was 0.2 µM, except the forward primer for mussels (0.4 µM). PCR cycling conditions were 15 min initial denaturation at 95 °C, 25 cycles at 95 °C, 62 °C and 72 °C for 30 s each, and a final elongation for 10 min at 72 °C. The primer pairs for mussels, scallops, and oysters with and without Illumina overhang adapter sequences were first used in singleplex PCR assays. Then, the seven primers (three forward and four reverse primers) listed in Table 2 were combined in a triplex assay. The identity of the PCR products was confirmed by melting curve analysis and/or agarose gel electrophoresis.

Table 2. Primers designed in this study.

Name	Sequence 5′→3′
mussel	
For_Mu	CCTTTTGCATAAGGGTTTTTCAAG
Rev1_Mu	CGAATAGTATCTAGCCGCCATTC
Rev2_Mu	GCAAATAGCATATCACTTTCACCTC
scallop	
For_Mu	TGCTAAGGTAGCTAAATTATGGCC
Rev_Mu	CTTCACGGGGTCTTCTCGTC
oyster	
For_Mu	GGTAGCGAAATTCCTTGCCTT
Rev_Mu	AAAGTTGCACGGGGTCTT
overhang	
Forward	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
Reverse	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG

2.6. Library Preparation and NGS

In general, samples were sequenced by using either the MiSeq® or the iSeq® platform (Illumina, San Diego, CA, USA). DNA extracts were diluted to a DNA concentration of 5 ng/µL. Extracts with a DNA concentration <5 ng/µL were used undiluted.

DNA library preparation was performed according to Dobrovolny et *al.* [47] with minor modifications (excess of $MgCl_2$, final concentration 3 mM; average library size: 278 bp; diluted libraries of the iSeq® system were denatured automatically on the instrument).

For the MiSeq® and iSeq® platform, the DNA library was adjusted to 4 and 1 nM, respectively, with 10 mM Tris-HCl, pH 8.6. After pooling individual DNA libraries (5 µL MiSeq®, 7 µL iSeq®), the DNA concentration was determined using Qubit® 2.0 fluorimeter.

All sequencing runs were performed using either the MiSeq® Reagent Kit v2 (300-cycles) or the iSeq® 100 i1 Reagent v2 (300-cycles) with a final loading concentration of 8 pM. The pooled DNA libraries contained a 5% PhiX spike-in.

Reference samples were sequenced in six replicates (three sequencing runs, two replicates per run), while DNA extract mixtures were sequenced in nine replicates (three sequencing runs, three replicates per run). Commercial food products were sequenced in triplicates (three sequencing runs, one replicate per run) and food products were sequenced at least once by using either the MiSeq® or the iSeq® platform.

2.7. NGS Data Analysis Using Galaxy

After paired-end sequencing, the resulting FastQ files, generated by the instrument control software, were used as input for data analysis. The sequencing output in FastQ format was then processed with an analysis pipeline as described previously by using Galaxy (version 19.01) [47]. The published amplicon analysis workflow was modified as follows: the target-specific primers were trimmed from both ends using the tool Cutadapt and reads were not clustered into Operational Taxonomic Units (OTUs) [57]. Completely identical sequences were collapsed into a single representative sequence with the tool Dereplicate to minimize the number of reads, and then compared against a customized database for bivalves (Supplementary Table S2) using BLASTn [58].

3. Results and Discussion

3.1. Barcode Region and Primer Systems

We aimed to develop a DNA metabarcoding method allowing the differentiation between species belonging to the bivalve families Pectinidae, Ostreidae, and Mytilidae. To be applicable in routine analysis, the method should allow identifying the economically most important bivalve species in raw and highly processed food products.

We started with searching for appropriate DNA barcode regions of about 150 bp in length, containing conserved parts at the ends and a variable part in between. Potential DNA barcode regions were found in the mitochondrial DNA, especially the mitochondrial 16S rDNA. Several metabarcoding studies have shown that the sequences of the 16S rDNA gene are suitable as barcodes for species identification. Since we have already used a barcode region of the mitochondrial 16S rDNA to identify mammals and poultry [47], this marker gene was chosen as the DNA barcode for our assay.

Since the DNA metabarcoding method for bivalves should be compatible with the DNA metabarcoding method for mammalian and poultry species published recently [47], the primers should anneal at the same temperature (62 °C). In addition, the PCR cycle number should be limited to 25 and DNA libraries should be sequenced with Illumina reagent kits in the 300-cycle format. Due to high sequence variability between closely related bivalve species, none of the primer sets designed enabled obtaining a PCR product for each of the bivalve species of interest. Thus, we continued by designing three primer sets, one for each of the three bivalve families, Pectinidae, Ostreidae, and Mytilidae. Primer pairs consisting of one forward and one reverse primer allowed amplifying the DNA barcode region in scallop and oyster species (Table 2). However, in the case of mussels, a primer set consisting of one forward primer and two reverse primers (Table 2) was necessary to obtain a PCR product for the mussel species listed in Table 1. Figure 1 shows an alignment of selected DNA barcode sequences for the commercially most relevant bivalve species. The alignment of the 90 bivalve species is shown in Supplementary Figure S1. Blue, green, and red bars indicate the binding sites of the primers for Pectinidae, Ostreidae and Mytilidae, respectively. With the three primer sets, PCR products differing in at least one base should be obtained for all bivalve species of interest.

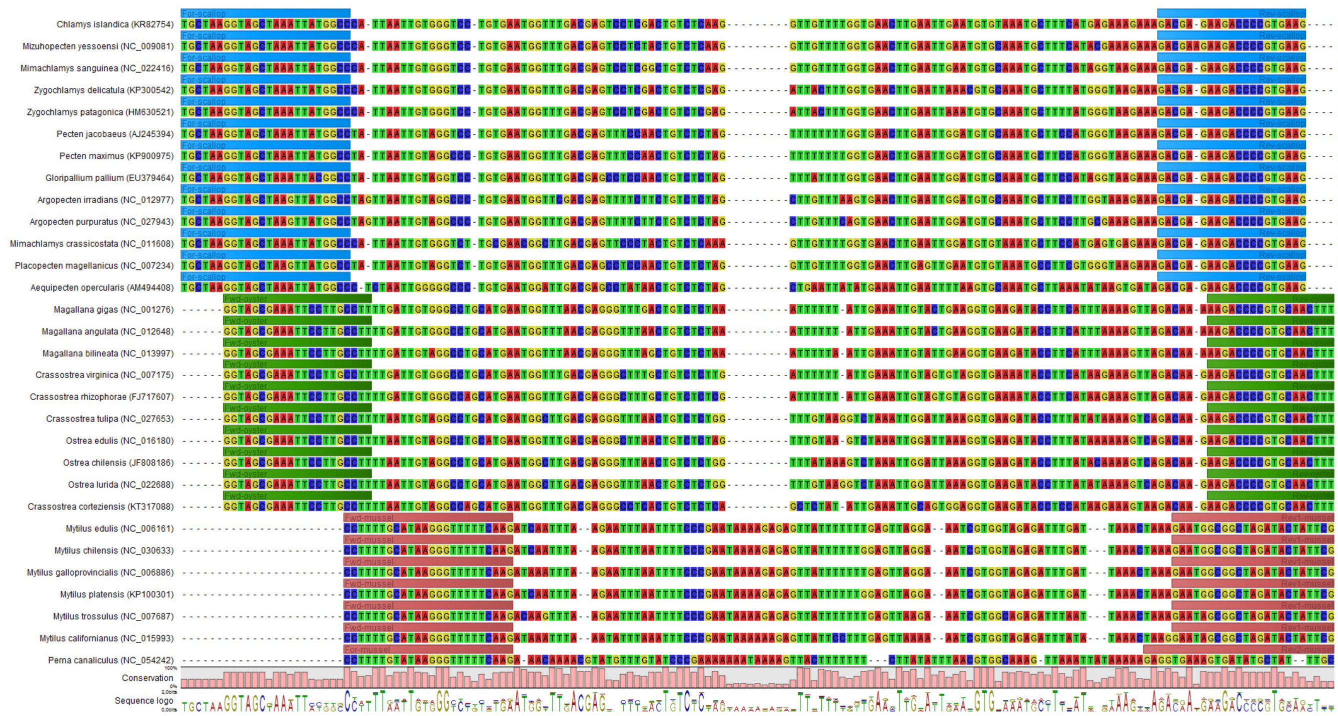

Figure 1. Multi-species sequence alignment of the mitochondrial 16S rDNA barcoding region for bivalve species. Colored bars indicate the binding sites of the primer sets for scallops (blue), oysters (green), and mussels (red, CLC Genomics Workbench software version 10.1.1, Qiagen, Hilden, Germany).

Further sequence alignments indicated that the DNA barcode region selected does not allow distinguishing between all species of the following genera: *Chlamys* spp., *Euvola* spp., *Pecten* spp., *Crassostrea* spp., *Magallana* spp., *Ostrea* spp. and *Saccostrea* spp. These species cannot be distinguished: *Chlamys rubida* and *Chlamys behringiana*; *Pecten albicans*, *Pecten fumatus*, *Pecten jacobaeus*, *Pecten keppelianus*, *Pecten novaezelandiae*, *Pecten sulcicostatus*, *Crassostrea hongkongensis,* and *Crassostrea rivularis*; *Ostrea angelica* and *Ostrea lurida;* as well as *Ostrea permollis* and *Ostrea puelchana*; and *Saccostrea echinata*, *Saccostrea glomerata*, and *Saccostrea mytiloides*. In addition, two mussel species, *Mytilus platensis* and *Mytilus chilensis*, can also not be distinguished (for *Mytilus platensis* only one DNA sequence entry was in the public databases provided by NCBI). However, differentiation at the genus level (*Chlamys* spp., *Pecten* spp., *Crassostrea* spp., *Ostrea* spp., *Mytilus* spp.) is sufficient according to the "Codex Alimentarius Austriacus" chapter B35 [56].

When we tested the primers in singleplex PCR assays, for each of the reference samples a PCR product of about 150 bp in length was obtained by increasing the concentration of the forward primer for mussels to 0.4 µM and keeping the concentration of the other six primers at 0.2 µM. In addition, we tested whether the seven primers could be combined to a triplex system. PCR products for the bivalve species of interest were obtained in one and the same vial by increasing the $MgCl_2$ concentration to a final concentration of 3 mM. Thus, we achieved our objective to perform the triplex PCR assay in combination with the previously published DNA metabarcoding assay for mammalian and poultry species [47].

3.2. Library Preparation, Pooling of Libraries, and Sequencing

Library preparation, pooling of 5 or 7 µL per normalized DNA library, and the sequencing process were performed as described previously [47]. However, in case of the pooling process, all DNA libraries were mixed in equal volumes as recommended by the manufacturer's instruction. In our previous study, different volumes from individual

DNA libraries were taken to achieve sufficient sequencing depth for minor components. For sample pooling to the maximum of 96 libraries, more than 100000 NGS reads per sample were expected to be obtained using the 300-cycle MiSeq® Reagent Kit v2.

Sequencing runs were performed in triplicate and the average run metrics were as follows: cluster density (969 K/mm^2) on the flow cell, cluster passing filter (70.22%) as well as the Q-scores (Q30) for read 1 and read 2 were 92.6% and 89.28%, respectively. A total of 5.02% of the total reads were identified as PhiX control sequences with an error rate of 1.49%.

3.3. Analysis of DNA Extracts from Reference Samples

PCR products were obtained for each of the reference samples and sequencing results for those samples are summarized in Table 3. The table shows mean values of the total number of raw reads, the total number of reads that passed the analysis pipeline in Galaxy as well as the total number and percentage of reads that were assigned correctly to the eleven species (based on six replicates).

No significant differences were observed in the total number of reads (before data analysis process) between these species, except *Mytilus galloprovincialis* (162843), *Perna canaliculus* (169631), and *Mytilus edulis* (134500). With the exception of *Perna canaliculus*, >70% of the reads passed the amplicon analysis workflow. All three mussel species, six scallop species and two oyster species could be identified with this workflow at a high rate (>97.5%), except *Mytilus edulis*.

Table 3. Results for DNA extracts from reference samples. Numbers are mean values (n = 6, three sequencing runs, two replicates per run).

Sample ID	Declaration on the Product		Species Identified	Total Number of Raw Reads	Total Number of Reads Passing the Workflow	Number of Reads Assigned Correctly	Percentage of Reads Assigned Correctly (%)
	Scientific/Latin Name	Product Description [Eng]					
O2	*Ostrea edulis*	Oyster	*Ostrea edulis*	78559	63491	61875	97.46
O3	*Crassostrea gigas**	Oyster	*Magallana gigas**	76143	65389	64125	98.07
M12	*Mytilus galloprovincialis*	Blue Mussel	*Mytilus galloprovincialis*	162843	150678	149315	99.09
M13	*Perna canaliculus*	New Zealand green-lipped mussel	*Perna canaliculus*	169631	104861	103350	98.56
M27	*Mytilus edulis*	Mussels in marinade	*Mytilus edulis*	134500	120686	105024	87.02
S42	*Mizuhopecten yessoensis*	Yesso scallop	*Mizuhopecten yessoensis*	75927	58069	57058	98.26
S46	*Pecten jacobaeus*	Great scallop	*Pecten* spp.	79472	61484	60514	98.42
S47	*Zygochlamys patagonica*	Scallop "á la Bretonne"	*Zygochlamys patagonica*	77747	59245	58429	98.62
S49	*Placopecten magellanicus*	Great scallop	*Placopecten magellanicus*	79131	61531	60886	98.95
S50	*Argopecten purpuratus*	Pacific scallop	*Argopecten purpuratus*	77383	55455	54588	98.44
S55	*Aequipecten opercularis*	Scallop in sauce	*Aequipecten opercularis*	79141	56064	55800	99.53

* former nomenclature, synonym for *Magallana gigas*.

3.4. Analysis of DNA Extract Mixtures

Six ternary DNA extract mixtures were analyzed containing the DNA of the three bivalve families Pectinidae, Ostreidae, and Mytilidae in ratios of 98.0:1.5:0.5 (*v*/*v*/*v*). The composition of the DNA extract mixtures and the results obtained by DNA metabarcoding are summarized in Table 4. The total number of raw reads ranged from 80856 to 159737 and the reads that passed the workflow were in the range from 65961 to 147196. For the main components (98.0%), the number of reads assigned correctly ranged from 62434 to 140147. In addition, both minor components (1.5% and 0.5%) could be identified. The number of reads assigned correctly was in the range from 1710 to 4356 and 555 to 1478, respectively.

Table 4. Results for ternary DNA extract mixtures representing the three bivalve families of interest. DNA extracts (5 ng/μL) were mixed in a ratio of 98.0:1.5:0.5 (*v*/*v*/*v*). Numbers are mean values ($n = 9$, three sequencing runs, three replicates per run).

Proportion			Total Number of Raw Reads	Total Number of Reads Passing the Workflow	Reads Assigned Correctly					
Species 1 (98%)	Species 2 (1.5%)	Species 3 (0.5%)			Species 1	(%)	Species 2	(%)	Species 3	(%)
Magallana gigas	*Mytilus galloprovincialis*	*Pecten* spp.	80856	69506	66430	95.57	1985	2.86	658	0.95
Magallana gigas	*Pecten* spp.	*Mytilus galloprovincialis*	89552	76669	73114	95.36	2182	2.85	894	1.17
Pecten spp.	*Magallana gigas*	*Mytilus galloprovincialis*	88971	69682	66291	95.13	1710	2.45	922	1.32
Pecten spp.	*Mytilus galloprovincialis*	*Magallana gigas*	84085	65961	62434	94.65	2281	3.46	555	0.84
Mytilus galloprovincialis	*Pecten* spp.	*Magallana gigas*	159737	147196	140147	95.21	4356	2.96	1478	1.00
Mytilus galloprovincialis	*Magallana gigas*	*Pecten* spp.	147443	136629	130986	95.87	3304	2.42	1156	0.85

In addition, we analyzed three DNA extract mixtures consisting of DNA from species belonging to one bivalve family (Table 5). The mixtures contained DNA from a scallop or mussel species, respectively. DNA from other bivalve species was present in a proportion of 1.0% each. Both species being present as main components, *Placopecten magellanicus* and *Perna canaliculus,* could be identified, with the number of reads assigned correctly ranging from 58156 to 77483. However, quite different numbers of reads were correctly assigned to the minor components, ranging from 626 (*Mizuhopecten yessoensis*) to 50391 (*Mytilus galloprovincialis*). *Aequipecten opercularis* was the only minor component that could not be detected.

Table 5. Results for DNA extract mixtures representing one bivalve family. DNA from minor components was present in a proportion of 1% each. In addition, results for a DNA extract mixture containing DNA from a squid species (*Sepiella inermis*) as main component (97.0%) and DNA from three bivalve species (1% each) is shown. Numbers are mean values (n = 9, three sequencing runs, three replicates per run).

Main Component	Minor Component (1.0% Each)	Total Number of Raw Reads	Total Number of Reads Passed the Workflow	Reads Assigned Correctly	Percentage of Reads Assigned Correctly (%)
Placopecten magellanicus				58156	88.86
	Mizuhopecten yessoensis			626	0.96
	Pecten spp.	83526 *	65446	817	1.25
	Zygochlamys patagonica			4534	6.93
	Argopecten purpuratus			663	1.01
	Aequipecten opercularis			35	0.05
Placopecten magellanicus				63628	95.41
	Magallana gigas	84282 *	66691	1298	1.95
	Ostrea edulis			1088	1.63
Perna canaliculus				77483	60.12
	Mytilus galloprovincialis	179227 *	128882	50391	39.10
	Mytilus edulis			824	0.64
Sepiella inermis					
	Placopecten magellanicus	78467	61415	31424	51.17
	Ostrea edulis			28162	45.86
	Perna canaliculus			806	1.31

* Number of values (n = 6, three sequencing runs, two replicates per run).

We analyzed a further DNA extract mixture containing DNA from the squid species *Sepiella inermis* as main component (97.0%) and DNA from the bivalve species *Placopecten magellanicus*, *Ostrea edulis,* and *Perna canaliculus* as minor components (1.0% each). As expected, in this mixture, the main component could not be detected because the primers are not suitable for amplification of the target region for *Sepiella inermis*. 31424, 28162, and 806 reads, respectively, were assigned correctly to the three bivalve species.

In our previous metabarcoding study [47], individual DNA libraries were pooled in different ratios to achieve sufficient sequencing depth for minor components. The present study demonstrates, that minor components down to a proportion of 0.5% could be identified and differentiated although DNA libraries were pooled by mixing them in equal volumes. DNA extracts from reference samples and DNA extract mixtures most frequently resulted in less than 100000 reads. However, for all samples on average >75000 raw reads were obtained, which turned out to be sufficient for reliable species identification.

3.5. Analysis of Commercial Seafood Samples

In order to investigate the applicability of the DNA metabarcoding method to foodstuffs, DNA extracts from 75 commercial food products were analyzed. According to declaration, eight samples (O1 and O4–O10) contained oyster species, 27 samples (M11, M14–M26, and M28–M40) mussel species, 15 samples (S41, S43–45, S48, S51–S55, and S56–S61) scallop species and 25 samples (Mi62–Mi86) were mixed-species seafood products (Table 6). The ingredient list of 30 out of 75 food products did not give any information on the bivalve species. A total of 39 samples were declared to contain "*Crassostrea gigas*", "*Mytilus galloprovincialis*", "*Mytilus chilensis*", "*Mytilus edulis*", "*Zygochlamys patagonica*", "*Chlamys opercularis*", "*Placopecten magellanicus*", "*Pecten maximus*", or "*Patinopecten yessoensis*". The remaining samples (n = 6) were labelled with "*Mytilus* spp." or "*Pecten* spp.".

Our results indicate that DNA metabarcoding by targeting the 16S rDNA barcode region of about 150 bp in length is applicable to complex and highly processed foodstuffs. The barcode region could be amplified and sequenced even in products such as Bouillabaisse, Paella, and instant noodle seafood. Oyster sauce was the only sample matrix for which PCR amplification and consequently sequencing failed. Failure of obtaining PCR products for oyster sauce has already been reported by Chin Chin et al. [50], most probably caused by excessive DNA fragmentation due to industrial processing.

Three oyster species (*Saccostrea malabonensis*, *Magallana bilineata*, *Magallana gigas*), three mussel species (*Mytilus galloprovincialis*, *Mytilus edulis*, *Perna canaliculus*), and three scallop species (*Aequipecten opercularis*, *Placopecten magellanicus*, *Pecten* spp.) were detected in food products (O4, O8, M17, M19, M23, M25, M26, M28, M31, M32, M35, M38–M40, S51, S56, S58–S60, Mi63, Mi65, Mi70, Mi71, Mi73–Mi76, Mi81, Mi83, Mi85, and Mi86) although they were not declared on the label.

In each of the six oyster products that could be subjected to sequencing (O1, O4–O8), *Magallana gigas* was identified. *Magallana gigas* is by far the predominant oyster species farmed in the EU [59].

In 21 products (M11, M16, M18, M21, M24, M33–M35, M37, M39, M40, Mi62, Mi64, Mi66, Mi69, Mi72, Mi77–Mi80, and Mi84), the mussel species *Mytilus galloprovincialis* was detected. In addition to *Mytilus galloprovincialis*, *Mytilus edulis* was identified (percentage of reads assigned correctly >1%) in 13 products (M24, M33, M34, M39, Mi62, Mi64, Mi66, Mi69, Mi72, Mi78–Mi80, and Mi84). In four products, *Mytilus edulis* could not be detected although it was declared on the label. *Mytilus galloprovincialis* and *Mytilus edulis* are the two mussel species most frequently cultivated in European mussel farms [59]. In none of the products declared to contain *Mytilus chilensis, Mytilus chilensis* was detected. Instead of *Mytilus chilensis,* imported to EU countries from Chile [60], *Mytilus galloprovincialis* and/or *Mytilus edulis* were identified. According to the multi-species sequence alignment shown in Figure 1, the barcode region should allow distinguishing the three Mytilus species.

Placopecten magellanicus and *Patinopecten yessoensis* were listed as ingredients in samples S41, S45, S54, and S57 and samples S48, S52, and S61, respectively. Our results confirmed the presence of these two species, except for sample S57. In sample S43, declared to contain *Pecten maximus,* the species *Mizuhopecten yessoensis* was detected. In sample S44 and S53, declared as *Pecten* spp., the species *Mizuhopecten yessoensis* was also identified. In line with previous studies, most products declared to contain "Jakobsmuschel" did not contain a species of the genus *Pecten* [15,18,19]. Instead, we identified *Placopecten magellanicus* or *Mizuhopecten yessoensis*.

Table 6. Results obtained for commercial seafood samples. Samples listed above the double line were sequenced with the MiSeq® (three sequencing runs, one replicate per run, numbers are mean values); samples listed below the double line were sequenced either with the MiSeq® or the iSeq®.

Sample ID	Declaration on the Product		Species Identified	Total Number of Raw Reads	Total Number of Reads Passed the Workflow	Reads Assigned Correctly	Percentage of Reads Assigned Correctly (%)
	Scientific/Latin Name	Product Description [Eng]					
O5	*Crassostrea gigas* [4]	Oyster in sunflower oil	*Magallana gigas* [4]	76930 [1]	65728	64369	97.93
O6	*Crassostrea gigas* [4]	Oyster in sunflower oil	*Magallana gigas* [4]	44848 [1]	38547	37610	97.57
O7	*Crassostrea gigas* [4]	Oyster in water	*Magallana gigas* [4]	76247	64917	63700	98.13
O8	not declared	Oyster sauce	*Saccostrea malabonensis* *Magallana bilineata*	14470	11658	5442 4652	46.68 39.91
M23	not declared	Mussel with sherry vinegar	*Mytilus galloprovincialis*	33517	30794	30358	98.58
M25	not declared	Mussel in marinade sauce	*Mytilus galloprovincialis*	163188	151688	150700	99.35
M26	not declared	Grilled blue	*Mytilus galloprovincialis*	163106	151608	150433	99.23

		mussel					
M29	*Mytilus galloprovincialis*	Blue mussel in tomato sauce	*Mytilus galloprovincialis* *Mytilus edulis*	153435	140475	132354 7937	94.22 5.65
M30	*Mytilus galloprovincialis*	Blue mussel A la mariniere	*Mytilus galloprovincialis* *Mytilus edulis*	185479	171890	170624 1156	99.26 0.67
M31	not declared	Blue mussel in organic marinade	*Mytilus galloprovincialis* *Mytilus edulis*	170303	158379	157015 1267	99.14 0.80
M32	not declared	Marinated blue mussel	*Mytilus galloprovincialis* *Mytilus edulis*	159181	144788	143399 1308	99.04 0.90
M33	*Mytilus chilensis*	Mussel in Escabeche	*Mytilus galloprovincialis* *Mytilus edulis*	167903	151219	118879 31737	78.61 20.99
M34	*Mytilus chilensis*	Mussel	*Mytilus galloprovincialis* *Mytilus edulis*	152112	138768	87964 49601	63.39 35.74
M36	*Mytilus galloprovincialis*	Blue mussel marinated	*Mytilus galloprovincialis* *Mytilus edulis*	176963	163721	162224 1323	99.09 0.81
M37	*Mytilus edulis*	Mussel in honey mustard sauce	*Mytilus galloprovincialis* *Mytilus edulis*	149364	136868	135249 1400	98.82 1.02
M38	not declared	Blue mussel in marinade	*Mytilus galloprovincialis* *Mytilus edulis*	138801	127244	125980 1056	99.01 0.83
S58	not declared	Rillettes de Saint-Jacques	*Aequipecten opercularis* *Mytilus galloprovincialis*	62787	44307	42716 1330	96.41 3.00
S59	not declared	Small scallop in galician sauce	*Aequipecten opercularis*	82550	59722	58296	97.61
Mi62	*Mytilus chilensis*	Seafood mix	*Mytilus galloprovincialis* *Mytilus edulis*	618324	569815	433439 134543	76.07 23.61
Mi63	not declared	Sauce with seafood	*Mytilus edulis* *Mytilus galloprovincialis*	152170	139306	73550 64729	52.80 46.47
Mi64	*Mytilus chilensis* *Mytilus edulis*	Seafood mix	*Mytilus galloprovincialis* *Mytilus edulis*	131285	119350	81590 37211	68.36 31.18
Mi65	not declared	Bouillabaisse Marseille	*Mytilus galloprovincialisMytilus edulis*	157311	143479	138535 4777	96.55 3.33
Mi66	*Mytilus chilensis*	Seafood mix	*Mytilus galloprovincialis* *Mytilus edulis*	152535	140047	92024 47415	65.71 33.86
Mi67	*Mytilus* spp.	Seafood mix	*Mytilus galloprovincialis* *Mytilus edulis*	76544	69081	48275 20459	69.88 29.62
Mi68	*Mytilus galloprovincialis*	Sea fruit salad in sunflower oil	*Mytilus galloprovincialis* *Mytilus edulis*	157861	145671	144468 1046	99.17 0.72
Mi69	*Mytilus chilensis*	Seafood mix	*Mytilus galloprovincialis* *Mytilus edulis*	140227	128007	85679 41686	66.93 32.57
Mi70	not declared	Sea fruit salad fantasy	*Mytilus galloprovincialis* *Mytilus edulis*	120677	106674	101121 5413	94.80 5.07
Mi71	not declared	Seafood mix	*Mytilus galloprovincialis* *Mytilus edulis*	160546	147278	79680 66675	54.10 45.27
Mi72	*Mytilus chilensis*	Seafood mix	*Mytilus galloprovincialis* *Mytilus edulis*	160059	146539	91557 54271	62.48 37.03
Mi73	not declared	Seafood mix	*Mytilus edulis* *Mytilus galloprovincialis*	150500	137634	78942 57608	57.36 41.86
Mi74	not declared	Seafood mix	*Mytilus galloprovincialis* *Mytilus edulis*	168841	155701	79035 75612	50.76 48.56
Mi75	not declared	Pizza Frutti di mare	*Mytilus galloprovincialis* *Mytilus edulis*	181822 [1]	172620	95184 71440	55.14 41.39
Mi76	not declared	Paella	*Mytilus galloprovincialis* *Mytilus edulis*	150431	139511	138335 1070	99.16 0.77
Mi77	*Mytilus edulis,* *Mytilus chilensis*	Paella	*Mytilus galloprovincialis* *Mytilus edulis*	141816	132092	130768 1242	99.00 0.94

Mi78	*Mytilus chilensis*	Seafood all´Olio	*Mytilus galloprovincialis* *Mytilus edulis*	134717	122906	73482 48774	59.79 39.68
Mi79	*Mytilus chilensis*	Seafood mix	*Mytilus galloprovincialis* *Mytilus edulis*	148773	137122	73035 63249	53.26 46.13
Mi80	*Mytilus chilensis*	Seafood mix	*Mytilus galloprovincialis* *Mytilus edulis*	136695	126608	88130 37970	69.61 29.99
Mi81	not declared	Sea fruit salad	*Mytilus galloprovincialis* *Mytilus edulis*	153499	142736	141578 1022	99.19 0.72
Mi82	*Zygochlamys patagonica* *Chlamys opercularis*	Scallop terrine	*Zygochlamys patagonica*	76554	59181	57329	96.87
Mi83	not declared	Terrine of salmon and great scallop	*Pecten* spp.	96596 [1]	76834	75476	98,23
Mi84	*Mytilus chilensis*	Seafood mix	*Mytilus galloprovincialis* *Mytilus edulis*	163885	150852	124468 25916	82.51 17.18
Mi85	not declared	Instant noodle seafood, mild	*Mytilus galloprovincialis*	15409	14118	13750	97.39
Mi86	not declared	Instant noodle seafood, spicy	*Mytilus galloprovincialis*	9787	8892	8473	95.29
O1	*Crassostrea gigas* [4]	Oyster	*Magallana gigas* [4]	139319 [2]	134073	133493	99.57
O4	not declared	Oyster	*Magallana gigas*	46089 [2]	40991	40279	98.26
O9	not declared	Oyster sauce		not evaluable [3]			
O10	not declared	Oyster sauce		not evaluable [3]			
M11	*Mytilus edulis*	Mussel	*Mytilus galloprovincialis*	23766 [2]	22546	22147	98.23
M14	*Mytilus* spp.	Blue mussel	*Mytilus galloprovincialis* *Mytilus edulis*	126880 [2]	119717	79522 39555	66.42 33.04
M15	*Mytilus* spp	Blue mussel	*Mytilus galloprovincialis*	227678	220699	220226	99.79
M16	*Mytilus edulis*	Bouchot mussel	*Mytilus galloprovincialis*	51292 [2]	49604	48832	98.44
M17	not declared	Grilled blue mussel	*Mytilus galloprovincialis* *Mytilus edulis*	9888 [2]	6750	3956 1998	58.61 29.60
M18	*Mytilus chilensis*	Blue mussel	*Mytilus galloprovincialis*	53710 [2]	51670	50733	98.19
M19	not declared	Blue mussel	*Mytilus galloprovincialis*	57238 [2]	54822	53829	98.19
M20	*Mytilus* spp.	Blue mussel	*Mytilus galloprovincialis*	72113 [2]	69576	68969	99.13
M21	*Mytilus edulis*	Mussel	*Mytilus galloprovincialis*	51328 [2]	49908	49459	99.10
M22	*Mytilus galloprovincialis*	Blue mussel	*Mytilus galloprovincialis* *Mytilus edulis*	115950[2]	110777	109262 1466	98.63 1.32
M24	*Mytilus chilensis*	Blue mussel in tomato sauce	*Mytilus galloprovincialis* *Mytilus edulis*	113942 [2]	107150	94449 12505	88.15 11.67
M28	not declared	Dry cat food with green lipped mussel	*Pecten* spp. *Mytilus galloprovincialis* *Perna canaliculus*	128693 [3]	126380	79764 40450 4712	63.11 32.01 3.73
M35	*Mytilus chilensis*	Mussel in tomato sauce	*Mytilus galloprovincialis*	197899 [3]	190771	189540	99.35
M39	*Mytilus chilensis*	Blue mussel	*Mytilus galloprovincialis* *Mytilus edulis*	182612 [3]	175982	96502 75204	54.84 42.73
M40	*Mytilus edulis*	Blue mussel	*Mytilus galloprovincialis*	182958 [3]	179399	178024	99.23
S41	*Placopecten magellanicus*	Deep-sea scallop	*Placopecten magellanicus*	143794 [2]	132140	131583	99.58
S43	*Pecten maximus*	Great scallop	*Mizuhopecten yessoensis*	122156 [2]	113706	113128	99.49
S44	*Pecten* spp.	Great scallop	*Mizuhopecten yessoensis*	2873135 [2]	2718126	2717426	99.97
S45	*Placopecten magellanicus*	Deep-sea scallop	*Placopecten magellanicus*	111673 [2]	107119	106632	99.55

S48	*Patinopecten yessoensis*	Great scallop/ Yesso scallop	*Mizuhopecten yessoensis*	47397 [2]	41076	407873	99.51
S51	not declared	Great scallop	*Placopecten magellanicus*	51565 [2]	45007	44915	99.80
S52	*Patinopecten yessoensis*	Great scallop	*Mizuhopecten yessoensis*	46673 [2]	39769	39627	99.64
S53	*Pecten* spp.	Great scallop	*Mizuhopecten yessoensis*	42857 [2]	36443	35265	96.77
S54	*Placopecten magellanicus*	Great scallop	*Placopecten magellanicus*	55475 [2]	48703	47915	98.38
S56	not declared	Great scallop	*Placopecten magellanicus*	1268169 [3]	1061137	1060653	99.95
S57	*Placopecten magellanicus*	Great scallop	*Pecten* spp.	174497 [3]	171299	170404	99.48
S60	not declared	Deep-sea scallop	*Placopecten magellanicus*	364474 [3]	350953	350869	99.98
S61	*Patinopecten yessoensis*	Great scallop	*Mizuhopecten yessoensis*	159145 [3]	152930	152849	99.95

[1] Mean of two replicates; [2] samples were analyzed with the MiSeq® instrument; [3] samples were analyzed with the iSeq® instrument; [4] former nomenclature, synonym for *Magallana gigas*.

4. Conclusions

The DNA metabarcoding method developed in this study allows the detection of species of Mytilidae (mussels), Pectinidae (scallops), and Ostreidae (oysters), the most important bivalve families for human consumption. By combining three forward and four reverse primers in a triplex PCR assay, the barcode region, a fragment of mitochondrial 16S rDNA, could be amplified in the species of interest.

The applicability of the novel DNA metabarcoding method was investigated by analyzing individual DNA extracts from eleven reference samples, ten DNA extract mixtures and DNA extracts from 75 commercial food products. In each of the eleven reference samples, the bivalve species was identified correctly. In DNA extract mixtures, not only the main component but also the minor components were detected correctly, with just a few exceptions. The analysis of commercial seafood products showed that the DNA metabarcoding method is applicable to complex and processed foodstuffs, allowing the identification of bivalves in, e.g., marinated form, in sauces, in seafood mixes and even in instant noodle seafood.

The DNA metabarcoding method runs on both the MiSeq® and iSeq® instrument of Illumina. Due to the compatibility of PCR and sequencing parameters, the DNA metabarcoding method can be combined with a DNA metabarcoding method for mammalian and poultry species published recently.

5. Patent

This manuscript has been submitted for grant of a European patent (application number: EP21204456.4).

Supplementary Materials: The following are available online at www.mdpi.com/article/10.3390/foods10112618/s1, Supplementary Table S1: Declaration, origin and processing condition of the 86 food products, Supplementary Table S2: Sequences included into the reverence database, Supplementary Figure S1: Multi-species sequence alignment of the mitochondrial 16S rDNA barcoding region for the bivalve species of interest.

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3. Supplementary Material

Appendix A

Supplementary Table S1 Declaration, origin and processing condition of the 86 commercial food products.

	Declaration on the product			*		
Sample ID	**Scientific/Latin name**	**Product description [Ger]**	**Product description [Eng]**	**Product origin**	**Purchase origin**	**Treatment**
O1	*Crassostrea gigas*	Felsenauster	Giant Oyster	France	delicacy shops	raw
O2	*Ostrea edulis*	Auster	Oyster	Denmark	fish market	raw
O3	*Crassostrea gigas*	Auster / Gillardeau	Oyster / Gillardeau	Denmark	fish market	raw
O4	not declared	Auster	Oyster	Pacific *	delicacy shops	raw
O5	*Crassostrea gigas*	Auster in Sonnenblumenöl	Oyster in sunflower oil	Korea	delicacy shops	processed
O6	*Crassostrea gigas*	Auster in Sonnenblumenöl	Oyster in sunflower oil	Korea	regional supermarket	processed
O7	*Crassostrea gigas*	Auster in Wasser	Oyster in water	Korea	delicacy shops	processed
O8	not declared	Austernsauce	Oyster sauce	Thailand	regional supermarket	processed
O9	not declared	Austernsauce	Oyster sauce	Thailand	delicacy shops	processed
O10	not declared	Austernsauce	Oyster sauce	China	delicacy shops	processed
M11	*Mytilus edulis*	Pfahlmuschel	Mussel	Spain	delicacy shops	processed
M12	*Mytilus galloprovincialis*	Miesmuschel	Blue Mussel	Spain	regional supermarket	frozen
M13	*Perna canaliculus*	Grünlippmuschel	New Zealand green-lipped mussel	New Zealand	delicacy shops	frozen
M14	*Mytilus spp.*	Miesmuschel	Blue Mussel	not declared	not declared	frozen
M15	*Mytilus edulis*	Miesmuschel	Blue Mussel	North Atlantic *	delicacy shops	raw
M16	*Mytilus edulis*	Bouchotmuschel	Bouchot mussel	France	fish market	raw
M17	not declared	Gegrillte Miesmuschel	Grilled blue mussel	Italy	delicacy shops	processed
M18	*Mytilus chilensis*	Miesmuschel	Blue Mussel	Spain	delicacy shops	frozen
M19	not declared	Miesmuschel	Blue Mussel	not declared	fish market	raw
M20	*Mytilus spp.*	Miesmuschel	Blue Mussel	not declared	fish market	raw
M21	*Mytilus edulis*	Muschel	Mussel	Denmark	delicacy shops	processed
M22	*Mytilus galloprovincialis*	Miesmuschel	Blue Mussel	Italy	delicacy shops	processed
M23	not declared	Muschel mit Sherry Essig	Mussel with sherry vinegar	Spain	delicacy shops	processed
M24	*Mytilus chilensis*	Miesmuschel in Tomatensauce	Blue Mussel in tomato sauce	Denmark	regional supermarket	processed
M25	not declared	Pfahlmuschel in Marinaden Sauce	Mussel in marinade sauce	Spain	regional supermarket	processed
M26	not declared	Gegrillte Miesmuschel	Grilled blue mussel	Italy	regional supermarket	processed
M27	*Mytilus chilensis*	Muschel in Marinade	Mussel in marinade	Spain	regional supermarket	processed
M28	not declared	Katzentrockenfutter mit Grünlippmuschel	Dry cat food with green lipped mussel	Germany	delicacy shops	processed
M29	*Mytilus galloprovincialis*	Miesmuschel in Tomatensauce	Blue mussel in tomato sauce	Spain	regional supermarket	processed

M30	*Mytilus galloprovincialis*	Miesmuschel a la mariniere	Blue mussel a la mariniere	Spain	regional supermarket	processed
M31	not declared	Miesmuschel in Bio-Marinade	Blue mussel in organic marinade	Germany	delicacy shops	processed
M32	not declared	Marinierte Miesmuschel	Marinated blue mussels	Spain	delicacy shops	processed
M33	*Mytilus chilensis*	Muschel in Escabeche	Mussel in Escabeche	Denmark	delicacy shops	processed
M34	*Mytilus chilensis*	Muschel	Mussel	Denmark	delicacy shops	processed
M35	*Mytilus chilensis*	Muschel in Tomatensauce	Mussel in tomato sauce	Denmark	delicacy shops	processed
M36	*Mytilus galloprovincialis*	Miesmuschel mariniert	Blue mussel marinated	Germany	delicacy shops	processed
M37	*Mytilus edulis*	Muschel in Honigsenfsauce	Mussel in honey mustard sauce	Denmark	delicacy shops	processed
M38	not declared	Miesmuschel in Marinade	Blue mussel in marinade	Spain	delicacy shops	processed
M39	*Mytilus chilensis*	Miesmuschel	Blue mussel	Germany	not declared	processed
M40	*Mytilus edulis*	Miesmuschel	Blue mussel	Netherlands	not declared	raw
S41	*Placopecten magellanicus*	Tiefseescallop	Deep-sea scallop	France	regional supermarket	cooled
S42	*Mizuhopecten yessoensis*	Japanische Kammmuschel	Yesso scallop	North Atlantic	fish market	cooled
S43	*Pecten maximus*	Jakobsmuschel	Great scallop	Pacific *	delicacy shops	cooled
S44	*Pecten spp.*	Jakobsmuschel	Great scallop	not declared	not declared	frozen
S45	*Placopecten magellanicus*	Tiefseescallop	Deep-sea scallop	not declared	not declared	frozen
S46	*Pecten jacobaeus*	Jakobsmuschel	Great scallop	Croatia	delicacy shops	frozen
S47	*Zygochlamys patagonica*	Jakobsmuschel "á la Bretonne"	Scallop "á la Bretonne"	France	delicacy shops	processed
S48	*Patinopecten yessoensis*	Jakobsmuschel/ Japanische Kammmuschel	Great scallop/ Yesso scallop	Pacific *	fish market	cooled
S49	*Placopecten magellanicus*	Jakobsmuschel	Great scallop	Pacific *	delicacy shops	cooled
S50	*Argopecten purpuratus*	Purpur Kammmuschel	Pacific scallop	Peru	delicacy shops	frozen
S51	not declared	Jakobsmuschel	Great scallop	not declared	fish market	cooled
S52	*Patinopecten yessoensis*	Jakobsmuschel	Great scallop	Pacific *	delicacy shops	cooled
S53	*Pecten sp.*	Jakobsmuschel	Great scallop	not declared	fish market	cooled
S54	*Placopecten magellanicus*	Jakobsmuschel	Great scallop	Pacific *	delicacy shops	cooled
S55	*Aequipecten opercularis*	Kammmuschel in Sauce	Scallop in sauce	Spain	delicacy shops	processed
S56	not declared	Jakobsmuschel	Great scallop	not declared	restaurant	processed
S57	*Placopecten magellanicus*	Jakobsmuschel	Great scallop	Austria	fish market	cooled
S58	not declared	Rillettes de Saint-Jacques	Rillettes de Saint-Jacques	France	delicacy shops	processed
S59	not declared	Kleine Pilgermuschel in galizischer Sauce	Small scallop in galician sauce	Spain	delicacy shops	processed

S60	not declared	Tiefseescallop	Deep-sea scallop	Germany	not declared	frozen
S61	*Patinopecten yessoensis*	Jakobsmuschel	Great scallop	Pacific *	delicacy shops	cooled
Mi62	*Mytilus chilensis*	Meeresfürchte Mischung	Seafood mix	France	regional supermarket	frozen
Mi63	not declared	Sauce mit Meeresfrüchten	Sauce with seafood	Italy	regional supermarket	processed
Mi64	*Mytilus chilensis, Mytilus edulis*	Meeresfrüchte Mischung	Seafood mix	Germany	delicacy shops	processed
Mi65	not declared	Bouillabaise Marseiller Art	Bouillabaise Marsille	Germany	delicacy shops	processed
Mi66	*Mytilus chilensis*	Meeresfrüchte Mischung	Seafood mix	France	regional supermarket	processed
Mi67	*Mytilus spp.*	Meeresfrüchte Mischung	Seafood mix	Chile	regional supermarket	processed
Mi68	*Mytilus galloprovincialis*	Meeresfrüchtesalat in Sonnenblumenöl	Sea fruit salad in sunflower oil	Italy	regional supermarket	processed
Mi69	*Mytilus chilensis*	Meeresfrüchte Mischung	Seafood mix	France	delicacy shops	processed
Mi70	not declared	Meeresfrüchtesalat Fantasie	Sea fruit salad fantasy	Italy	regional supermarket	processed
Mi71	not declared	Meeresfrüchte Mix	Seafood mix	Italy	regional supermarket	processed
Mi72	*Mytilus chilensis*	Meeresfrüchte Mix	Seafood mix	Croatia	delicacy shops	processed
Mi73	not declared	Meeresfrüchte Mix	Seafood mix	not declared	not declared	unknown
Mi74	not declared	Meeresfrüchte Mix	Seafood mix	not declared	not declared	unknown
Mi75	not declared	Pizza Frutti di Mare	Pizza Frutti di mare	Austria	restaurant	processed
Mi76	not declared	Paella	Paella	Germany	delicacy shops	processed
Mi77	*Mytilus edulis, Mytilus chilensis*	Paella	Paella	Germany	regional supermarket	processed
Mi78	*Mytilus chilensis*	Meeresfüchte all´Olio	Seafood all´Olio	France	regional supermarket	processed
Mi79	*Mytilus chilensis*	Meeresfrüchte Mix	Seafood mix	Spain	delicacy shops	processed
Mi80	*Mytilus chilensis*	Meeresfrüchte Mix	Seafood mix	Austria	delicacy shops	processed
Mi81	not declared	Meeresfrüchtesalat	Sea fruit salad	Italy	delicacy shops	processed
Mi82	*Zygochlamys patagonica, Chlamys opercularis*	Jakobsmuschelterrine	Scallop terrine	France	delicacy shops	processed
Mi83	not declared	Terrine vom Lachs und Jakobsmuschel	Terrine of salmon and great scallop	Austria	delicacy shops	processed
Mi84	*Mytilus chilensis*	Meeresfrüchte Mischung	Seafood mix	Germany	delicacy shops	processed
Mi85	not declared	Instant Nudeln Seafood, mild	Instant noodle seafood, mild	Korea	delicacy shops	processed
Mi86	not declared	Instant Nudeln Seafood, scharf	Instant noodle seafood, spicy	Korea	delicacy shops	processed

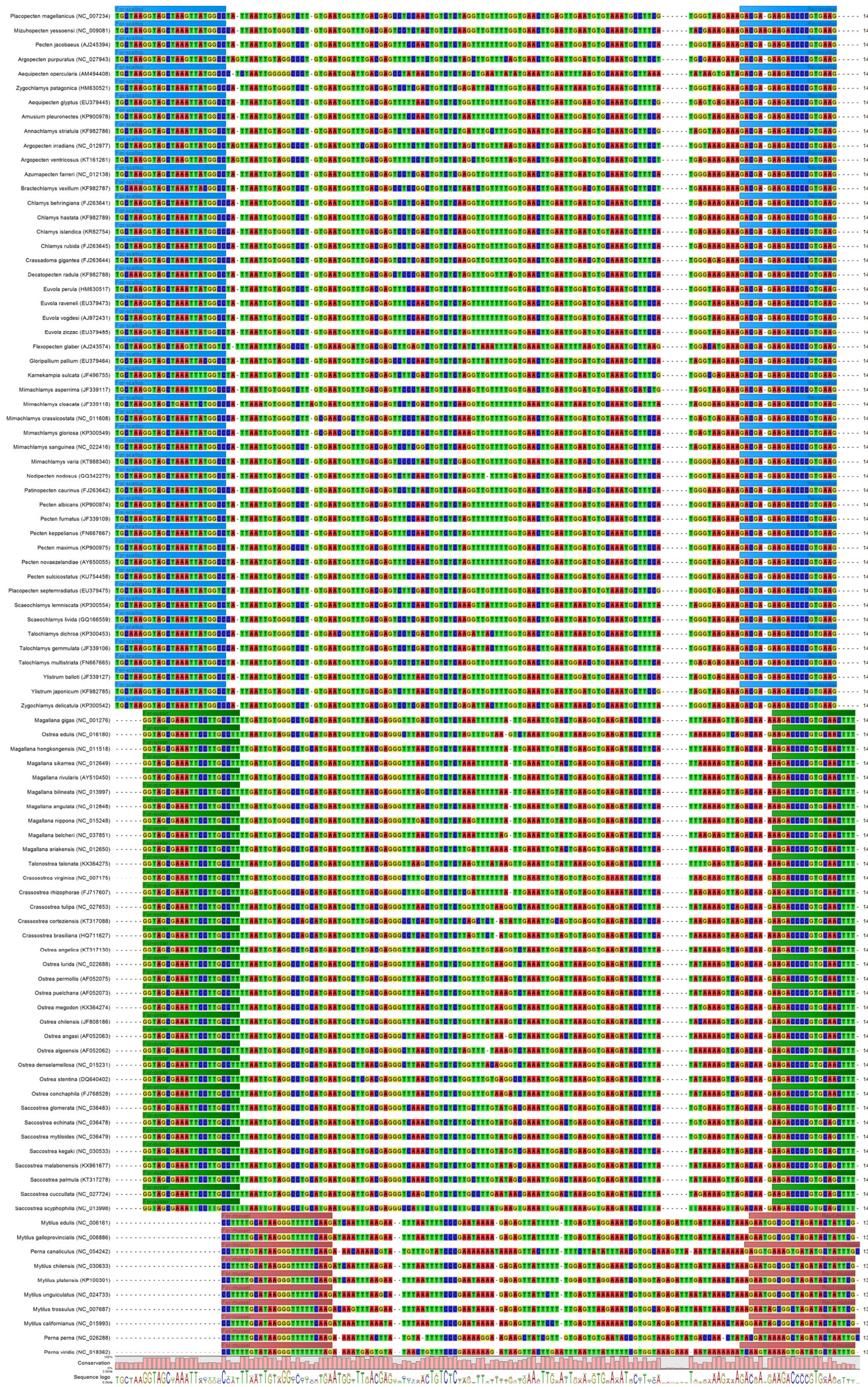
* In case the country of production was unknown, the fishing region was specified

Supplementary Table S2 Sequences included into the reference database.

Scientific name of species	Accession No	Scientific name of species	Accession No
mussels		**scallops**	
Mytilus chilensis	NC_030633	*Euvola vogdesi*	AJ972431
Perna perna	NC_026288	*Mimachlamys crassicostata*	NC_011608
Mytilus unguiculatus	NC_024733	*Gloripallium pallium*	EU379464
Perna viridis	NC_018362	*Flexopecten glaber*	AJ243574
Mytilus californianus	NC_015993	*Pecten jacobaeus*	AJ245394
Mytilus trossulus	NC_007687	*Pecten novaezelandiae*	AY650055
Mytilus galloprovincialis	NC_006886	*Euvola raveneli*	EU379473
Mytilus edulis	NC_006161	*Aequipecten opercularis*	AM494408
Perna canaliculus	NC_054242	*Euvola perula*	HM630517
Mytilus platensis	KP100301	*Nodipecten nodosus*	GQ342275
		Scaeochlamys livida	GQ166559
oysters		*Pecten keppelianus*	FN667667
Magallana bilineata	NC_013997	*Talochlamys multistriata*	FN667665
Magallana gigas	NC_001276	*Patinopecten caurinus*	FJ263642
Crassostrea virginica	NC_007175	*Chlamys behringiana*	FJ263641
Magallana hongkongensis	NC_011518	*Placopecten septemradiatus*	EU379475
Magallana angulata	NC_012648	*Pecten maximus*	KP900975
Magallana sikamea	NC_012649	*Pecten albicans*	KP900974
Magallana ariakensis	NC_012650	*Zygochlamys delicatula*	KP300542
Ostrea denselamellosa	NC_015231	*Chlamys hastata*	KF982789
Magallana nippona	NC_015248	*Ylistrum japonicum*	KF982785
Ostrea edulis	NC_016180	*Pecten fumatus*	JF339109
Ostrea lurida	NC_022688	*Talochlamys gemmulata*	JF339106
Crassostrea tulipa	NC_027653	*Zygochlamys patagonica*	HM630521
Ostrea angasi	AF052063	*Argopecten purpuratus*	NC_027943
Magallana belcheri	NC_037851	*Argopecten irradians*	NC_012977
Crassostrea rhizophorae	FJ717607	*Azumapecten farreri*	NC_012138
Crassostrea brasiliana	HQ711627	*Mizuhopecten yessoensi*	NC_009081
Talonostrea talonata	KX364275	*Placopecten magellanicus*	NC_007234
Crassostrea corteziensis	KT317088	*Euvola ziczac*	EU379485
Magallana rivularis	AY510450	*Pecten sulcicostatus*	KU754458
Ostrea angelica	KT317130	*Chlamys islandica*	KR827548
Ostrea permollis	AF052075	*Argopecten ventricosus*	KT161261
Ostrea chilensis	JF808186	*Mimachlamys varia*	KT988340
Ostrea algoensis	AF052062	*Amusium pleuronectes*	KP900978
Ostrea puelchana	AF052073	*Mimachlamys sanguinea*	NC_022416
Ostrea megodon	KX364274	*Talochlamys dichroa*	KP300543
Saccostrea cuccullata	NC_027724	*Mimachlamys gloriosa*	KP300549
Saccostrea palmula	KT317278	*Mimachlamys cloacata*	JF339118
Saccostrea malabonensis	KX961677	*Mimachlamys asperrima*	JF339117
Saccostrea scyphophilla	NC_013998	*Annachlamys striatula*	KF982786

Saccostrea glomerata	NC_036483	*Decatopecten radula*	KF982788
Saccostrea kegaki	NC_030533	*Bractechlamys vexillum*	KF982787
Saccostrea echinata	NC_036478	*Aequipecten glyptus*	EU379445
Saccostrea mytiloides	NC_036479	*Scaeochlamys lemniscata*	KP300554
		Chlamys rubida	FJ263645
		Karnekampia sulcata	JF496755
		Crassadoma gigantea	FJ263644
		Ylistrum balloti	JF339127

Supplementary Figure S1 Multi-species sequence alignment of the mitochondrial 16S rDNA barcoding region for the bivalve species of interest. Colored bars indicate the binding sites of the primer sets for scallops (blue), oysters (green) and mussels (red, CLC Genomics Workbench 10.1.1 (Qiagen)).



Placopecten magellanicus (NC_007234)
Mizuhopecten yessoensi (NC_009081)
Pecten jacobaeus (AJ245394)
Argopecten purpuratus (NC_027943)
Aequipecten opercularis (AM494408)
Zygochlamys patagonica (HM630521)
Aequipecten glyptus (EU379445)
Amusium pleuronectes (KP900978)
Annachlamys striatula (KF982786)
Argopecten irradians (NC_012977)
Argopecten ventricosus (KT161261)
Azumapecten farreri (NC_012138)
Bractechlamys vexillum (KF982787)
Chlamys behringiana (FJ263641)
Chlamys hastata (KF982789)
Chlamys islandica (KR82754)
Chlamys rubida (FJ263645)
Crassadoma gigantea (FJ263644)
Decatopecten radula (KF982788)
Euvola perula (HM630517)
Euvola raveneli (EU379473)
Euvola vogdesi (AJ972431)
Euvola ziczac (EU379485)
Flexopecten glaber (AJ243574)
Gloripallium pallium (EU379464)
Karnekampia sulcata (JF496755)
Mimachlamys asperrima (JF339117)
Mimachlamys cloacata (JF339118)
Mimachlamys crassicostata (NC_011608)
Mimachlamys gloriosa (KP300549)
Mimachlamys sanguinea (NC_022416)
Mimachlamys varia (KT988340)
Nodipecten nodosus (GQ342275)
Patinopecten caurinus (FJ263642)
Pecten albicans (KP900974)
Pecten fumatus (JF339109)
Pecten keppelianus (FN667667)
Pecten maximus (KP900975)
Pecten novaezelandiae (AY650055)
Pecten sulcicostatus (KU754458)
Placopecten septemradiatus (EU379475)
Scaeochlamys lemniscata (KP300554)
Scaeochlamys livida (GQ166559)
Talochlamys dichroa (KP300453)
Talochlamys gemmulata (JF339106)
Talochlamys multistriata (FN667665)
Ylistrum balloti (JF339127)
Ylistrum japonicum (KF982785)
Zygochlamys delicatula (KP300542)
Magallana gigas (NC_001276)
Ostrea edulis (NC_016180)
Magallana hongkongensis (NC_011518)
Magallana sikamea (NC_012649)
Magallana rivularis (AY510450)
Magallana bilineata (NC_013997)
Magallana angulata (NC_012648)
Magallana nippona (NC_015248)
Magallana belcheri (NC_037851)
Magallana ariakensis (NC_012650)
Talonostrea talonata (KX364275)
Crassostrea virginica (NC_007175)
Crassostrea rhizophorae (FJ717607)
Crassostrea tulipa (NC_027653)
Crassostrea corteziensis (KT317088)
Crassostrea brasiliana (HQ711627)
Ostrea angelica (KT317130)
Ostrea lurida (NC_022688)
Ostrea permollis (AF052075)
Ostrea puelchana (AF052073)
Ostrea megodon (KX364274)
Ostrea chilensis (JF808186)
Ostrea angasi (AF052063)
Ostrea algoensis (AF052062)
Ostrea denselamellosa (NC_015231)
Ostrea stentina (DQ640402)
Ostrea conchaphila (FJ768528)
Saccostrea glomerata (NC_036483)
Saccostrea echinata (NC_036478)
Saccostrea mytiloides (NC_036479)
Saccostrea kegaki (NC_030533)
Saccostrea malabonensis (KX961677)
Saccostrea palmula (KT317278)
Saccostrea cucullata (NC_027724)
Saccostrea scyphophilla (NC_013998)
Mytilus edulis (NC_006161)
Mytilus galloprovincialis (NC_006886)
Perna canaliculus (NC_054242)
Mytilus chilensis (NC_030633)
Mytilus platensis (KP100301)
Mytilus unguiculatus (NC_024733)
Mytilus trossulus (NC_007687)
Mytilus californianus (NC_015993)
Perna perna (NC_026288)
Perna viridis (NC_018362)
Conservation
Sequence logo

4. Further systems

In the following, systems are described in slightly more detail that have not been published yet but were part of the development and patent (in the following chapter described in more detail). The state of development of the different systems varies. These systems will be published in a renowned journal after further experiments and validation. The approach and results were very similar to the published metabarcoding systems of bivalve families Mytilidae (mussels), Pectinidae (scallops), and Ostreidae (oysters). Therefore, a lot of information refers to the already published metabarcoding system, as the development of all systems was conducted in parallel and simultaneously.

4.1 Sampling

For the identification of crustaceans, molluscs, land snails, and Venus shells samples were collected from regional supermarkets, fish markets and delicacy shops in Austria from summer 2018 to winter 2020, as described in the publication of Gense et al. [95] for bivalves metabarcoding system. All samples were given an individual number (ID number), with the letter “Cr” referring to crustaceans, “Ce” for cephalopods, “Sn” for land snails, “Ve” for Venus shells, and “Mi” to mixed-species seafood (Table 1). The food products were either fresh, deep-frozen, or in processed condition. After sampling the seafood product samples were stored at - 20°C until extraction.

Out of 126 samples, 38 samples were used as reference samples; eighteen for crustaceans, eleven for cephalopods, three for land snails, and six for Venus shells (Table 2). The reference samples were verified by subjecting DNA extracts to Sanger sequencing (Microsynth, Balgach, Switzerland). The obtained sequences were checked against the public databases provided by the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA). For Sanger sequencing, the newly designed forward and reverse primers listed in Table 3 were applied.

Table 1 Declaration, origin and processing condition of the 126 commercial seafood products.

ID Number	Declaration on the product Scientific/Latin name	Product description [Ger]	Product description [Eng]	Purchase origin	Treatment
Cr1	*Nephrops norvegicus*	Kaisergranat/ Scampi	Norway lobster	delicacy shops	frozen
Cr2	*Procambarus clarkii*	Louisiana Flusskrebs	Louisiana red crayfish	fish market	cooled
Cr3	*Panulirus argus*	Karibik-Languste	Caribbean spiny lobster	fish market	cooled
Cr4	*Homarus americanus*	Amerikanischer Hummer	American lobster	fish market	frozen
Cr5	*Callinectes* spp.	Blaukrabbe	Blue crab	delicacy shops	processed
Cr6	*Paralithodes camtschaticus*	Kamtschatkakrabbe/ Königskrabbe	Red king crab	fish market	frozen
Cr7	*Chionoecetes opilio*	Schneekrabbe	Snow crab	delicacy shops	frozen
Cr8	*Homarus gammarus*	Europäischer Hummer	European lobster	fish market	frozen
Cr9	*Pleoticus muelleri*	Argentinische Rotgarnele	Argentine red shrimp	regional supermarket	frozen
Cr10	*Penaeus monodon*	(Black) Tiger Garnele	Giant tiger prawn	regional supermarket	processed
Cr11	*Macrobrachium rosenbergii*	Rosenberg-Süßwassergarnele	Giant river prawn	fish market	cooled
Cr12	*Pandalus borealis*	Kaltwassergarnele	Northern prawn	regional supermarket	cooled
Cr13	*Crangon crangon*	Nordseekrabbe	Brown shrimp	fish market	cooled
Cr14	*Litopenaeus vannamei*	White Tiger Garnele	White shrimp	regional supermarket	frozen
Cr15	*Macrobrachium rosenbergii*	Rosenberg-Süßwassergarnele	Giant river prawn	delicacy shops	cooled
Cr16	*Litopenaeus vannamei*	White Tiger Garnele	White shrimp	regional supermarket	frozen
Cr17	*Metapenaeus monoceros*	Garnele	Speckled shrimp	delicacy shops	frozen
Cr18	*Heterocarpus reedi*	Garnele	Chilean nylon shrimp	delicacy shops	cooled
Cr19	*Penaeus monodon*	Tiger- Garnele	Giant tiger prawn	not declared	frozen
Cr20	*Macrobrachium rosenbergii*	Rosenberg-Süßwassergarnele	Giant river prawn	not declared	frozen
Cr21	*Penaeus monodon*	Bio Black Tiger Garnele	Tiger shrimp	not declared	frozen
Cr22	*Crangon crangon*	Nordseekrabbe	Brown shrimp	fish market	cooled
Cr23	*Crangon crangon*	Nordseekrabbe	Brown shrimp	fish market	cooled
Cr24	*Aristaeopsis edwardsiana*	Carabineros	Giant scarlet shrimp	delicacy shops	cooled
Cr25	*Pleoticus muelleri*	Argentinische Rotgarnele	Argentine red shrimp	regional supermarket	frozen
Cr26	*Penaeus occidentalis*	Pazifische Garnele	Western white shrimp	fish market	frozen
Cr27	*Penaeus notialis*	Weiße Garnele	Southern pink shrimp	fish market	frozen
Cr28	*Penaeus borealis*	Grönlandgarnele	Northern prawn	fish market	frozen
Cr29	Dendrobranchiata	Rote Garnele	Red shrimp	not declared	processed
Cr30	*Metapenaeus monoceros*	Garnele	Speckled shrimp	delicacy shops	frozen
Cr31	*Penaeus merguiensis* *Metapenaeus ensis*	Bio Garnele	Bio shrimp	regional supermarket	frozen

Cr32	*Litopenaeus vannamei*	Cocktail-Garnele	Cocktail shrimp	regional supermarket	frozen
Cr33	*Crangon crangon*	Nordseekrabbe	Brown shrimp	regional supermarket	frozen
Cr34	*Litopenaeus vannamei*	Garnelenkranz	Shrimp wreath	delicacy shops	cooled
Cr35	*Litopenaeus vannamei*	Pacific Pawan	Pacific Pawan	delicacy shops	frozen
Cr36	*Litopenaeus vannamei*	Pacific Pawan	Pacific Pawan	delicacy shops	frozen
Cr37	Not declared	Shrimpcocktail Florida	Shrimp cocktail Florida	fish market	processed
Cr38	Not declared	Hummersuppe	Lobster soup	fish market	processed
Cr39	Not declared	Krabbensuppe	Crab soup	fish market	processed
Cr40	*Pandalus borealis*	Eismeerkrabben	Northern prawn	regional supermarket	processed
Cr41	*Paralomis granulosa*	Krabbenfleisch	Crab met	delicacy shops	processed
Cr42	*Cancer Pagurus*	Krabbencreme	Crab cream	delicacy shops	processed
Cr43	*Portunus* spp.	Schwimmkrabbenfleisch	Swim crab met	delicacy shops	processed
Cr44	Not declared	Hummerbutter	Lobster butter	delicacy shops	processed
Cr45	*Penaeus merguiensis*	Hanami Pawan Cracker	Hanami Pawan cracker	delicacy shops	processed
Cr46	*Portunus pelagicus*	Krabbenfleisch	Crab cream	delicacy shops	processed
Cr47	Not declared	Ramen mit Garnele	Ramen with prawns	delicacy shops	processed
Cr48	Dendrobranchiata	Dried baby shrimps	Dried baby shrimps	delicacy shops	processed
Cr49	Dendrobranchiata	Dried Shrimps	Dried Shrimps	delicacy shops	processed
Cr50	Not declared	Krabbensuppe	Crab soup	delicacy shops	processed
Cr51	Not declared	Hummer-Rahm-Suppe	Lobster cream soup	delicacy shops	processed
Cr52	*Homarus americanus* *Pandalus borealis*	Hummerfond	Lobster fond	delicacy shops	processed
Cr53	*Litopenaeus vannamei*	Garnele	Shrimp	delicacy shops	frozen
Cr54	*Litopenaeus vannamei*	Garnele	Shrimp	delicacy shops	frozen
Cr55	*Homarus americanus*	Hummer	Lobster	delicacy shops	frozen
Ce1	*Loligo edulis*	Kalmar	Squid	delicacy shops	frozen
Ce2	*Octopus vulgaris*	Oktopus	Octopus	fish market	cooled
Ce3	*Loligo chinensis*	Kalmar	Squid	fish market	cooled
Ce4	*Loligo duvaucii*	Kalmar	Squid	regional supermarket	frozen
Ce5	*Sepiella japonica*	Sepia	Sepia	regional supermarket	frozen
Ce6	*Octopus vulgaris*	Oktopus	Octopus	delicacy shops	cooled
Ce7	*Sepia officinalis*	Tintenfisch	Cuttlefish	not declared	frozen
Ce8	*Loligo vulgaris*	Kalmar	Squid	not declared	frozen
Ce9	*Octopus aegina*	Oktopus	Sand bird octopus	delicacy shops	frozen
Ce10	*Loligo opalescens*	Kalmar	Squid	not declared	frozen
Ce11	*Sepiella inermis*	Sepia	Sepia	not declared	frozen
Ce12	*Octopus maya*	Oktopus	Octopus	not declared	frozen
Ce13	*Sepiella inermis*	Sepia	Sepia	not declared	frozen

Ce14	*Loligo edulis*	Kalmar	Squid	not declared	frozen
Ce15	*Eledone moschata*	Moscardini (Moschuskrake)	Musky octopus	fish market	processed
Ce16	*Loligo chinensis*	Kalmar	Squid	delicacy shops	cooled
Ce17	*Loligo gahi*	Kalmar	Squid	delicacy shops	cooled
Ce18	Not declared	Kalmar	Squid	regional supermarket	processed
Ce19	Not declared	Octopus Carpaccio	Octopus carpaccio	delicacy shops	processed
Ce20	*Uroteuthis duvaucii*	Tintenfischtuben	Cuttlefish tubes	delicacy shops	frozen
Ce21	*Dosidicus gigas*	Tintenfisch	Cuttlefish	delicacy shops	processed
Ce22	*Dosidicus gigas*	Tintenfisch	Cuttlefish	delicacy shops	processed
Ce23	*Dosidicus gigas*	Tintenfisch	Cuttlefish	delicacy shops	processed
Ce24	Not declared	Tintenfisch	Cuttlefish	delicacy shops	processed
Ce25	*Octopus membranaceus*	Moschuskrake	Musky octopus	regional supermarket	processed
Ce26	Not declared	Kalamari Ringe	Calamari rings	not declared	processed
Sn1	*Helix lucorum*	Weinbergschnecke	Roman snail	regional supermarket	frozen
Sn2	Not declared	Achatschnecke	Agate snail	not declared	frozen
Sn3	*Helix lucorum*	Weinbergschnecke	Roman snail	regional supermarket	frozen
Sn4	Not declared	Schneckenkapsel	snail capsule	not declared	processed
Sn5	Not declared	Weinbergschnecke	Roman snail	delicacy shops	processed
Sn6	Not declared	Weinbergschnecke	Roman snail	delicacy shops	processed
Sn7	Not declared	Achatschnecke	Agate snail	delicacy shops	processed
Ve1	*Ensis ensis*	Schwertmuschel	common razor clam	delicacy shops	cooled
Ve2	*Ruditapes philippinarum*	Venusmuschel	Venus shell	delicacy shops	cooled
Ve3	Not declared	Herzmuschel	Cockle	delicacy shops	processed
Ve4	*Meretrix lyrata*	Venusmuschel	Venus shell	delicacy shops	frozen
Ve5	*Callista Chione*	Venusmuschel	Venus shell	not declared	frozen
Ve6	*Solen marginatus*	Schwertmuschel	common razor clam	delicacy shops	cooled
Ve7	*Ruditapes philippinarum*	Venusmuschel	Venus shell	delicacy shops	cooled
Ve8	*Cerastoderma edule*	Herzmuschel	Cockle	fish market	cooled
Ve9	*Chamelea gallina*	Venusmuschel	Venus shell	fish market	cooled
Ve10	*Cerastoderma edule*	Herzmuschel	Cockle	delicacy shops	cooled
Ve11	*Ruditapes philippinarum*	Schwertmuschel	carpet shell	delicacy shops	cooled
Ve12	*Ensis directus*	Meerscheide	common razor clam	fish market	cooled
Ve13	*Venus spp.*	Venusmuschel	Venus shell	fish market	cooled
Ve14	*Meretrix lyrata*	Venusmuschel	Venus shell	delicacy shops	frozen
Ve15	Not declared	Venusmuschel	Venus shell	delicacy shops	processed
Ve16	Not declared	Vongole	Vongole	fish market	cooled

Mi62	*Litopenaeus vannamei* *Pleoticus muelleri* *Uroteuthis duvaucii* *Dosidicus gigas* *Sepia pharaonis* *Sepia aculeata* *Illex argentinus* *Illex illecebrosus* *Nototodarus sloanii*	Meeresfrüchte Mix	Seafood Mix	regional supermarket	frozen
Mi63	Not declared	Sauce mit Meeresfrüchten	Sauce with seafood	regional supermarket	processed
Mi64	*Litopenaeus vannamei* *Mytilus chilensis* *Mytilus edulis* *Ilex argentinus*	Meeresfrüchte Mix	Seafood Mix	delicacy shops	processed
Mi65	Not declared	Suppe Bouillabaisse Marseille Art	Soup Bouillabaisse Marseille style	delicacy shops	processed
Mi66	*Litopenaeus vannamei* *Mytilus chilensis* *Ilex argentinus*	Meeresfrüchte Mix	Seafood Mix	regional supermarket	processed
Mi67	*Mytilus spp.* *Loligo gahi,* *Litopenaeus vannamei*	Meeresfrüchte Mix	Seafood Mix	regional supermarket	processed
Mi68	*Dosidicus gigas* *Octopus membranaceus* *Mytilus galloprovincialis* *Litopenaeus vannamei*	Meeresfrüchtesalat in Sonnenbluemenöl	Sea fruit salad in sunflower oil	regional supermarket	processed
Mi69	*Mytilus chilensis* *Solenocera melantho* *Loligo duvaucii* *Uroteuthis chinensis* *Spisula subtruncata*	Meeresfrüchte Mix	Seafood Mix	delicacy shops	processed
Mi70	Not declared	Meeresfrüchtesalat Fantasia	Sea fruit salad fantasy	regional supermarket	processed
Mi71	Not declared	Meeresfrüchte Mix	Seafood Mix	regional supermarket	processed
Mi72	*Loligo gahi* *Mytilus chilensis* *Solenocera melantho* *Paphia undulata*	Meeresfrüchte Mix	Seafood Mix	delicacy shops	processed
Mi73	Not declared	Meeresfrüchte Mix	Seafood Mix	not declared	unknown
Mi74	Not declared	Meeresfrüchte Mix	Seafood Mix	not declared	unknown
Mi75	Not declared	Pizza Frutti di mare	Pizza Frutti di mare	restaurant	processed
Mi76	Not declared	Paella	Paella	delicacy shops	processed
Mi77	*Mytilus edulis* *Mytilus chilensis* *Penaeidae*	Paella	Paella	regional supermarket	processed
Mi78	*Mytilus chilensis,* *Illex illecebrosus,* *Litopenaeus vannamei*	Meeresfrüchte all 'Olio	Seafood all 'Olio	regional supermarket	processed
Mi79	*Mytilus chilensis* *Paphia undulata* *Penaeus vannamei*	Meeresfrüchte Mix	Seafood Mix	delicacy shops	processed

Mi80	*Mytilus chilensis* *Metapenaeus monoceros* *Loligo duvaucii* *Sepia aculeata* *Paphia undulata*	Meeresfrüchte Mix	Seafood Mix	delicacy shops	processed
Mi84	*Litopenaeus vannamei* *Uroteuthis chinensis* *Octopus vulgaris* *Mytilus chilensis*	Meeresfrüchte Mix	Seafood Mix	delicacy shops	processed
Mi85	Not declared	Instant Nudeln Seafood, mild	Instant noodle seafood, mild	delicacy shops	processed
Mi86	Not declared	Instant Nudeln Seafood, scharf	Instant noodle seafood, spicy	delicacy shops	processed

Table 2 Seafood species used for development of DNA metabarcoding system.

Scientific name	Commercial name (German)	Commercial name (English)
	Krustentier	Crustacean
Nephrops norvegicus	Norwegischer Hummer	Norway lobster
Homarus americanus	Amerikanischer Hummer	American lobster
Homarus gammarus	Europäischer Hummer	European lobster
Panulirus argus	Karibik-Languste	Caribbean spiny lobster
Procambarus clarkii	Louisianakrebs	Louisiana crayfish
Callinectes spp.	Blaukrabbe	Blue crab
Paralithodes camtschaticus	Kamtschatkakrabbe	Red king crab
Monomia gladiator	Blaukrabbe	Gladiator swimming crab
Chionoecetes spp.	Schneekrabbe	Snow crab
Crangon crangon	Nordseekrabbe	Brown shrimp
Pleoticus muelleri	Argentinische Rotgarnele	Argentine red shrimp
Penaeus monodon	schwarze Tigergarnele	Giant tiger prawn
Macrobrachium rosenbergii	Rosenberg Süßwassergarnele	Giant river prawn
Pandalus borealis	Eismeergarnele	Northern prawn
Penaeus vannamei	Weißbein-Garnele	Whiteleg shrimp
Heterocarpus spp.	Garnele	Chilean nylon shrimp
Aristaeopsis edwardsiana	Atlantische rote Riesengarnele	Scarlet shrimp
Penaeus duorarum	Nördliche rosa Garnele	Northern pink shrimp
	Tintenfisch	Cephalopod
Octopus vulgaris	Gemeiner Krake	Common octopus
Uroteuthis (Photololigo) chinensis	Kalmar	Mitre squid
Uroteuthis (Photololigo) duvaucii	Kalmar	Indian squid
Sepia pharaonis	Pharao-Tintenfisch	Pharaoh cuttlefish
Octopus cyanea	Großer blauer Krake	Cyane's octopus
Todarodes pacificus	Japanischer Flugkalmar	Japanese flying squid

Amphioctopus aegina	Oktopus	Sanbird octopus
Doryteuthis opalescens	Schließaugenkalmar	Opalescent inshore squid
Sepiella inermis	Stachellose Sepiette	Spineless cuttlefish
Octopus maya	Mexikanischer Vieraugenkrake	Mexican four-eyed octopus
Doryteuthis (Amerigo) gahi	Kalmar	Patagonian squid
	Landschnecke	Land snail
Helix lucorum	Gestreifte Weinbergschnecke	Turkish snail
Helix promatia	Weinbergschnecke	Roman snail/ Burgundy snail
Achatina reticulata	Achatschnecke	African land snail
	Venusmuschel	Venus shell
Ensis spp.	Schwertmuschel	Sword razor
Ruditapes philippinarum	Japanische Teppichmuschel	Manila clam
Meretrix lyrata	Weiße Venusmuschel	White clam
Callista chione	Braune Venusmuschel	Brown callista
Cerastoderma edule	Gemeine Herzmuschel	Common cockle
Chamelea gallina	Gemeine Venusmuschel	Striped Venus Clam

Table 3 Primers designed in this study for different seafood species.

Name	Sequence 5´->3´
Crustacean	
For1_Cr	GGGGGACGATAAGACCCTATAAA
For2_Cr	TGGGAAGACAAGACCCTATAAA
Rev_Cr	ATTACGCTGTTATCCCTAAAGTAACTT
Cephalopod	
For_Ce	GGGACGAGAAGACCCTAWTGA *
Rev_Ce	ATTACGCTGTTATCCCTATGGTAACT
Land Snail	
For_Sn	TGACTGTGCAAAGGTAGCATAAT
Rev1_Sn	AAGTTTCTAGGGTCTTCTCGTCT
Rev2_Sn	AAACTCTAAGGGTCTTCTCGTCT
Venus shell	
For1_Ve	TTGGCCTTTAATTGGGGTCC
For2_Ve	GGTAGCGCGATAATTTGTCTCTTAA
For3_Ve	CTTAATTGGAGAAGGGTATGAATGG
For4_Ve	GTTTAACGGCCGCAGTTGTC
For5_Ve	TACCGCAGGGATAACAGCG
Rev1_Ve	GCTCGACAGGGTCTTCTCGTCT

Rev2_Ve	CAAAAATTCAGGTTTTTCACTTG
Rev3_Ve	TCATGTAAGAAATTAAAAAACGAACAG
Overhang	
Forward	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
Reverse	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG

* W stands for nucleotide A and T

4.2 DNA Extraction

The DNA extraction and quantification were done as described in Gense et al. [95]. To validate all systems, a dilution and series of mixtures should also be prepared as described earlier [95].

4.3 Reference Sequence

As a DNA barcode system for crustacean and cephalopods a 220 bp, for land snails a 155 bp, and for Venus shells a 140 bp fragment of mitochondrial 16S rDNA gene was taken under the term described before [95]. Reference sequences were handled as described previously. All 1034 resulting reference sequences are listed in Table 4. The primer system, library preparation (including die PCR conditions), sequencing process, NGS data analysis by using Galaxy software were applied in the same way as mentioned in the publication for the bivalve metabarcoding system. Although not all samples could be analysed with Galaxy software yet, this is one part of further research.

Table 4 Sequences included into the reference database.

Accession No	Scientific name of species	Accession No	Scientific name of species	Accession No	Scientific name of species
Crustacean		Crustacean		Crustacean	
AB058620	Varuna litterata	NC_039671	Panulirus argus	GQ302745	Heterocarpus gibbosus
AF107616	Hemisquilla ensigera	NC_039112	Munida isos	JX403852	Funchalia villosa
AF107615	Gonodactylus smithii	NC_044425	Scyllarides squammosus	JX403847	Hemipenaeus carpenter
AF107611	Pullosquilla thomassini	NC_016925	Cambaroides similis	JX403849	Mesopenaeus tropicalis
AF107609	Chorisquilla trigibbosa	NC_037695	Charybdis bimaculata	JF899809	Pelagopenaeus balboae
MT335860	Chionoecetes opilio	NC_033507	Cambarus robustus	JF899806	Penaeus hathor
AB220027	Chionoecetes opilio	NC_039640	Thalamita sima	JF899805	Metapenaeopsis provocatoria
AB188686	Telmessus acutidens	NC_024438	Thranita crenata	NC_039154	Aristeus virilis
AB236927	Lithodes aequispinus	NC_033508	Orconectes luteus	KJ486492	Aristeus alcock

AB248090	Panulirus echinatus	NC_030768	Orconectes punctimanus	KF983532	Penaeus aztecus
AF192870	Jasus paulensis	NC_029721	Orconectes sanbornii	KF023186	Heterocarpus abulbus
AF192869	Jasus caveorum	NC_024029	Cherax crassimanus	JX403862	Penaeus setiferus
AF175246	Parastacus pilimanus	NC_023482	Cherax preissii	JX403860	Cerataspis monstrosus
MG551495	Parastacus brasiliensis	KF051309	Munida spinosa	JX403857	Pleoticus robustus
AF175243	Parastacus defossus	MK847971	Munida asprosoma	JX403854	Aristaeopsis edwardsiana
AF175233	Parastacus nicoleti	MK847969	Munida leagora	JX403853	Solenocera necopina
AF133678	Gonodactylus graphurus	AY351105	Munida alonsoi	KP059272	Parapenaeus indicus
AF192873	Jasus lalandii	AY351177	Munida taenia	KP059270	Parapenaeus cayrei
AF192872	Jasus paulensis	MK847957	Munida gordoae	KP059273	Parapenaeus fissurus
AF425333	Lopholithodes mandtii	AY351190	Munida zebra	KP059274	Parapenaeus investigatoris
AF425331	Lithodes santolla	AY351119	Munida distiza	KP059271	Parapenaeus fissuroides
AF425330	Lithodes maja	AY351158	Munida psamathe	KP059269	Parapenaeus australiensis
AF337979	Jasus edwardsii	AY351181	Munida thoe	KP059267	Parapenaeus americanus
AF337976	Panulirus regius	AY351130	Munida guttata	KP059268	Parapenaeus ruberoculatus
AF337973	Panulirus pascuensis	AY351170	Munida stia	NC_040855	Heterocarpus ensifer
AF337969	Panulirus laevicauda	AY351150	Munida ommata	KP059284	Kishinouyepenaeopsis cornuta
AF337964	Panulirus gracilis	MK847958	Munida roshanei	KP059283	Parapenaeus sextuberculatus
AF337963	Panulirus guttatus	MK847944	Munida compressa	KP059281	Parapenaeus perezfarfantae
AF337960	Panulirus inflatus	AY351113	Munida clinata	KP059280	Parapenaeus murrayi
AF339156	Panulirus femoristriga	MK847964	Munida chydaea	KP059278	Parapenaeus longipes
AY227446	Chionoecetes bairdi	MK847941	Munida compacta	KP059276	Parapenaeus lanceolatus
AF425339	Paralomis granulosa	AY351122	Munida eclepsis	KP059275	Parapenaeus kensleyi
AF502949	Scyllarus arctus	AY351185	Munida tyche	KT372710	Heterocarpus chani
AF502955	Palinurus elephas	MK847953	Munida philippinensis	KP725531	Heterocarpus woodmasoni
AJ784020	Episesarma mederi	AY351107	Munida armilla	KP725530	Heterocarpus sibogae
NC_033504	Austropotamobius torrentium	KY230470	Munida mesembria	KP725528	Heterocarpus dorsalis
AM410525	Cycloachelous granulatus	AY351166	Munida spilota	KP721230	Metapenaeopsis andamanensis
AY151824	Eriocheir recta	KY230468	Munida benguela	KP725527	Heterocarpus corona
AY351244	Cervimunida johni	MK847934	Munida endeavourae	KP721226	Metapenaeopsis coniger
DQ388058	Achelous floridanus	MK847961	Munida agave	KP081666	Macrobrachium idella
DQ388053	Portunus sayi	MK458566	Munida idyia	KX162742	Trachysalambria brevisuturae
DQ388054	Portunus anceps	AY351143	Munida militaris	KX162734	Trachysalambria aspera
DQ377978	Palinurus gilchristi	MF490158	Munida flinti	KX162733	Trachysalambria albicoma
DQ377977	Palinurus mauritanicus	AY351115	Munida congesta	NC_040987	Euphausia superba
DQ377975	Palinurus charlestoni	AY351163	Munida rubridigitalis	KU133278	Solenocera hextii
AY947836	Pseudosquilla ciliata	KF182521	Munida iris	KU133288	Hymenopenaeus equalis
AY351259	Pleuroncodes monodon	MF490159	Munida microphthalma	KT959496	Rimapenaeus constrictus
DQ388060	Portunus ventralis	AY351164	Munida rufiantennulata	KT952497	Crangon crangon

DQ388061	Achelous spinicarpus	KF182522	Munida pusilla	KX162771	Trachypenaeus anchoralis
DQ407681	Callinectes toxotes	KY230472	Munida remota	KX162770	Megokris pescadoreensis
DQ407680	Callinectes danae	AY351141	Munida leptosyne	KX162768	Trachysalambria longipes
DQ407679	Callinectes ornatus	AY351162	Munida rosula	KX162764	Trachysalambria starobogatovi
DQ407678	Callinectes marginatus	LC464553	Munida munin	KX162758	Trachysalambria nansei
DQ407677	Callinectes affinis	JN800548	Munida valida	KX162757	Trachysalambria malaiana
DQ407673	Callinectes rathbunae	AY351153	Munida proto	KX162755	Trachysalambria dentata
DQ407674	Callinectes bocourti	AY351142	Enriquea leviantennata	KX162749	Trachysalambria parvispina
DQ407672	Callinectes similis	LC430735	Munida multilineata	LC464546	Crangon uritai
DQ407671	Callinectes bellicosus	AY351152	Munida pagesi	LC341266	Pandalus borealis
DQ407669	Callinectes arcuatus	KY230475	Munida stomifera	LC150203	Metapenaeus monoceros
EU186116	Metanephrops armatus	AF436050	Munida quadrispina	LC121756	Pandalus nipponensis
EU186114	Metanephrops mozambicus	AY351183	Munida tiresias	KY419832	Hadropenaeus lucasii
EU186115	Metanephrops japonicus	AY351159	Munida psylla	KY316150	Ganjampenaeopsis uncta
EU186113	Metanephrops andamanicus	MK847965	Munida heteracantha	KX530803	Solenocera annectens
EU186112	Metanephrops velutinus	FJ462645	Paralomis formosa	KX574332	Solenocera melantho
EU186111	Metanephrops sagamiensis	FJ462646	Paralomis spinosissima	MH045067	Parapenaeopsis stylifera
EU186109	Metanephrops binghami	KY426326	Paralomis birsteini	MG772559	Penaeus japonicus
EF599137	Parastacus pugnax	KY426327	Paralomis hirtella	MG001109	Penaeus brevirostris
EF060259	Paranephrops zealandicus	MF460387	Scyllarus subarctus	MG001088	Penaeus notialis
DQ407682	Callinectes exasperatus	FJ174908	Scyllarus pygmaeus	MG001081	Penaeus duorarum
FJ174903	Palinurus barbarae	JN701734	Scyllarus chacei	MF490229	Penaeus schmitti
NC_022736	Sagmariasus verreauxi	FJ174909	Scyllarus caparti	MF490228	Artemesia longinaris
EU882877	Metanephrops rubellus	JN701732	Scyllarus americanus	MF490143	Penaeus subtilis
EU186128	Metanephrops challengeri	FN295576	Episesarma palawanense	NC_012060	Penaeus stylirostris
EU186125	Metanephrops neptunus	FN295575	Episesarma singaporense	NC_009626	Penaeus vannamei
EU186124	Metanephrops australiensis	KP712873	Austropotamobius fulcisianus orientalis	NC_006880	Macrobrachium rosenbergii
EU186123	Metanephrops arafurensis	FJ152150	Achelous tumidulus	NC_002184	Penaeus monodon
EU186121	Metanephrops boschmai	MG515565	Achelous asper	MK470792	Pandalus hypsinotus
EU186117	Metanephrops formosanus	DQ388067	Achelous sebae	MK430861	Ganjampenaeopsis uncta
EU186118	Metanephrops sinensis	MG515559	Portunus acuminatus	MK000270	Pandalus jordani
FJ462647	Lithodes ferox	MG515578	Achelous tuberculatus	NC_027602	Macrobrachium bullatum
FJ224280	Oratosquillina interrupta	MG515567	Achelous iridescens	NC_026885	Penaeus penicillatus
FJ224282	Odontodactylus japonicus	MG515580	Portunus xantusii	NC_026884	Penaeus merguiensis
FJ224271	Miyakella nepa	DQ388065	Achelous depressifrons	NC_026834	Metapenaeus ensis
FJ224263	Erugosquilla woodmasoni	DQ388063	Achelous rufiremus	NC_017600	Acetes chinensis
FJ224255	Clorida decorata	DQ388057	Achelous gibbesii	NC_015073	Macrobrachium nipponense
FJ224261	Dictyosquilla foveolata	MG515573	Portunus minimus	NC_012738	Penaeus californiensis
FJ224251	Anchisquilla fasciata	MG515576	Achelous stanfordi	NC_012217	Macrobrachium lanchesteri

FJ174906	Scyllarides herklotsii	MG515566	Achelous brevimanus	NC_039964	Pleoticus muelleri
FJ174904	Palinurus delagoae	MG515560	Portunus affinis	NC_039179	Metapenaeus affinis
GU727618	Astacus astacus	MG515561	Achelous angustus	NC_039169	Hymenopenaeus neptunus
FM208780	Portunus hastatus	DQ388062	Achelous binoculus	NC_031366	Penaeus indicus
FM208751	Achelous ordwayi	MH168220	Oratosquillina inornata	NC_039153	Aristaeomorpha foliacea
FM208763	Carcinus maenas	MH168238	Oratosquillina asiatica	NC_030280	Solenocera crassicornis
FM208752	Portunus inaequalis	MH168217	Oratosquillina anomala	NC_030277	Mierspenaeopsis hardwickii
FJ965952	Astacoides madagascariensis	MH168218	Oratosquillina perpensa	NC_040140	Penaeus latisulcatus
FM207657	Erimacrus isenbecki	MH168226	Erugosquilla graham	MG821354	Penaeus semisulcatus
FJ871141	Hemisquilla australiensis	MH168223	Busquilla quadraticauda	AF401305	Penaeus silasi
FJ871139	Austrosquilla tsangi	MH168213	Kempella stridulans	MG001049	Penaeus isabelae
FJ462648	Lithodes confundens	AF133678	Gonodactylaceus graphurus	NC_039168	Sicyonia lancifer
HM138821	Fallosquilla fallax	MW019425	Gonodactylaceus randalli	NC_029457	Metapenaeopsis dalei
HM138820	Echinosquilla guerinii	CAU74327	Carcinus aestuarii	MK971537	Metapenaeopsis gerardoi
HM138819	Coronis scolopendra	KF220514	Menippe rumphii	KP059279	Parapenaeus longirostris
HM138818	Chorisquilla tweediei	MK971425	Menippe nodifrons	MK470790	Pandalus eous
HM138817	Chorisquilla hystrix	HM637973	Menippe adina	LC431729	Pandalus miyakei
HM138816	Chorisquilla excavata	JX514567	Procambarus liberorum	MK470785	Pandalus japonicas
HM138815	Busquilla plantei	AY214438	Procambarus toltecae	LC431728	Pandalus glabrus
HM138814	Alima pacifica	EF012344	Procambarus curdi	MK470796	Pandalus teraoi
HM138813	Alima orientalis	AY214435	Procambarus digueti	MK470793	Pandalus ivanovi
HM138812	Alachosquilla vicina	EF012345	Procambarus nigrocinctus	MK470784	Pandalus coccinatus
HM138822	Gonodactylellus espinosus	JF737390	Procambarus versutus	MK470791	Pandalus formosanus
HM138823	Gonodactylellus affinis	EU433916	Procambarus gibbus	LC431732	Pandalus princeps
HM138833	Kempella mikado	EU433911	Cambarus pecki	MK470789	Pandalus chani
HM138832	Hemisquilla californiensis	JX514566	Procambarus geminus	LC431731	Pandalus houyuu
HM138831	Haptosquilla trispinosa	LC430785	Charybdis acuta	LC431730	Pandalus capillus
HM138830	Haptosquilla glyptocercus	KC163442	Creaserinus fodiens	MK470786	Pandalus longirostris
HM138828	Gonodactylus platysoma	KC163480	Fallicambarus jeanae	MK470787	Pandalus ochotensis
HM138827	Gonodactylaceus falcatus	KC163523	Creaserinus gordoni	AB244633	Pandalus latirostris
HM138825	Gonodactylus childi	KC163501	Creaserinus caesius	MK500702	Metapenaeus brevicornis
HM138824	Gonodactylellus annularis	KC163463	Fallicambarus dissitus	MK500704	Metapenaeus dobsoni
HM138842	Odontodactylus scyllarus	KC163518	Creaserinus danielae	MK470779	Heterocarpus hayashii
HM138841	Odontodactylus latirostris	KC163509	Fallicambarus oryktes	MK470780	Heterocarpus fascirostratus
HM138840	Odontodactylus havanensis	KC163506	Fallicambarus byersi	NC_050168	Palaemon adspersus
HM138839	Odontodactylus cultrifer	KC163511	Creaserinus burrisi	NC_050266	Palaemon serratus
HM138838	Neogonodactylus oerstedii	KC163450	Creaserinus gilpini	NC_045090	Palaemon sinensis
HM138837	Neogonodactylus bredini	KC163479	Fallicambarus harpi	NC_039373	Palaemon capensis
HM138836	Neogonodactylus bahiahondensis	KC163444	Fallicambarus macneesei	NC_038117	Palaemon annandalei

HM138835	Lysiosquillina sulcata	KC163468	Fallicambarus petilicarpus	NC_029240	Palaemon gravieri
HM138854	Squilla rugosa	KC163434	Fallicambarus wallsi	NC_027601	Palaemon serenus
HM138852	Raoulserenea hieroglyphica	KC163486	Fallicambarus strawni	NC_012566	Palaemon carinicauda
HM138851	Raoulserenea oxyrhyncha	KC163474	Fallicambarus devastator	KT959474	Palaemon pugio
HM138845	Pseudosquillopsis marmorata	KC163432	Fallicambarus houstonensis	KF923713	Palaemon pandaliformis
HM138850	Raoulserenea ornata	KC163472	Fallicambarus hortoni	MT340087	Palaemon elegans
HM138849	Raoulserenea komaii	FM208749	Arenaeus cribrarius	MT340091	Palaemon longirostris
HM138843	Protosquilla folini	JX514521	Cambarus tenebrosus	JQ042296	Palaemon peringueyi
NC_041153	Ibacus alticrenatus	JX514504	Cambarus deweesae	KP725611	Palaemon debilis
JN701692	Scyllarides nodifer	JX514513	Cambarus striatus	KF923720	Palaemon carteri
MN817127	Scyllarides haanii	JX514506	Cambarus graysoni	JN674344	Palaemon ritteri
JN701689	Scyllarides brasiliensis	JX514537	Cambarus monongalensis	KC515036	Palaemon orientis
NC_050686	Taku spinosocarinatus	JX514511	Cambarus pyronotus	DQ194924	Macrobrachium gracilirostre
JN566222	Jasus frontalis	AF235988	Cambarus maculatus	KT959473	Palaemon vulgaris
HM637974	Menippe mercenaria	JX514524	Cambarus coosawattae	JQ042295	Palaemon serrifer
JF737171	Procambarus paeninsulanus	JX514509	Cambarus latimanus	MT340090	Palaemon varians
JQ229876	Puerulus sewelli	JX514517	Cambarus strigosus	JQ042297	Palaemon macrodactylus
JQ229873	Panulirus polyphagus	JX514556	Cambarus parrishi	KC515035	Palaemon tonkinensis
MN817128	Panulirus longipes	JX514532	Cambarus bouchardi	JQ042294	Palaemon xiphias
MT533488	Panulirus penicillatus	JX514525	Cambarus fasciatus	KF923729	Palaemon ivonicus
JN701738	Panulirus interruptus	JX514508	Cambarus harti	JN674340	Palaemon pacificus
JN701739	Panulirus marginatus	JX514555	Cambarus nerterius	JN674352	Palaemon atrinubes
JN701700	Ibacus peronii	JX514539	Cambarus setosus	KF923725	Palaemon intermedius
JN701698	Ibacus chacei	JX514531	Cambarus batchi	KC515043	Palaemon concinnus
KF220508	Charybdis hellerii	JX514507	Cambarus halli	KF923716	Palaemon yuna
KC237200	Faxonella clypeata	JX514533	Cambarus crinipes	JQ042306	Palaemon antennarius
KC163440	Fallicambarus kountzeae	JX514541	Cambarus unestami	JN674333	Palaemon dolospinus
JX123471	Arenaeus mexicanus	JX514557	Cambarus reburrus	KF923714	Palaemon gracilis
JX514540	Cambarus tartarus	AY853664	Cambarus gentry	JN674354	Palaemon mundusnovus
JQ407454	Chionoecetes tanneri	JX514518	Cambarus hubbsi	KF923712	Palaemon suttkusi
JQ229907	Thenus unimaculatus	DQ411733	Cambarus friaufi	JQ042299	Palaemon zariquieyi
JQ229878	Thenus indicus	JX514538	Cambarus obeyensis	JN674353	Macrobrachium australiense
KM074036	Haptosquilla hamifera	JX514522	Cambarus cracens	JN674345	Palaemon semmelinkii
KJ132573	Lithodes turritus	JX514530	Cambarus asperimanus	JN674337	Palaemon litoreus
KF828212	Bouchardina robisoni	JX514554	Cambarus hobbsorum	LC425608	Palaemon septemtrionalis
KF828201	Troglocambarus maclanei	JX514523	Cambarus williami	JN674335	Palaemon guangdongensis
KF828192	Hobbseus yalobushensis	JX514502	Cambarus howardi	KF923715	Palaemon hancocki
KF828190	Hobbseus prominens	JX514510	Cambarus obstipus	KC515037	Palaemon vietnamicus
KF220510	Charybdis lucifera	JX514526	Cambarus girardianus	JQ042303	Palaemon texanus
KF828189	Hobbseus petilus	JX514534	Cambarus cryptodytes	JN674339	Palaemon ortmanni

KX237946	Faxonella creaseri	JX514528	Cambarus speciosus	JQ042302	Palaemon turcorum
KU130124	Thranita danae	JX514503	Cambarus sciotensis	KF923718	Palaemon kadiakensis
KT365606	Monomia petrea	JX514553	Cambarus georgiae	EU493137	Macrobrachium asperulum
KT001544	Neogonodactylus wennerae	JX514561	Cambarus pristinus	GU987057	Macrobrachium australe
KR026905	Xiphonectes pseudohastatoides	JX514529	Cambarus aculabrum	MH253292	Macrobrachium olfersii
KT001545	Gonodactylellus viridis	JX514505	Cambarus englishi	GU929448	Macrobrachium jelskii
KT001543	Gonodactylaceus ternatensis	DQ411732	Cambarus brachydactylus	KJ544746	Macrobrachium villosimanus
KR153534	Belosquilla laevis	JX514552	Cambarus cumberlandensis	KP763693	Macrobrachium equidens
KX238053	Procambarus okaloosae	JX514536	Cambarus dubius	JX466930	Macrobrachium potiuna
KX238050	Procambarus morrisi	JX514512	Cambarus reflexus	MK782955	Macrobrachium malcolmsonii
KX238049	Procambarus milleri	JX514520	Cambarus rusticiformis	KC515041	Macrobrachium superbum
KX238048	Procambarus mancus	JX514559	Cambarus scotti	KM610135	Macrobrachium striatum
KX238046	Procambarus lunzi	JX514551	Cambarus coosae	JF310722	Macrobrachium latidactylus
KX237948	Hobbseus cristatus	JX514535	Cambarus distans	JQ805822	Macrobrachium hancocki
KX237996	Procambarus acutissimus	JX514527	Cambarus longirostris	GU929449	Macrobrachium acanthurus
KX237973	Faxonius pagei	JX514519	Cambarus hubrichti	DQ194931	Macrobrachium inflatum
KY236044	Manningia pilaensis	MG563792	Monomia lucida	JQ805801	Macrobrachium crenulatum
KX279350	Pontastacus leptodactylus	MH168232	Faughnia serenei	KM101474	Macrobrachium carcinus
KX238091	Procambarus zonangulus	MH168201	Harpiosquilla melanoura	GU929463	Macrobrachium americanum
KX238089	Procambarus youngi	MH168202	Harpiosquilla annandalei	EU493143	Macrobrachium latimanus
KX238075	Procambarus seminolae	KJ920795	Cherax communis	FM986629	Macrobrachium mammillodactylus
KX238063	Procambarus pycnogonopodus	EU977342	Cherax cuspidatus	JQ805812	Macrobrachium faustinum
KX238054	Procambarus orcinus	KJ920829	Cherax paniaicus	AY377842	Macrobrachium heterochirus
KX238055	Procambarus pallidus	KJ920812	Cherax lorentzi	KP756688	Macrobrachium scabriculum
MH168208	Alima maxima	KJ920772	Cherax albertisii	KM101476	Macrobrachium digueti
MF490148	Scyllarides deceptor	AF135973	Cherax rotundus	KF383310	Macrobrachium tenellum
KY524476	Monomia argentata	KM039077	Cherax leckii	KP037053	Macrobrachium idae
KY524461	Xiphonectes pulchricristatus	KJ920825	Cherax murido	JX025200	Macrobrachium japonicum
NC_042240	Paralithodes platypus	KM039082	Cherax wasselli	EU493150	Macrobrachium formosense
KY426330	Lopholithodes foraminatus	KM039078	Cherax parvus	JQ390474	Macrobrachium dienbienphuense
KY236047	Faughnia formosae	KJ920828	Cherax pallidus	EU493139	Macrobrachium placidulum
KY236046	Faughnia profunda	KM039079	Cherax cartalacoolah	JQ362449	Macrobrachium sintangense
KY236045	Bathysquilla crassispinosa	KJ920806	Cherax longipes	JQ359750	Macrobrachium niphanae
NC_006992	Eriocheir sinensis	KJ920765	Cherax rhynchotus	KF383311	Macrobrachium totonacum
NC_006916	Harpiosquilla harpax	KY654091	Cherax pulcher	JF491346	Macrobrachium tuxtlaense
NC_006281	Callinectes sapidus	KJ920835	Cherax peknyi	KF383313	Macrobrachium vicconi
NC_006081	Squilla mantis	AF135972	Cherax setosus	KF383314	Macrobrachium villalobosi
NC_005037	Portunus trituberculatus	KJ920813	Cherax misolicus	KM101468	Macrobrachium amazonicum

NC_004251	Panulirus japonicus	KY654088	Cherax warsamsonicus	JF774072	Macrobrachium canarae
MN334534	Cancer pagurus	KJ920836	Cherax solus	JQ362452	Macrobrachium tratense
MT750295	Chionoecetes japonicus	KY654087	Cherax snowden	JQ362454	Macrobrachium forcipatum
NC_012567	Scylla tranquebarica	KJ920785	Cherax buitendijkae	JQ390475	Macrobrachium hirsutimanus
NC_012565	Scylla serrata	KJ920784	Cherax boschmai	GU929445	Macrobrachium borellii
NC_011598	Eriocheir hepuensis	KJ920760	Cherax nucifraga	GU929446	Macrobrachium brasiliense
NC_011597	Eriocheir japonica	KJ920752	Cherax barrette	JF310709	Macrobrachium aemulum
NC_011243	Cherax destructor	MH168227	Oratosquilla fabricii	JF310717	Macrobrachium handschini
>NC_007444	Squilla empusa	DQ006549	Astacopsis tricornis	JF310719	Macrobrachium horstii
NC_007443	Lysiosquillina maculata	FJ152163	Thalamita admete	GU929447	Macrobrachium ferreirai
NC_007442	Gonodactylus chiragra	KT959518	Faxonius virilis	DQ194911	Macrobrachium lanatum
NC_016015	Panulirus homarus	KJ132641	Thranita prymna	JF310725	Macrobrachium novaehollandiae
NC_015607	Homarus americanus	AF044240	Astacopsis franklinii	EF588319	Macrobrachium tolmerum
NC_014854	Panulirus ornatus	KX268737	Cambaroides schrenckii	HM352432	Macrobrachium iheringi
NC_014342	Oratosquilla oratoria	EU433912	Orconectes australis	JF310728	Macrobrachium saigonense
NC_014339	Panulirus stimpsoni	LC469670	Thalamita chaptalii	HM352428	Macrobrachium nattereri
NC_013246	Charybdis japonica	LC469673	Zygita longifrons	HM352430	Macrobrachium aracamuni
NC_012572	Scylla paramamosain	LC469671	Thalamita picta	HM352433	Macrobrachium inpa
NC_012569	Scylla olivacea	LC469672	Thalamita seurati	HM352435	Macrobrachium depressimanum
NC_022937	Cherax quadricarinatus	LC430787	Thranita pelsarti	HM352446	Macrobrachium surinamicum
NC_022936	Cherax cainii	EU433913	Orconectes barri	HM352462	Macrobrachium denticulatum
NC_021458	Paralithodes brevipes	JX514565	Faxonius ronaldi	GQ487497	Macrobrachium pilimanus
NC_020029	Paralithodes camtschaticus	JX514564	Faxonius neglectus	HM352464	Macrobrachium ohione
NC_020022	Scyllarides latus	EU433917	Orconectes compressus	EF501999	Macrobrachium hainanense
NC_016926	Procambarus clarkii	EU433919	Orconectes forceps	EU493138	Macrobrachium lepidactyloides
NC_020021	Procambarus fallax	EU433914	Orconectes pellucidus	EU493146	Macrobrachium jaroense
NC_020020	Homarus gammarus	NC_041211	Neoeriocheir leptognathus	EU493145	Macrobrachium esculentum
NC_024440	Thenus orientalis	AF279826	Penaeus kerathurus	DQ194910	Macrobrachium maculatum
NC_024202	Lithodes nintokuae	AF279824	Penaeus marginatus	DQ194912	Macrobrachium edentatum
NC_023481	Cherax cairnsensis	AF279823	Penaeus longistylus	DQ194926	Macrobrachium grandimanus
NC_023480	Cherax dispar	AF279822	Penaeus plebejus	DQ194947	Macrobrachium malayanum
NC_023479	Cherax quinquecarinatus	AF105044	Metapenaeopsis liui	DQ194949	Macrobrachium meridionale
NC_023478	Cherax robustus	AF105043	Metapenaeopsis lamellata	DQ194953	Macrobrachium neglectum
NC_022938	Cherax monticola	AF105040	Metapenaeopsis acclivis	DQ194954	Macrobrachium platycheles
NC_022939	Cherax glaber	AF105042	Metapenaeopsis commensalis	DQ194950	Macrobrachium naso
NC_026224	Cherax holthuisi	AY622201	Atypopenaeus stenodactylus	DQ194957	Macrobrachium placidum
NC_026215	Astacopsis gouldi	AY601738	Aristeus antillensis	DQ194960	Macrobrachium yui
NC_026209	Portunus pelagicus	AY601736	Solenocera vioscai	DQ194961	Macrobrachium shokitai

NC_025957	Paranephrops planifrons
NC_025958	Nephrops norvegicus
NC_025581	Ibacus ciliatus
NC_024632	Charybdis feriata
NC_025323	Metanephrops sibogae
NC_028024	Panulirus cygnus
NC_027608	Metanephrops thomsoni
NC_026561	Faxonius limosus
NC_027178	Squilloides leptosquilla
NC_026226	Cherax bicarinatus
NC_026560	Austropotamobius pallipes
NC_026559	Cherax tenuimanus
NC_026227	Cherax boesemani
NC_036132	Charybdis (Charybdis) natator
NC_033510	Procambarus acutus
NC_033509	Pacifastacus leniusculus
NC_030255	Munida gregaria
NC_028627	Panulirus versicolor
NC_029720	Faxonius rusticus
NC_028225	Portunus sanguinolentus
NC_028447	Procambarus alleni
NC_050675	Metacarcinus magister
NC_041155	Puerulus angulatus
NC_040124	Lupocycloporus gracilimanus
NC_037173	Monomia gladiator
NC_037155	Varuna yui
Cephalopods	
AF110075	Loligo forbesii
AB270953	Nototodarus sloanii
AB193802	Sepia lorigera
AB193801	Sepia pardex
AB192323	Sepia kobiensis
AB191138	Rossia pacifica
AB191136	Berryteuthis magister
AJ252763	Eledone massyae
AF369957	Sepia robsoni

NC_050695	Trachysalambria curvirostris
AY264908	Penaeus chinensis
AY264907	Penaeus canaliculatus
NC_040139	Metapenaeopsis barbata
AF279828	Penaeus esculentus
AY622202	Heteropenaeus longimanus
EF601684	Atypopenaeus dearmatus
AY622218	Funchalia taaningi
AY622217	Xiphopenaeus kroyeri
AY622216	Trachypenaeopsis mobilispinis
AY622215	Rimapenaeus similis
AY622214	Megokris granulosus
AY622210	Parapenaeus politus
FR849633	Solenocera membranacea
FJ435649	Solenocera koelbeli
NC_038069	Alcockpenaeopsis hungerfordii
FJ435641	Batepenaeopsis tenella
EU548178	Pandalus platyceros
FJ435639	Metapenaeus moyebi
NC_042173	Metapenaeus joyneri
EU868698	Pandalus montagui
HQ241514	Heterocarpus parvispina
HM014402	Penaeus brasiliensis
GU972651	Aristeus antennatus
GQ302761	Heterocarpus laevigatus
GQ302759	Heterocarpus lepidus
Cephalopods	
MT025991	Octopus americanus
MG548981	Narrowteuthis nesisi
NC_020348	Ommastrephes bartramii
NC_017749	Sepiella japonica
NC_017746	Uroteuthis (Photololigo) edulis
NC_012840	Doryteuthis (Amerigo) opalescens
NC_011581	Architeuthis dux
NC_009734	Dosidicus gigas
NC_009690	Sepia esculenta

AY377852	Macrobrachium sundaicum
AY858836	Macrobrachium rude
AY730051	Macrobrachium lamarrei
AY730052	Macrobrachium sankolli
AY730054	Macrobrachium gangeticum
KX162763	Trachysalambria palaestinensis
NC_016184	Euphausia pacifica
GQ890518	Euphausia lucens
MG677874	Euphausia vallentini
DQ356240	Euphausia triacantha
AF177180	Euphausia longirostris
AF177178	Euphausia similis
MG677872	Euphausia recurve
MG677869	Euphausia krohni
DQ356239	Euphausia frigida
MG677867	Euphausia gibboides
DQ079713	Euphausia eximia
MG677865	Euphausia americana
MG677873	Euphausia tenera
MG677871	Euphausia pseudogibba
MG677868	Euphausia hemigibba
MG677866	Euphausia brevis
KF182582	Hymenopenaeus debilis
KC515042	Nematopalaemon tenuipes
Cephalopods	
KC792312	Octopus maya
HQ733953	Illex illecebrosus
GQ412305	Nototodarus gouldi
EU735264	Gonatopsis octopedatus
EU735218	Illex coindetii
EU735238	Berryteuthis anonychus
EU735210	Gonatus fabricii
KR259947	Lusepiola birostrata
KJ605235	Octopus tetricus

AF110081	Loligo reynaudii	NC_007896	Amphioctopus fangsiao	KF854042	Uroteuthis (Photololigo) sibogae
AF110079	Doryteuthis (Amerigo) pealeii	X79585	Loligo vulgaris	KF854023	Doryteuthis (Doryteuthis) pleii
AF110076	Doryteuthis (Amerigo) gahi	NC_044093	Octopus mimus	KF854021	Doryteuthis sanpaulensis
AY293671	Sepiola rondeletii	NC_029747	Octopus conispadiceus	KF854020	Doryteuthis (Amerigo) surinamensis
AY293670	Sepiola robusta	NC_029723	Octopus bimaculoides	KF373761	Octopus hubbsorum
AY293669	Sepiola intermedia	NC_028189	Uroteuthis (Photololigo) chinensis	MG010490	Macrotritopus defilippi
AY293668	Adinaefiola ligulata	NC_027729	Uroteuthis (Photololigo) duvaucelii	MF040832	Octopus insularis
AM088007	Sepia smithi	NC_026908	Illex argentinus	LC121083	Loliolus (Nipponololigo) sumatrensis
AM088005	Sepia elliptica	NC_022959	Sepia aculeata	LC121068	Sepia stellifera
AJ252765	Eledone palari	NC_022693	Sepiella inermis	LC121066	Sepia recurvirostra
AJ252764	Eledone moschata	NC_022468	Sepia lycidas	LC121063	Sepia madokai
EU735193	Sepiola affinis	NC_022467	Sepia latimanus	LC121061	Sepia kobiensis
EU735192	Rossia palpebrosa	NC_022466	Sepia apama	NC_029702	Amphioctopus aegina
AY681045	Gonatus madokai	NC_021146	Sepia pharaonis	NC_007895	Sepia officinalis
AY681038	Gonatus kamtschaticus	NC_028034	Loliolus (Nipponololigo) beka	NC_007894	Sepioteuthis lessoniana
AY616973	Eledone cirrhosa	AF110072	Alloteuthis subulata	NC_006354	Todarodes pacificus
AY377630S	Sepia elegans	AB270952	Nototodarus hawaiiensis	NC_006353	Octopus vulgaris
AY293676	Rossia bipillata	X79578	Sepia orbignyana	NC_002507	Heterololigo bleekeri
AY293672	Sepiola atlantica	X79586	Sepia papuensis	MT712046	Octopus sinensis
KF266732	Lolliguncula (Lolliguncula) panamensis	X79577	Rossia macrosoma	LC121047	Amphioctopus rex
AJ311110	Octopus maorum	AF110084	Lolliguncula (Lolliguncula) brevis	AB192324	Sepia peterseni
MK450541	Octopus fitchi	EU735243	Lolliguncula (Loliolopsis) diomedeae	NC_016423	Bathyteuthis abyssicola
NC_049899	Amphioctopus neglectus	AF110085	Afrololigo mercatoris	NC_016425	Semirossia patagonica
NC_026724	Loliolus (Nipponololigo) uyii	NC_015896	Octopus minor	NC_023257	Cistopus taiwanicus
NC_030208	Loliolus (Nipponololigo) japonica	NC_028547	Octopus bimaculatus	NC_010636	Sthenoteuthis oualaniensis
AB191107	Enteroctopus dofleini	NC_038213	Octopus variabilis	NC_007893	Watasenia scintillans
GQ226031	Sasakiopus salebrosus	AB191115	Octopus cyanea	EU735265	Gonatopsis okutanii
AY545105	Octopus berrima	AB191114	Callistoctopus ornatus	AY293656	Uroteuthis (Aestuariolus) noctiluca
NC_036351	Amphioctopus marginatus	MG999649	Enteroctopus megalocyathus	AB675085	Sepioteuthis australis
LC121044	Amphioctopus membranaceus	AJ311108	Amphioctopus kagoshimensis		
LC121036	Amphioctopus exannulatus	AF110090	Sepioteuthis sepioidea		
Land snails		Land snails		Land snails	
NC_041247	Helix pomatia	KF246970	Caracolus caracollus	MF564117	Helix vladika
NC_024601	Achatina fulica	KF246999	Lacteoluna selenina	KF246957	Pleurodonte discolor

NC_021747	Helix aspersa	KF247012	Cernuella cisalpine	KF246963	Pleurodonte lychnuchus
KT211748	Helix thessalica	KF247015	Cochlicella acuta	GQ402402	Erctella mazzullii
KR705016	Helix pomatia	KF247017	Disculella maderensis	GQ402397	Erctella cephalaeditana
KR705014	Helix lucorum	KF247020	Dialeuca nemoraloides	KF246958	Pleurodonte formosa
KR705012	Helix nicaeensis	KF247021	Monadenia fidelis	KR705018	Helix christophi
JQ619236	Achatina reticulata	NC_001816	Cepaea nemoralis	KR705019	Helix nordmanni
EU912833	Helix aspersa maxima	KF247040	Sphincterochila candidissima	KF246965	Pleurodonte nucleola
GQ402391	Helix aperta	KF247041	Microphysula ingersolli	KF246967	Pleurodonte parilis
KR705013	Helix albescens	KF247043	Helicodonta obvoluta	KF246969	Gonostomopsis auridens
MH130070	Tyrrhenaria ceratina	NC_030723	Cernuella virgata		
Venus shell		Venus shell		Venus shell	
AF122977	Tridacna mbalavuana	NC_012767	Meretrix petechialis	HF970473	Ensis macha
AB751362	Siliqua alta	NC_008452	Acanthocardia tuberculata	EU169034	Siliqua minima
AB751329	Megangulus zyonoensis	LT630276	Tridacna maxima	GQ166561	Ensis leei
AB751328	Megangulus venulosus	NC_023384	Lutraria rhynchaena	DQ459296	Polititapes durus
AB751311	Donax faba	NC_022924	Meretrix lyrata	KP052743	Cerastoderma glaucum
AB751310	Donax cuneatus	NC_022709	Arctica islandica	KF611752	Donax longissimus
AB751309	Donax kiusiuensis	NC_017616	Solen strictus	KC429308	Solen vaginoides
DQ160001	Mactra quadrangularis	NC_016891	Paratapes undulatus	KC429301	Venus verrucosa
AJ548775	Ensis ensis	NC_016890	Paratapes textilis	JN969934	Ezocallista brevisiphonata
AM085110	Chamelea gallina	NC_016889	Paphia amabilis	KR422892	Procardium indicum
AJ548774	Spisula subtruncata	NC_016665	Solen grandis	KR422893	Cardium maxicostatum
AJ548772	Callista chione	NC_036766	Lutraria maxima	KR422891	Cardium costatum
AJ294950	Polititapes rhomboides	NC_035987	Donax vittatus	KR422879	Acanthocardia paucicostata
AJ417845	Venerupis corrugata	NC_035986	Donax variegatus	KR422878	Acanthocardia echinata
AJ294950	Polititapes aureus	NC_035985	Donax trunculus	KR422877	Acanthocardia aculeata
DQ459295	Venus crebrisulca	NC_035984	Donax semistriatus	KP055816	Ruditapes philippinarum
DQ459280	Mercenaria campechiensis	NC_035757	Ruditapes decussatus	KR422901	Corculum cardissa
NC_051506	Antigona lamellaris	NC_035728	Cerastoderma edule	KX713257	Spisula solida
DQ459260	Ameghinomya antiqua	NC_026558	Tridacna squamosa	NC_046518	Scrobicularia plana
DQ356382	Callista erycina	NC_031332	Ruditapes philippinarum	KX713231	Mactra violacea
DQ356381	Venerupis aspera	NC_025510	Mactra chinensis	KX713203	Chamelea striatula
DQ356380	Paphia philippiana	NC_039945	Tridacna derasa	KX713217	Ensis siliqua
JF808192	Venus casina	NC_048487	Mercenaria mercenaria	KR422986	Serripes groenlandicus
HF970514	Ensis terranovensis	NC_050683	Tridacna gigas	MN068431	Tridacna elongatissima
GQ166567	Mactra stultorum	NC_021375	Mactra antiquata	MN068543	Tridacna rosewateri
NC_014579	Paphia euglypta	NC_016174	Meretrix lamarckii		
NC_013188	Meretrix meretrix	NC_014809	Meretrix lusoria		

Similar to the bivalve metabarcoding system, the crustacean, cephalopod, land snail, and Venus shell system should be compatible with the recently published mammalian and poultry systems [94]. For this purpose, seafood species were separated into respective groups (crustacean, cephalopod, land snail, and Venus shell) on basis of their genetic diversity. The primers were then created for each system, as described previously for the molluscs and are listed in Table 3. For the crustaceans, two forward (For_Cr1 and For_Cr2) and one reverse (Rev_Cr) primers; for the cephalopods (For_Ce) one forward and one reverse (Rev_Ce) primers; for the land snails one forward (For_Sn) and two reverse primers (Rev1_Sn and Rev2_Sn); and for the Venus shell four forward (For1_ Ve, For2_ Ve, For3_ Ve, For4_Ve, and For5_Ve,) and three reverse (Rev1_Ve Rev2_Ve, and Rev3_Ve) primers were designed manually. Each system was tested separately, under the same conditions as described above for bivalves, mammals and poultry [94,95]. Primer concentrations and addition of $MgCl_2$ were optimised individually for each system. The amounts used for the respective systems are listed in Table 5.

Table 5 Primer and magnesium amounts per seafood system.

System	Concentration froward primer	Concentration reverse primer	Addition of $MgCl_2$
Crustacean	0.2 mM (respectively)	0.4 mM	3 mM
Cephalopod	0.2 mM	0.4 mM	3 mM
Land snail	0.4 mM	0.2 mM (respectively)	3 mM
Venus shell	0.2 mM (respectively)	0.2 mM (respectively for Rev2_Ve and Rev3_Ve) 0.4 mM (for Rev1_Ve)	4.5 mM

In the following, the alignments of the individual systems are shown. For a better overview, the crustacean system has been subdivided into crayfish and shrimps. For the alignments, commercially important species were selected and aligned each time: Figure 8 for crayfish (crustacean) species; Figure 9 for shrimp (crustacean) species; Figure 10 for cephalopod species; Figure 11 for land snail species; and Figure 12 for Venus shell species. In total 802 DNA barcodes were collated for the reference database of crustacean, 118 DNA barcodes for cephalopod, 35 DNA barcodes for land snail, and 79 DNA barcodes for Venus shell species. A part of the sequence alignments are shown below (Figure 8 till Figure 12).

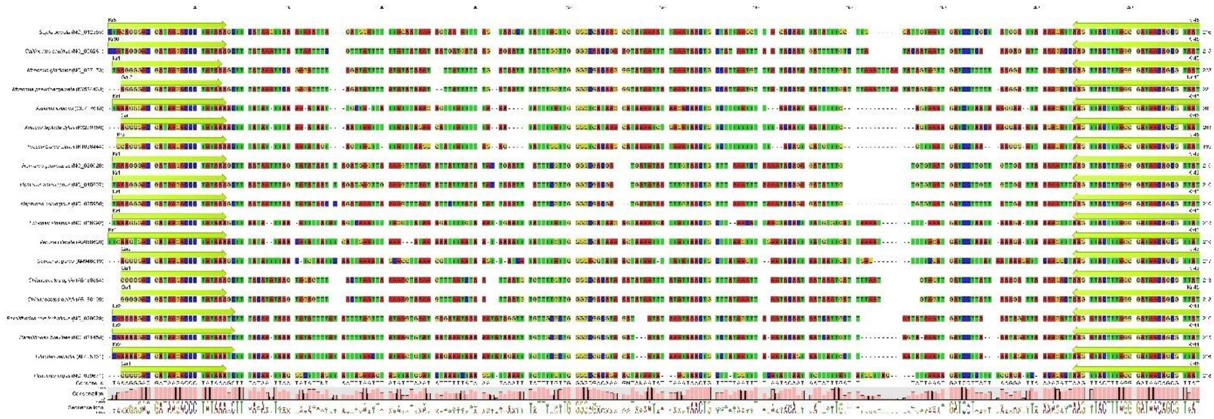


Figure 8 Multi-species sequence alignment of the mitochondrial 16S rDNA barcoding region for crayfish species (crustacean). Coloured arrows indicate the binding sites of the primer sets (CLC Genomics Workbench software version 10.1.1, Qiagen, Hilden, Germany).

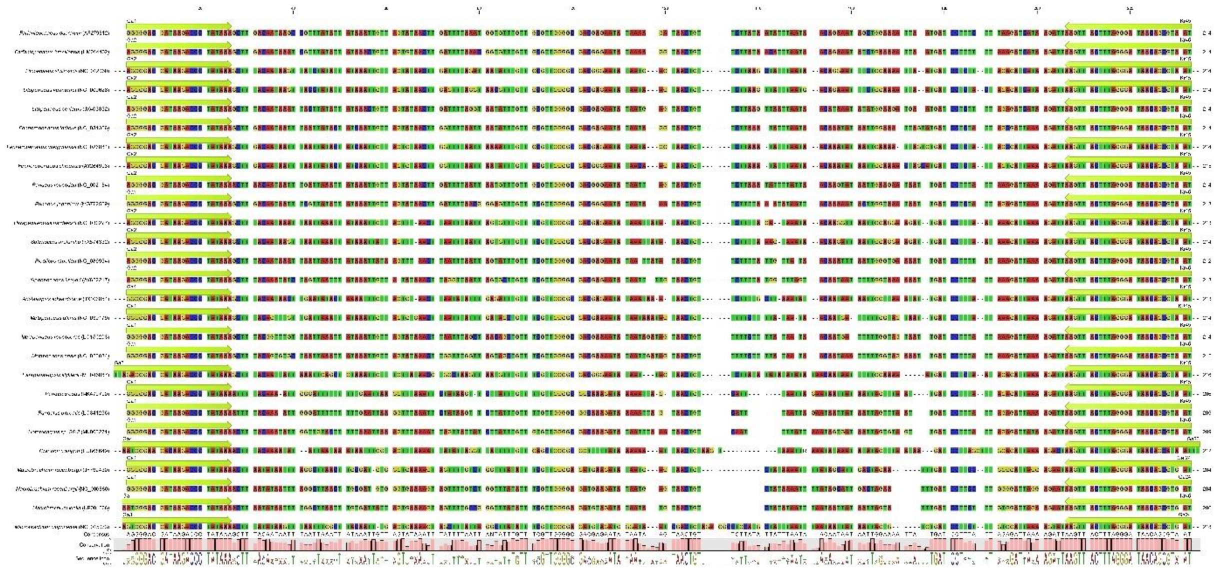


Figure 9 Multi-species sequence alignment of the mitochondrial 16S rDNA barcoding region for shrimp species (crustacean). Coloured arrows indicate the binding sites of the primer sets (CLC Genomics Workbench software version 10.1.1).

Figure 10 Multi-species sequence alignment of the mitochondrial 16S rDNA barcoding region for cephalopod species. Coloured arrows indicate the binding sites of the primer sets (CLC Genomics Workbench software version 10.1.1).

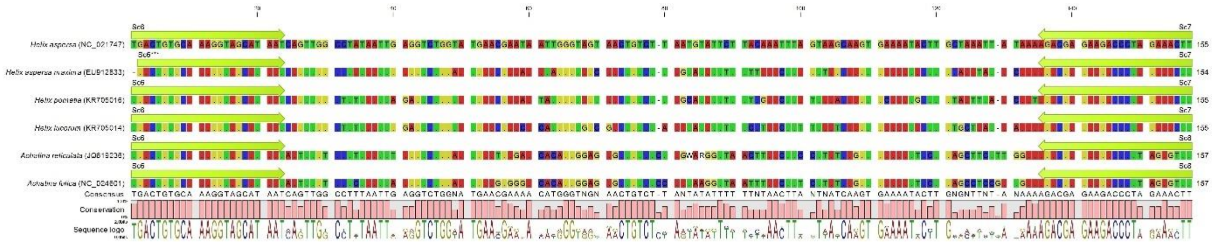


Figure 11 Multi-species sequence alignment of the mitochondrial 16S rDNA barcoding region for land snail species. Coloured arrows indicate the binding sites of the primer sets (CLC Genomics Workbench software version 10.1.1).

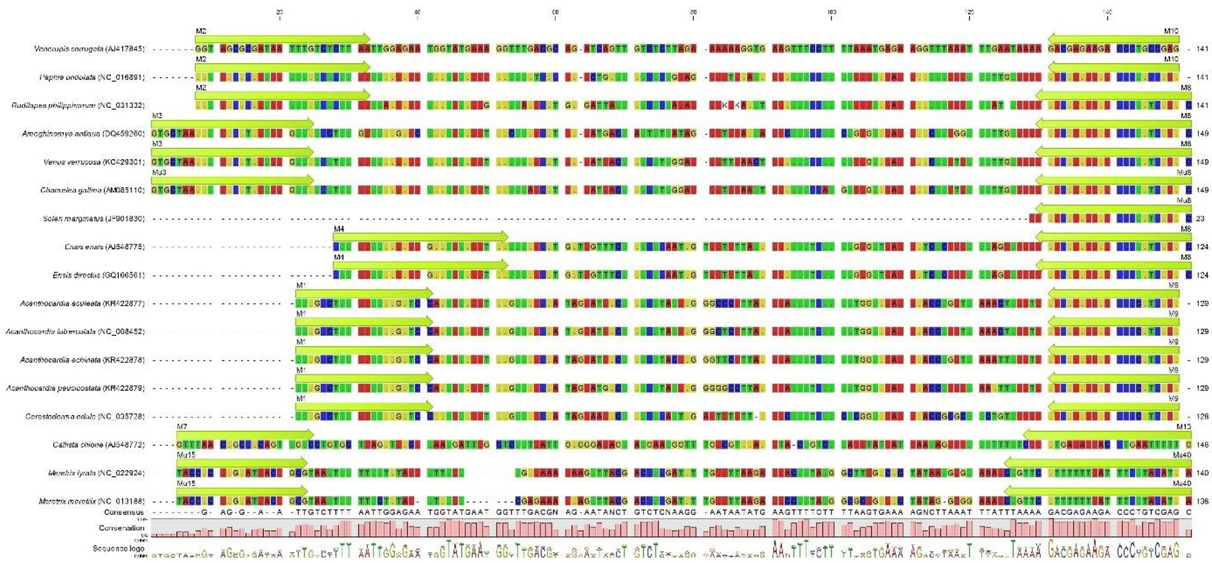


Figure 12 Multi-species sequence alignment of the mitochondrial 16S rDNA barcoding region for Venus shell species. Coloured arrows indicate the binding sites of the primer sets (CLC Genomics Workbench software version 10.1.1).

An extended alignment shows that some species are indistinguishable in this special DNA barcode region. These species are for crustacean: *Charybdis (Charybdis) lucifera* and *Charybdis (Charybdis) heller; Chionoecetes bairdi* and *Chionoecetes opilio; Lithodes santolla* and *Lithodes confundens; Metanephrops sagamiensis* and *Metanephrops andamanicus; Raoulserenea hieroglyphica* and *Raoulserenea ornate; Palinurus barbarae*, *Palinurus gilchristi* and *Palinurus delagoae; Parapenaeus kensleyi*, *Parapenaeus lanceolatus*, *Parapenaeus sextuberculatus* and *Parapenaeus fissuroides indicus, Parapenaeus australiensis* and *Parapenaeus ruberoculatus; Solenocera crassicornis* and *Solenocera koelbeli; Heterocarpus gibbosus* and *Heterocarpus corona; Trachysalambria dentata* and *Trachypenaeus curvirostris; Megokris pescadoreensis* and *Megokris granulosus; Melicertus canaliculatus* and *Macrobrachium japonicum; Solenocera koelbeli* and *Solenocera crassicornis; Metapenaeus dobsoni* and *Metapenaeus brevicornis;* Fenneropenaeus silasi and Fenneropenaeus penicillatus; *Pandalopsis longirostris, Pandalus capillus* and *Pandalus princeps; Pandalopsis ochotensis* and *Pandalus houyuu; Heterocarpus corona* and *Heterocarpus gibbosus*; *Heterocarpus hayashii* and *Heterocarpus parvispina; Heterocarpus fascirostratus* and *Heterocarpus woodmasoni; Palaemon adspersus* and *Palaemon adspersus*.

The following species were not differentiable for the cephalopods: *Sepia kobiensis* and *Sepia stellifera; Sepiola affinis*, *Sepiola intermedia* and *Sepiola robusta; Uroteuthis chinensis* and *Illex illecebrosus; Enteroctopus dofleini* and *Octopus conispadiceus; Octopus minor* and *Octopus variabilis.*

The land snail species were all distinguishable at this 16S rDNA target region.

Not discriminable were following Venus shell species: *Tridacna gigas* and *Tridacna derasa; Meretrix meretrix* and *Meretrix petechialis; Ensis directus* and *Ensis terranovensis; Mactra violacea* and *Mactra antiqua.*

As biologists are constantly performing genome analyses and new knowledge becomes included in the taxonomy, it cannot be ruled out that the undistinguishable species are one and the same species. It is also unclear to which extent these indistinguishable species play a role in human nutrition.

4.4 Duplex System

The idea was to create in one attempt a duplex mollusc system using cephalopods and land snail primers. For this purpose, a PCR was conducted with the primer of cephalopod and land snails together. For that, primers were taken in aliquots with these species. However, there were problems in library preparation, because primer dimers were formed, which disrupted the sequencing run. Therefore, the planned system had to be dropped.

Since seafood mixtures usually include both crustaceans and cephalopods, the idea was to combine the crustacean system with the cephalopod system, as CruTin system. For this purpose, primers of these two systems were combined and ran in a conventional PCR with their respective species. Afterwards, the primers were checked to find out whether the existing crustacean and cephalopod systems are specific, similar to the bivalves system [95]. Both approaches, the combination and the specificity, show promising results. A sequencing run was also performed and produced interesting outcomes. However, these have not yet been analysed with the Galaxy software of customized database for crustaceans and cephalopods.

Similar experiments were conducted out with the combination of crustacean and land snail systems. These results showed interesting results as well. However, an application is not practicable, as there is currently no dish in which crustaceans are served with land snails. Due to the lack of application, this system was dropped.

Since the Venus shells are currently running with eight primers, a combination with another system was not relevant. Further research is needed to determine how and under which conditions this system can be optimised.

4.5 Sequencing results

Some of the commercial seafood products were successfully sequenced. Table 5 shows the results of sequencing by MiSeq®. Not all products were sequenced and analysed, yet. As shown in Table 5, the samples can be identified as various seafood species. The sequencing was successful and a sufficient number of sequences were generated to allow a successful analysis using CLC genomic workbench’s software. As this little analysis has only limited possibilities and some quality parameters cannot be adjusted, a more detailed analysis has to be carried out using the Galaxy software and then compared against a customized database for crustaceans, cephalopods, land snails, and Venus shells. This will be part of future research.

Table 6 Results for commercial seafood samples. Samples were sequenced with the MiSeq® and additionally analysed by using CLC genomic workbench software.

ID Number	Declaration on the Product: Scientific name of species	Declaration on the Product: Common name (Eng)	Species identified by CLC software
Cr1	*Nephrops norvegicus*	Norway lobster	*Nephrops norvegicus*
Cr2	*Procambarus clarkii*	Louisiana crayfish	*Procambarus clarkii*
Cr3	*Panulirus argus*	Caribbean spiny lobster	*Panulirus argus*
Cr4	*Homarus americanus*	American lobster	*Homarus americanus*
Cr5	Not declared	Blue crab	*Monomia gladiator*
Cr6	*Paralithodes camtschaticus*	Red king crab	*Paralithodes camtschaticus*
Cr7	*Chionoecetes opilio*	Snow crab	*Chionoecetes opilio* *Chionoecetes bairdi*
Cr8	*Homarus gammarus*	European lobster	*Homarus gammarus*
Cr9	*Pleoticus muelleri*	Argentine red shrimp	*Pleoticus muelleri*
Cr10	*Penaeus monodon*	Giant tiger prawn	*Penaeus monodon*
Cr11	*Macrobrachium rosenbergii*	Giant river prawn	*Macrobrachium rosenbergii*
Cr12	*Pandalus borealis*	Northern prawn	*Pandalus borealis*
Cr13	*Crangon crangon*	Brown shrimp	*Crangon crangon*
Cr14	*Litopenaeus vannamei*	White shrimp	*Penaeus vannamei*
Cr15	*Macrobrachium rosenbergii*	Giant river prawn	*Penaeus monodon*
Cr16	*Litopenaeus vannamei*	White shrimp	*Penaeus vannamei*
Cr17	*Metapenaeus monoceros*	Speckled shrimp	*Mierspenaeopsis hardwickii* *Ganjampenaeopsis uncta*
Cr18	*Heterocarpus reedi*	Chilean nylon shrimp	*Heterocarpus spp.*
Cr19	*Penaeus monodon*	Giant tiger prawn	*Penaeus vannamei*
Cr20	*Macrobrachium rosenbergii*	Giant river prawn	*Penaeus monodon*
Cr21	*Penaeus monodon*	Tiger shrimp	*Penaeus monodon*
Cr22	*Crangon crangon*	Brown shrimp	*Crangon crangon*
Cr23	*Crangon crangon*	Brown shrimp	*Crangon crangon*
Cr24	*Aristaeopsis edwardsiana*	Giant scarlet shrimp	*Aristaeopsis edwardsiana*
Cr25	*Pleoticus muelleri*	Argentine red shrimp	*Pleoticus muelleri*
Cr26	*Penaeus occidentalis*	Western white shrimp	*Pleoticus muelleri*
Cr27	*Penaeus notialis*	Southern pink shrimp	*Penaeus duorarum*
Cr28	*Penaeus borealis*	Northern prawn	*Pandalus borealis*
Cr29	Dendrobranchiata	Red shrimp	*Penaeus vannamei*

Cr30	*Metapenaeus monoceros*	Speckled shrimp	not analysed yet
Cr31	*Penaeus merguiensis* *Metapenaeus ensis*	White shrimp	not analysed yet
Cr32	*Litopenaeus vannamei*	White shrimp	not analysed yet
Cr33	*Crangon crangon*	Brown shrimp	not analysed yet
Cr34	*Litopenaeus vannamei*	White shrimp	not analysed yet
Cr35	*Litopenaeus vannamei*	White shrimp	not analysed yet
Cr36	*Litopenaeus vannamei*	White shrimp	not analysed yet
Cr37	Not declared	Shrimp cocktail Florida	not analysed yet
Cr38	Not declared	Lobster soup	not analysed yet
Cr39	Not declared	Crab soup	not analysed yet
Cr40	*Pandalus borealis*	Northern prawn	not analysed yet
Cr41	*Paralomis granulosa*	Crab met	not analysed yet
Cr42	*Cancer Pagurus*	Crab cream	not analysed yet
Cr43	*Portunus* spp.	Swim crab met	not analysed yet
Cr44	Not declared	Lobster butter	not analysed yet
Cr45	*Penaeus merguiensis*	Hanami Pawan cracker	not analysed yet
Cr46	*Portunus pelagicus*	Crab cream	not analysed yet
Cr47	Not declared	Shrimp Ramen	not analysed yet
Cr48	Dendrobranchiata	Dried baby shrimps	not analysed yet
Cr49	Dendrobranchiata	Dried Shrimps	not analysed yet
Cr50	Not declared	Crab soup	not analysed yet
Cr51	Not declared	Lobster cream soup	not analysed yet
Cr52	*Homarus americanus* *Pandalus borealis*	Lobster fond	not analysed yet
Cr53	*Litopenaeus vannamei*	Shrimp	not analysed yet
Cr54	*Litopenaeus vannamei*	Shrimp	not analysed yet
Cr55	*Homarus americanus*	Lobster	not analysed yet
Ce1	*Loligo edulis*	Squid	*Illex illecebrosus* *Uroteuthis (Photololigo) chinensis*
Ce2	*Octopus vulgaris*	Octopus	*Octopus vulgaris*
Ce3	*Loligo chinensis*	Squid	*Doryteuthis (Amerigo) gahi*
Ce4	*Loligo duvaucii*	Squid	*Loligo spp.*
Ce5	*Sepiella japonica*	Sepia	*Sepiella inermis*
Ce6	*Octopus vulgaris*	Octopus	*Octopus vulgaris*
Ce7	*Sepia officinalis*	Cuttlefish	*Sepia pharaonis*
Ce8	*Loligo vulgaris*	Squid	*Doryteuthis (Amerigo) gahi*
Ce9	*Octopus aegina*	Sand bird octopus	*Amphioctopus aegina*

Ce10	*Loligo opalescens*	Squid	*Doryteuthis opalescens*
Ce11	*Sepiella inermis*	Sepia	*Sepiella inermis*
Ce12	*Octopus maya*	Octopus	*Octopus maya*
Ce13	*Sepiella inermis*	Sepia	*Sepiella inermis*
Ce14	*Loligo edulis*	Squid	*Uroteuthis chinensis* *Ilex illecebrosus*
Ce15	*Eledone moschata*	Musky octopus	*Amphioctopus aegina*
Ce16	*Loligo chinensis*	Squid	*Uroteuthis chinensis* *Ilex illecebrosus*
Ce17	*Loligo gahi*	Squid	*Doryteuthis (Amerigo) gahi*
Ce18	Not declared	Calamari	*Doryteuthis (Amerigo) gahi*
Ce19	Not declared	Octopus carpaccio	*Octopus cyanea*
Ce20	*Uroteuthis duvaucii*	Cuttlefish	*Uroteuthis duvaucii*
Ce21	*Dosidicus gigas*	Cuttlefish	*Dosidicus gigas*
Ce22	*Dosidicus gigas*	Cuttlefish	*Dosidicus gigas*
Ce23	*Dosidicus gigas*	Cuttlefish	*Todarodes pacificus*
Ce24	Not declared	Cuttlefish	*Todarodes pacificus*
Ce25	*Octopus membranaceus*	Musky octopus	*Amphioctopus membranaceus*
Ce26	Not declared	Calamari	*Dosidicus gigas*
Sn1	*Helix lucorum*	Roman snail	*Helix spp.*
Sn2	Not declared	Agate snail	*Helix spp.*
Sn3	*Helix lucorum*	Roman snail	*Helix spp.*
Sn4	Not declared	snail capsule	*Helix thessalica*
Sn5	Not declared	Roman snail	*Helix spp.*
Sn6	Not declared	Roman snail	*Helix aspersa maxima*
Sn7	Not declared	Agate snail	*Achatina fulica*
Ve1	*Ensis ensis*	common razor clam	*Ensis* spp.
Ve2	*Ruditapes philippinarum*	Venus shell	*Ruditapes philippinarum*
Ve3	Not declared	Cockle	*Cerastoderma edule*
Ve4	*Meretrix lyrata*	Venus shell	*Meretrix lyrata*
Ve5	*Callista Chione*	Venus shell	no evaluation
Ve6	*Ensis ensis*	common razor clam	*Ensis* spp.
Ve7	*Ruditapes philippinarum*	Venus shell	*Ruditapes philippinarum*
Ve8	*Cerastoderma edulis*	Cockle	*Cerastoderma edulis*
Ve9	*Chamelea gallina*	Venus shell	*Chamelea gallina*
Ve10	*Cerastoderma edule*	Cockle	*Cerastoderma edulis*
Ve11	*Ruditapes philippinarum*	carpet shell	*Ruditapes philippinarum*
Ve12	*Ensis ensis*	common razor clam	*Ensis spp.*
Ve13	*Venus spp.*	Venus shell	*Ruditapes philippinarum*
Ve14	*Meretrix lyrata*	Venus shell	*Metrix lyrate*

Ve15	Not declared	Venus shell	*Chamelea gallina*
Ve16	Not declared	Vongole	*Ruditapes philippinarum*
Mi62	*Litopenaeus vannamei* *Pleoticus muelleri* *Uroteuthis duvaucii* *Dosidicus gigas* *Sepia pharaonis* *Sepia aculeata* *Ilex argentinus* *Ilex illecebrosus* *Nototodarus sloanii*	Seafood Mix	not analysed yet
Mi63	Not declared	Sauce with seafood	not analysed yet
Mi64	*Litopenaeus vannamei* *Mytilus chinensis* *Mytilus edulis* *Ilex argentinus*	Seafood Mix	not analysed yet
Mi65	Not declared	Soup Bouillabaisse Marseille style	not analysed yet
Mi66	*Litopenaeus vannamei* *Mytilus chinensis* *Ilex argentinus*	Seafood Mix	not analysed yet
Mi67	*Mytilus spp.* *Loligo gahi,* *Litopenaeus vannamei*		not analysed yet
Mi68	*Dosidicus gigas* *Octopus membranaceus* *Mytilus galloprovincialis* *Litopenaeus vannamei*	Sea fruit salad in sunflower oil	not analysed yet
Mi69	*Mytilus chilensis* *Solenocera melantho* *Loligo duvaucii* *Uroteuthis chinensis* *Spisula subtruncata*	Seafood Mix	not analysed yet
Mi70	Not declared	Sea fruit salad fantasy	not analysed yet
Mi71	Not declared	Seafood Mix	not analysed yet
Mi72	*Loligo gahi* *Mytilus chilensis* *Solenocera melantho* *Paphia Undulata*	Seafood Mix	not analysed yet
Mi73	Not declared	Seafood Mix	not analysed yet
Mi74	Not declared	Seafood Mix	not analysed yet
Mi75	Not declared	Pizza Frutti di mare	not analysed yet
Mi76	Not declared	Paella	not analysed yet
Mi77	*Mytilus edulis* *Mytilus chilensis* *Penaeidae*	Paella	not analysed yet

Mi78	*Mytilus chilensis,* *Illex illecebrosus,* *Litopenaeus vannamei*	Seafood all 'Olio	not analysed yet
Mi79	*Mytilus chilensis* *Paphia undulata* *Penaeus vannamei*	Seafood Mix	not analysed yet
Mi80	*Mytilus chilensis* *Metapenaeus monoceros* *Loligo duvaucii* *Sepia aculeata* *Paphia undulata*	Seafood Mix	not analysed yet
Mi84	*Litopenaeus vannamei* *Uroteuthis chilensis* *Octopus vulgaris* *Mytilus chilensis*	Seafood Mix	not analysed yet
Mi85	Not declared	Instant noodle seafood, mild	not analysed yet
Mi86	Not declared	Instant noodle seafood, spicy	not analysed yet

Fifty-five of the samples, according to their labelling, belong to crustaceans, twenty-six to the cephalopods, seven to the land snails, sixteen to the Venus shells and twenty-two samples are mixed species from different seafood species. In some products, the Latin species names were not declared but could still be assigned to the individual systems because the German name was written.

In the case of crustaceans, the sequencing results demonstrated inconsistencies with the declarations on the product (Cr15, Cr17, Cr19, Cr20, Cr26 and Cr27). Cr15 and Cr20 were probably confusions in the product chain because the species which were found by sequencing are almost much more expensive than the species that were labelled (e.g., *Macrobrachium rosenbergii* approx. 80€/kg and *Penaeus monodon* 180€/kg). Cr26 and Cr27 could also be a mistake, as the two species are similar in price. Cr19 is classic food fraud because a high expensive species is labelled on the product. The sample Cr17 is a very interesting one. The sample could not clearly be identified to belong to a specific species. It could be matched to the indicated species with mismatches. Explanation could be no entry exists for this species yet in NCBI. Further research will be needed to determine the species of this sample.

In the case of cephalopods, a high number of samples demonstrate an inconsistency. According to the "Bundesministerium für Soziales, Gesundheit, Pflege und Konsumentenschutz" (Federal Ministry for Social Affairs, Health, Care and Consumer Protection) in Austria, the reason for this could be that the species are too similar and that it is not possible to distinguish between them. Rough differences are still possible (eight-legged (octopus) from 10-legged (squid) species), but within that, it becomes very difficult. To complicate the situation, all cephalopods are caught in the wild and are rarely farmed by aquaculture.

A different situation is found for snails. Some sequences are unable to be matched to specific species. The cause may be that there are not enough entries in the NCBI. The entries in the NCBI are constantly being expanded. It is possible that within a year (or longer) several land snail sequences are added, which then match our sequences better. This would allow an accurate determination at the species level.

The Venus shells were a challenge in themselves. There are many differences in bivalves' families, not only morphologically, but also in genetic diversity. For this reason, various primers were developed to cover a large number of species. In the case of the Venus shells, however, there were no declarative differences. The sample Ve5 was not satisfactorily analysed. It was a problematic sample, as it did not contain any information about its origin. It would be useful to have a new sample of this species, but in Austria, such a specimen is a delicacy and difficult to collect. All other samples, which could be analysed, were assigned correctly to the respective species.

4.6 Further Outlook

The results show that the metabarcoding systems for crustaceans, cephalopods, land snails, and Venus shells is suitable for discrimination of seafood at the species level. The developed seafood systems are summarised in Figure 13. Of course, further experiments are needed to optimize all systems. A validation as in the case of the metabarcoding system of bivalves has to be done, even analysing down to which concentration crustaceans can be analysed in food. It would also be interesting to know whether the same results can be reached by sequencing with iSeq®. Furthermore, it would be very interesting to check if the system of crustacean is compatible with the system of mollusc as a duplex approach. The first results in this direction were promising. The database must be constantly updated, as new and up-to-date sequences (especially RefSeq) are always being added to NCBI. Additionally, processed foods such as seafood mix, salat and pastes or highly processed foods such as soups, butter, creams, and sauce should be analysed. A large part has been completed, but there are still some parts of the research that are not fully finished. This will be part of a future project.

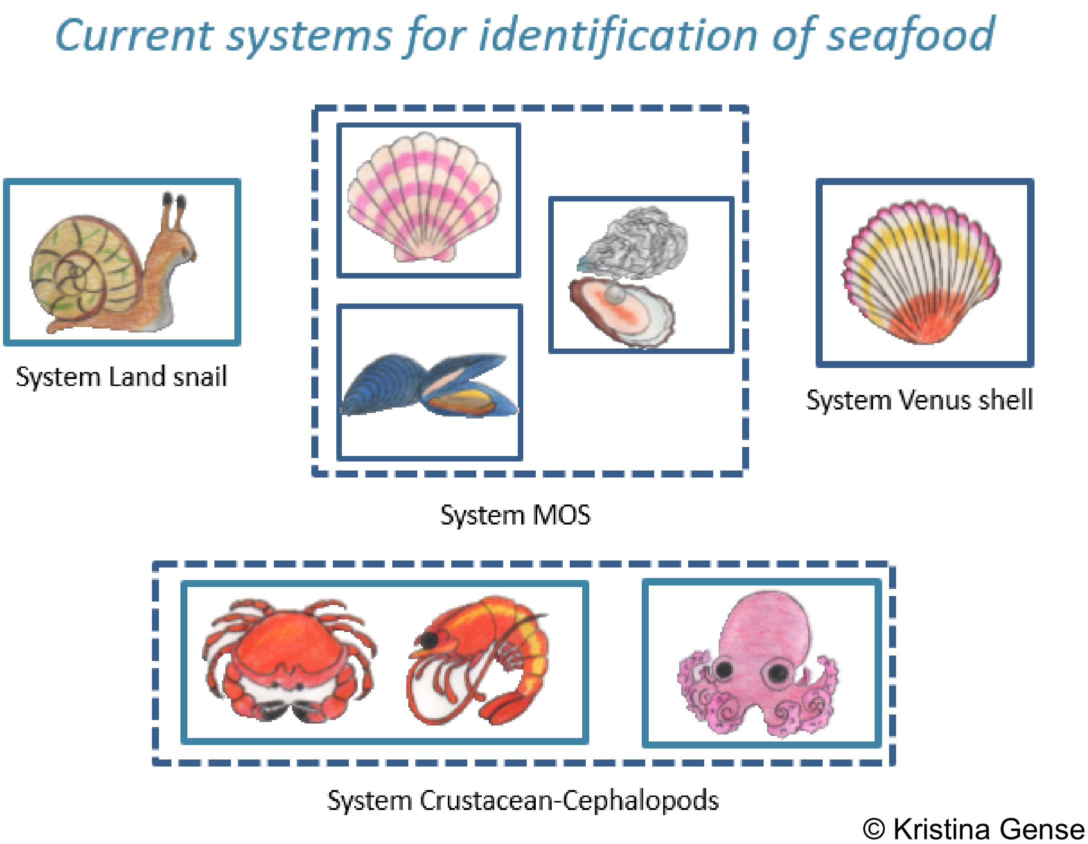


Figure 13 Current systems for seafood identification.

5. Patent

The developed metabarcoding systems were submitted to the European Patent Office.

Patent name: DNA BASED IDENTIFICATION OF SEAFOOD SPECIES IN SAMPLES

Publication number: 21204456.4.

Publication date: 25th October 2021

The abridged version of the patent application is inserted on the following pages (FIELD OF THE INVENTION, BACKGROUND OF THE INVENTION, SUMMARY OF THE INVENTION, CLAIMS, and ABSTRACT). The complete manuscript (of 151 pages) of this invention report can be found online (e.g., https://worldwide.espacenet.com/patent/).

DNA BASED IDENTIFICATION OF SEAFOOD SPECIES IN SAMPLES

FIELD OF THE INVENTION

The present invention relates to the technical field of DNA barcoding, particularly to a method for the identification of seafood species in samples comprising the steps of isolating DNA from a sample, amplifying fragments of the isolated DNA using specific primers, sequencing of the amplified DNA fragments, and identifying the seafood species through sequence comparison with reference sequences. Further provided herein is a primer library and a kit.

BACKGROUND OF THE INVENTION

Food adulteration is a worldwide problem in various food products, e.g., in farm animal, wild animal, seafood, and also plant products. The term of food adulteration is not uniformly defined but in general, it describes misdeclaration of food intending to gain an economic benefit without limits (Robson, K. et al, 2021). Seafood has a high risk of fraud and seafood products are often mislabelled. According to Pardo, M. et al. (2016), up to 27 % of the seafood is mislabelled worldwide. Food adulteration includes, but is not limited to, replacement (a (valuable) ingredient is replaced by one of a lower value), relabelled or incorrectly labelled food. Incorrect labelling can result when different local names are used for the same species, when the same name is used for different species, or due to translation errors.

However, correct labelling of seafood products is important for traceability issues, protection of endangered species, mitigation of illegal fishing, and for individual reasons of end consumers (Rodríguez, E.M. and Ortea, I., 2017).

Correct declaration of seafood is regulated in the European Union. Thereby, international and national regulations exist to ensure legal trade in seafood and seafood products. The EU directive 1379/2013 regulates market organization of fishery and aquaculture products, including correct declaration of seafood. To comply with legal regulations, labels must include both the local trade name in the official language(s) and the correct scientific Latin name (Regulation (EU) No 1379/2013; Regulation (EU) No 1169/2011).

Regardless of clear and strict requirements for species declaration, incorrect labelling of e.g., bivalve products, has repeatedly been detected in Europe (Näumann,

G. et al., 2012; Fernandes, T. et al., 2020). In German and Swiss studies, more than half of the products declared to contain “Jakobsmuschel” (or “Jacobsmuschel“) were labelled incorrectly. Although the German name “Jakobsmuschel” (or “Jacobsmuschel“) may only be used for scallop species of the genus *Pecten*, species of other genera (particularly *Placopecten* and *Mizuhopecten*) were identified in these products (Näumann, G. et al., 2012; Stephan, R. et al., 2014).

Compliance with regulations is especially important since seafood is gaining importance in human nutrition. In 2019, 107.6 billion US $ were made with the marketing of seafood (crustaceans and molluscs), compared to 8.1 billion US $ 30 years ago. In 2019, 1.03 million tons of mussels, scallops, and oysters were caught in nature and more than 10 million tons were cultivated in aquaculture, earning a profit of millions of US dollars. Worldwide, 6.1 million tonnes of crustaceans were caught and 10.5 million tonnes were cultivated in 2019. In the same year, 6.4 million tonnes of molluscs were caught and 17.6 million tonnes were cultivated.

Crustaceans and molluscs are divided into numerous genera comprising a high number of species with a worldwide distribution. A class of molluscs are for example bivalves, wherein *Mytilidae* (mussels), *Pectinidae* (scallops), and *Ostreidae* (oysters) are the most important bivalve species for human consumption. Each of these bivalve species is divided into several genera comprising a high number of species which makes correct identification of seafood species difficult using known methods.

In the case of seafood, especially for bivalves, morphological characteristics such as shell, colour and size may allow correct species classification. However, after shell removal or mechanical processing, classification by morphology may be hampered or even be impossible (Espiñeira, M. et al., 2009; Fernández, A. et al., 2000).

Recently, matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) has been shown to be suitable for accurate species identification of scallops (Stephan, R. et al., 2014). However, MALDI-TOF MS instruments are expensive and do not allow high throughput analysis. Therefore, this methodology is less applicable for routine analyses and for the fast identification of several seafood species.

DNA metabarcoding methods have been recently developed for the identification of mammalian and poultry species in food (Dobrovolny S. et al., 2019).

However, methods which provide comprehensive information on the plurality of seafood species which are present in a food sample are still missing. Thus, there is an urgent need in the field for improved means of identifying several different seafood species in complex and processed foodstuffs suitable for food authentication in routine analysis.

SUMMARY OF THE INVENTION

It is the objective of the present invention to provide improved means and methods for the identification of several different seafood species in food samples.

The objective is solved by the subject matter of the present invention.

The present invention provides a method which is highly suitable for the identification of seafood species of different origin and processing degree in complex food samples. The inventors of the present invention surprisingly discovered a library of primers which can be incorporated in a fast and reliable metabarcoding method for the identification of a plurality of seafood species in food samples.

According to the invention, there is provided a method for identifying seafood species in a sample, comprising the steps of

a) isolating DNA from the sample,
b) amplifying fragments of said DNA with one or more primer sets (ps) selected from the group of
   i. ps 1 for identifying seafood species of the family of Crustacean comprising one or more primer pairs of one forward primer selected from any one of SEQ ID NOs: 1 and 2, and the reverse primer SEQ ID NO: 15 and/or the reverse complement sequences of the primer sequences;
   ii. ps 2 for identifying seafood species of the family of Cephalopods comprising one or more primer pairs of one forward primer selected from any one of SEQ ID NOs: 3 to 5, and the reverse primer SEQ ID NO: 16 and/or reverse complement sequences of the primer sequences;
   iii. ps 3 for identifying seafood species of the family of Gastropoda comprising one or more primer pairs of the forward primer SEQ ID NO: 6, and one reverse primer selected from any one of SEQ ID NOs: 17 to 18 and/or reverse complement sequences of the primer sequences;
   iv. ps 4 for identifying seafood species of the family of Veneridae comprising one or more primer pairs of one forward primer selected from any one of SEQ ID NOs: 7 to 11, and one reverse primer selected from any one of SEQ ID NOs: 19 to 21 and/or reverse complement sequences of the primer sequences;

v. ps 5 for identifying seafood species of the family of Ostreidae comprising the forward primer SEQ ID NO: 12 and the reverse primer SEQ ID NO: 22 and/or reverse complement sequences of the primer sequences;

vi. ps 6 for identifying seafood species of the family of Pectinidae comprising the forward primer SEQ ID NO: 13 and the reverse primer SEQ ID NO: 23 and/or reverse complement sequences of the primer sequences; and

vii. ps 7 for identifying seafood species of the family of Mytilidae comprising one or more primer pairs of the forward primer SEQ ID NO: 14, and one reverse primer selected from any one of SEQ ID NOs: 24 to 25 and/or reverse complement sequences of the primer sequences,

c) sequencing the amplified DNA fragments of step b), and

d) identifying the seafood species by comparison of the sequences obtained by steps a) to c) with reference sequences of seafood species.

Specifically, amplifying of fragments of DNA in step b) is performed by a polymerase chain reaction (PCR), preferably by a PCR comprising 25-30 cycles, more preferably by 25 cycles, and preferably at an annealing temperature of 60-65 °C, more preferably at an annealing temperature of 62°C.

Specifically, amplifying fragments of the DNA in step b) is performed with at least 1, 2, 3, 4, 5, 6, or 7 primer sets.

Specifically, the amplified DNA fragments of step b) are 16S rDNA fragments, preferably the amplified DNA fragments comprise 120bp-220bp of the 16S rDNA.

Specifically, the reference sequences of seafood species comprise the DNA sequence of the 16S rDNA of said seafood species.

More specifically, the reference sequences are SEQ ID NOs: 28 to 1153.

Specifically, the identified species of the family of Crustacean are selected from the following Group 1: *Varuna litterata, Hemisquilla ensigera, Gonodactylus smithii, Pullosquilla thomassini, Chorisquilla trigibbosa, Telmessus acutidens, Lithodes aequispinus, Panulirus echinatus, Jasus paulensis, Jasus caveorum, Parastacus pilimanus, Parastacus brasiliensis, Parastacus defossus, Parastacus nicoleti, Gonodactylus graphurus, Jasus lalandii, Lopholithodes mandtii, Lithodes spp., Lithodes maja, Jasus edwardsii, Panulirus regius, Panulirus pascuensis, Panulirus laevicauda, Panulirus gracilis, Panulirus guttatus, Panulirus femoristriga, Chionoecetes* spp., *Paralomis granulosa, Panulirus* spp., *Scyllarus arctus, Palinurus elephas, Episesarma mederi, Austropotamobius torrentium, Cycloachelous granulatus, Eriocheir recta,* *Cervimunida johni, Achelous floridanus, Portunus sayi, Portunus anceps,, Palinurus mauritanicus, Palinurus charlestoni, Pseudosquilla ciliata, Pleuroncodes monodon, Portunus ventralis, Achelous spinicarpus, Callinectes toxotes, Callinectes danae, Callinectes ornatus, Callinectes marginatus, Callinectes affinis, Callinectes rathbunae, Callinectes bocourti, Callinectes similis, Callinectes bellicosus, Callinectes arcuatus, Metanephrops armatus, Metanephrops mozambicus, Metanephrops japonicus, Metanephrops spp., Metanephrops binghami, Parastacus pugnax, Paranephrops zealandicus, Callinectes exasperatus, Palinurus* spp., *Sagmariasus verreauxi, Metanephrops rubellus, Metanephrops challengeri, Metanephrops neptunus, Metanephrops australiensis, Metanephrops arafurensis, Metanephrops boschmai, Metanephrops formosanus, Metanephrops sinensis, Lithodes ferox, Oratosquillina interrupta, Odontodactylus japonicus, Miyakella nepa, Erugosquilla woodmasoni, Clorida decorata, Dictyosquilla foveolata, Anchisquilla fasciata, Scyllarides herklotsii, Astacus astacus, Portunus hastatus, Achelous ordwayi, Carcinus maenas, Portunus inaequalis, Astacoides madagascariensis, Erimacrus isenbecki, Hemisquilla australiensis, Austrosquilla tsangi, Fallosquilla fallax, Echinosquilla guerinii, Coronis scolopendra, Chorisquilla tweediei, Chorisquilla hystrix, Chorisquilla excavate, Busquilla plantei, Alima pacifica, Alima orientalis, Alachosquilla vicina, Gonodactylellus espinosus, Gonodactylellus affinis, Kempella mikado, Hemisquilla californiensis, Haptosquilla trispinosa, Haptosquilla glyptocercus, Gonodactylus platysoma, Gonodactylaceus falcatus, Gonodactylus childi, Gonodactylellus annularis, Odontodactylus scyllarus, Odontodactylus latirostris, Odontodactylus havanensis, Odontodactylus cultrifer, Neogonodactylus oerstedii, Neogonodactylus bredini, Neogonodactylus bahiahondensis, Lysiosquillina sulcata, Squilla rugosa, Raoulserenea* spp., *Raoulserenea oxyrhyncha, Pseudosquillopsis marmorata, Raoulserenea komaii, Protosquilla folini, Ibacus alticrenatus, Scyllarides nodifer, Scyllarides haanii, Scyllarides brasiliensis, Taku spinosocarinatus, Jasus frontalis, Procambarus paeninsulanus, Puerulus sewelli, Panulirus polyphagus, Panulirus longipes., Panulirus interruptus, Panulirus marginatus, Ibacus peronii, Ibacus chacei, Faxonella clypeata, Fallicambarus kountzeae, Arenaeus mexicanus, Cambarus tartarus, Chionoecetes tanneri, Thenus unimaculatus, Thenus indicus, Haptosquilla hamifera, Lithodes turritus, Bouchardina robisoni, Troglocambarus maclanei, Hobbseus yalobushensis, Hobbseus prominens, Charybdis* spp., *Hobbseus petilus, Faxonella creaseri, Thranita danae., Monomia petrea, Neogonodactylus wennerae, Xiphonectes pseudohastatoides, Gonodactylellus* *viridis, Gonodactylaceus ternatensis, Belosquilla laevis, Procambarus okaloosae, Procambarus morrisi, Procambarus milleri, Procambarus mancus, Procambarus lunzi, Hobbseus cristatus, Procambarus acutissimus, Faxonius pagei, Manningia pilaensis, Pontastacus leptodactylus, Procambarus zonangulus, Procambarus youngi, Procambarus seminolae, Procambarus pycnogonopodus, Procambarus orcinus, Procambarus pallidus, Alima maxima, Scyllarides deceptor, Monomia argentata, Xiphonectes pulchricristatus, Paralithodes platypus, Lopholithodes foraminatus, Faughnia formosae, Faughnia profunda, Bathysquilla crassispinosa, Eriocheir sinensis, Harpiosquilla harpax, Callinectes sapidus, Squilla mantis, Portunus trituberculatus, Panulirus japonicus, Cancer pagurus, Chionoecetes japonicus, Scylla tranquebarica, Scylla serrata, Eriocheir japonica, Eriocheir hepuensis, Cherax destructor, Squilla empusa, Lysiosquillina maculata, Gonodactylus chiragra, Panulirus homarus, Homarus americanus, Panulirus ornatus, Oratosquilla oratoria, Panulirus stimpsoni, Charybdis japonica, Scylla paramamosain, Scylla olivacea, Cherax quadricarinatus, Cherax cainii, Paralithodes brevipes, Paralithodes camtschaticus, Scyllarides latus, Procambarus clarkii, Procambarus fallax, Homarus gammarus, Thenus orientalis, Lithodes nintokuae, Cherax cairnsensis, Cherax dispar, Cherax quinquecarinatus, Cherax robustus, Cherax monticola, Cherax glaber, Cherax holthuisi, Astacopsis gouldi, Portunus pelagicus, Paranephrops planifrons, Nephrops norvegicus, Ibacus ciliatus, Charybdis feriata, Metanephrops sibogae, Panulirus cygnus, Metanephrops thomsoni, Faxonius limosus, Squilloides leptosquilla, Cherax bicarinatus, Austropotamobius pallipes, Cherax tenuimanus, Cherax boesemani, Charybdis (Charybdis) natator, Procambarus acutus, Pacifastacus leniusculus, Munida gregaria, Panulirus versicolor, Faxonius rusticus, Portunus sanguinolentus, Procambarus alleni, Metacarcinus magister, Puerulus angulatus, Lupocycloporus gracilimanus, Monomia gladiator, Varuna yui, Panulirus argus, Munida isos, Scyllarides squammosus, Cambaroides similis, Charybdis bimaculata, Cambarus robustus, Thalamita sima, Thranita crenata, Orconectes luteus, Orconectes punctimanus, Orconectes sanbornii, Cherax* spp.*, Cherax crassimanus, Cherax preissii, Munida spinosa, Munida asprosoma, Munida leagora, Munida alonsoi, Munida taenia, Munida gordoae, Munida zebra, Munida distiza, Munida psamathe, Munida thoe, Munida guttata*, *Munida stia, Munida ommata, Munida roshanei, Munida compressa, Munida clinata, Munida chydaea, Munida compacta, Munida eclepsis, Munida tyche, Munida philippinensis, Munida armilla, Munida mesembria, Munida spilota, Munida benguela, Munida endeavourae, Munida agave, Munida idyia, Munida* *militaris, Munida flinti, Munida congesta, Munida rubridigitalis, Munida iris, Munida microphthalma, Munida rufiantennulata, Munida pusilla, Munida remota, Munida leptosyne, Munida rosula, Munida munin, Munida valida, Munida proto, Enriquea leviantennata, Munida multilineata, Munida pagesi, Munida stomifera, Munida quadrispina, Munida tiresias, Munida psylla, Munida heteracantha, Paralomis formosa, Paralomis spinosissima, Paralomis birsteini, Paralomis hirtella, Scyllarus subarctus, Scyllarus pygmaeus, Scyllarus chacei, Scyllarus caparti, Scyllarus americanus, Episesarma palawanense, Episesarma singaporense, Austropotamobius fulcisianus orientalis, Achelous tumidulus, Achelous asper, Achelous sebae, Portunus acuminatus, Achelous tuberculatus, Achelous iridescens, Portunus xantusii, Achelous depressifrons, Achelous rufiremus, Achelous gibbesii, Portunus minimus, Achelous stanfordi, Achelous brevimanus, Portunus affinis, Achelous angustus, Achelous binoculus, Oratosquillina inornata, Oratosquillina asiatica, Oratosquillina anomala, Oratosquillina perpensa, Erugosquilla graham, Busquilla quadraticauda, Kempella stridulans, Gonodactylaceus graphurus, Gonodactylaceus randalli, Carcinus aestuarii, Menippe rumphii, Menippe nodifrons, Menippe* spp.*, Procambarus liberorum, Procambarus toltecae, Procambarus curdi, Procambarus digueti, Procambarus nigrocinctus, Procambarus versutus, Procambarus gibbus, Cambarus pecki, Procambarus geminus, Charybdis acuta, Creaserinus fodiens, Fallicambarus jeanae, Creaserinus gordoni, Creaserinus caesius, Fallicambarus dissitus, Creaserinus danielae, Fallicambarus oryktes, Fallicambarus byersi, Creaserinus burrisi, Creaserinus gilpini, Fallicambarus harpi, Fallicambarus macneesei, Fallicambarus petilicarpus, Fallicambarus wallsi, Fallicambarus strawni, Fallicambarus devastator, Fallicambarus houstonensis, Fallicambarus hortoni, Arenaeus cribrarius, Cambarus* spp.*, Cambarus deweesae, Cambarus striatus, Cambarus graysoni, Cambarus monongalensis, Cambarus pyronotus, Cambarus maculatus, Cambarus latimanus, Cambarus strigosus, Cambarus parrishi, Cambarus bouchardi, Cambarus fasciatus, Cambarus harti, Cambarus nerterius, Cambarus setosus, Cambarus batchi, Cambarus halli,, Cambarus unestami, Cambarus reburrus, Cambarus gentry, Cambarus hubbsi, Cambarus friaufi, Cambarus obeyensis, Cambarus cracens, Cambarus asperimanus, Cambarus hobbsorum, Cambarus williami, Cambarus howardi, Cambarus obstipus, Cambarus girardianus, Cambarus cryptodytes, Cambarus sciotensis, Cambarus georgiae, Cambarus pristinus, Cambarus aculabrum, Cambarus englishi, Cambarus brachydactylus, Cambarus cumberlandensis, Cambarus dubius, Cambarus reflexus, Cambarus scotti, Cambarus longirostris, Cambarus* *hubrichti, Monomia lucida, Faughnia serenei, Harpiosquilla melanoura, Harpiosquilla annandalei, Cherax cuspidatus, Cherax paniaicus, Cherax lorentzi, Cherax albertisii, Cherax rotundus, Cherax leckii, Cherax murido, Cherax wasselli, Cherax parvus, Cherax pallidus, Cherax cartalacoolah, Cherax rhynchotus, Cherax pulcher, Cherax peknyi, Cherax setosus, Cherax misolicus, Cherax warsamsonicus, Cherax snowden, Cherax boschmai, Cherax nucifraga, Cherax barrette, Oratosquilla fabricii, Astacopsis tricornis, Thalamita admete, Faxonius virilis, Thranita prymna, Astacopsis franklinii, Cambaroides schrenckii, Orconectes australis, Thalamita chaptalii, Zygita longifrons, Thalamita picta, Thalamita seurati, Thranita pelsarti, Orconectes barri, Faxonius ronaldi, Faxonius neglectus, Orconectes compressus, Orconectes forceps, Orconectes pellucidus, Neoeriocheir leptognathus, Penaeus kerathurus, Penaeus marginatus, Penaeus longistylus, Penaeus plebejus, Metapenaeopsis liui, Metapenaeopsis lamellata, Metapenaeopsis acclivis, Metapenaeopsis commensalis, Atypopenaeus stenodactylus, Aristeus antillensis, Solenocera vioscai, Penaeus chinensis, Penaeus spp., Metapenaeopsis barbata, Penaeus esculentus, Heteropenaeus longimanus, Atypopenaeus dearmatus, Funchalia taaningi, Xiphopenaeus kroyeri, Trachypenaeopsis mobilispinis, Rimapenaeus similis, Parapenaeus politus, Solenocera membranacea, Alcockpenaeopsis hungerfordii, Batepenaeopsis tenella, Pandalus platyceros, Metapenaeus moyebi, Metapenaeus joyneri, Pandalus montagui, Penaeus brasiliensis, Aristeus antennatus, Heterocarpus laevigatus, Heterocarpus lepidus, Funchalia villosa, Hemipenaeus carpenter, Mesopenaeus tropicalis, Pelagopenaeus balboae, Penaeus hathor, Metapenaeopsis provocatoria, Aristeus virilis, Aristeus alcock, Penaeus aztecus, Heterocarpus abulbus, Penaeus setiferus, Cerataspis monstrosus, Pleoticus robustus, Aristaeopsis edwardsiana, Solenocera necopina,Parapenaeus cayrei, Parapenaeus fissurus, Parapenaeus investigatoris, Parapenaeus fissuroides, Parapenaeus americanus, Heterocarpus ensifer, Kishinouyepenaeopsis cornuta, Parapenaeus perezfarfantae, Parapenaeus murrayi, Parapenaeus longipes, Parapenaeus spp., Heterocarpus chani, Heterocarpus sibogae, Heterocarpus dorsalis, Metapenaeopsis andamanensis, Metapenaeopsis coniger, Macrobrachium idella, Trachysalambria brevisuturae, Trachysalambria aspera, Trachysalambria albicoma, Euphausia superba, Solenocera hextii, Hymenopenaeus equalis, Rimapenaeus constrictus, Crangon crangon, Trachypenaeus anchoralis, Megokris spp., Trachysalambria longipes, Trachysalambria starobogatovi, Trachysalambria nansei, Trachysalambria malaiana, Trachysalambria spp.,* *Trachysalambria parvispina, Crangon uritai, Pandalus borealis, Metapenaeus monoceros, Pandalus nipponensis, Hadropenaeus lucasii, Ganjampenaeopsis uncta, Solenocera annectens, Solenocera melantho, Parapenaeopsis stylifera, Penaeus japonicus, Penaeus brevirostris, Penaeus notialis, Penaeus duorarum, Penaeus schmitti, Artemesia longinaris, Penaeus subtilis, Penaeus stylirostris, Penaeus vannamei, Macrobrachium rosenbergii, Penaeus monodon, Pandalus hypsinotus, Heterocarpus* spp.*, Pandalus jordani, Macrobrachium bullatum, Penaeus merguiensis, Metapenaeus ensis, Acetes chinensis, Macrobrachium nipponense, Penaeus californiensis, Macrobrachium lanchesteri, Pleoticus muelleri, Metapenaeus affinis, Hymenopenaeus neptunus, Penaeus indicus, Aristaeomorpha foliacea, Solenocera* spp.*, Mierspenaeopsis hardwickii, Penaeus latisulcatus, Penaeus semisulcatus, Penaeus isabelae, Sicyonia lancifer, Metapenaeopsis dalei, Metapenaeopsis gerardoi, Parapenaeus longirostris, Pandalus eous, Pandalus miyakei, Pandalus japonicas, Pandalus glabrus, Pandalus teraoi, Pandalus ivanovi, Pandalus coccinatus, Pandalus formosanus, Pandalus chani, Pandalus* spp.*, Pandalus longirostris, Pandalus latirostris, Metapenaeus* spp.*, Palaemon* spp.*, Palaemon serratus, Macrobrachium nipponense, Palaemon capensis, Palaemon sinensis ,Palaemon annandalei, Palaemon gravieri, Palaemon serenus, Palaemon carinicauda, Palaemon pugio, Palaemon pandaliformis, Palaemon elegans, Palaemon longirostris, Palaemon peringueyi, Palaemon debilis, Palaemon carteri, Palaemon ritteri, Palaemon orientis, Macrobrachium gracilirostre, Palaemon vulgaris, Palaemon serrifer, Palaemon varians, Palaemon macrodactylus, Palaemon tonkinensis, Palaemon xiphias, Palaemon ivonicus, Palaemon pacificus, Palaemon atrinubes, Palaemon intermedius, Palaemon concinnus, Palaemon yuna, Palaemon antennarius, Palaemon dolospinus, Palaemon gracilis, Palaemon mundusnovus, Palaemon suttkusi, Palaemon zariquieyi, Macrobrachium australiense, Palaemon semmelinkii, Palaemon litoreus, Palaemon septemtrionalis, Palaemon guangdongensis, Palaemon hancocki, Palaemon vietnamicus, Palaemon texanus, Palaemon ortmanni, Palaemon turcorum, Palaemon kadiakensis, Macrobrachium asperulum, Macrobrachium australe, Macrobrachium olfersii, Macrobrachium jelskii, Macrobrachium villosimanus, Macrobrachium equidens, Macrobrachium potiuna, Macrobrachium malcolmsonii, Macrobrachium superbum, Macrobrachium striatum, Macrobrachium latidactylus, Macrobrachium hancocki, Macrobrachium acanthurus, Macrobrachium inflatum, Macrobrachium crenulatum, Macrobrachium carcinus, Macrobrachium americanum, Macrobrachium latimanus, Macrobrachium* *mammillodactylus, Macrobrachium faustinum, Macrobrachium heterochirus, Macrobrachium scabriculum, Macrobrachium digueti, Macrobrachium tenellum, Macrobrachium idae, Macrobrachium formosense, Macrobrachium dienbienphuense, Macrobrachium placidulum, Macrobrachium sintangense, Macrobrachium niphanae, Macrobrachium totonacum, Macrobrachium tuxtlaense, Macrobrachium vicconi, Macrobrachium villalobosi, Macrobrachium amazonicum, Macrobrachium canarae, Macrobrachium tratense, Macrobrachium forcipatum, Macrobrachium hirsutimanus, Macrobrachium borellii, Macrobrachium brasiliense, Macrobrachium aemulum, Macrobrachium handschini, Macrobrachium horstii, Macrobrachium ferreirai, Macrobrachium lanatum, Macrobrachium novaehollandiae, Macrobrachium tolmerum, Macrobrachium iheringi, Macrobrachium saigonense, Macrobrachium nattereri, Macrobrachium aracamuni, Macrobrachium inpa, Macrobrachium depressimanum, Macrobrachium surinamicum, Macrobrachium denticulatum, Macrobrachium pilimanus, Macrobrachium ohione, Macrobrachium hainanense, Macrobrachium lepidactyloides, Macrobrachium jaroense, Macrobrachium esculentum, Macrobrachium maculatum, Macrobrachium edentatum, Macrobrachium grandimanus, Macrobrachium malayanum, Macrobrachium meridionale, Macrobrachium neglectum, Macrobrachium platycheles, Macrobrachium naso, Macrobrachium placidum, Macrobrachium yui, Macrobrachium shokitai, Macrobrachium sundaicum, Macrobrachium rude, Macrobrachium lamarrei, Macrobrachium sankolli, Macrobrachium gangeticum, Trachysalambria palaestinensis, Euphausia pacifica, Euphausia lucens, Euphausia vallentini, Euphausia triacantha, Euphausia longirostris, Euphausia similis, Euphausia recurve, Euphausia krohni, Euphausia frigida, Euphausia gibboides, Euphausia eximia, Euphausia americana, Euphausia tenera, Euphausia pseudogibba, Euphausia hemigibba, Euphausia brevis, Hymenopenaeus debilis* and *Nematopalaemon tenuipes* (Group 1).

Specifically, the identified species of the family of Cephalopods are selected from the following Group 2: *Loligo forbesii, Nototodarus sloanii, Sepia* spp.*, Sepia lorigera, Sepia pardex, Rossia pacifica, Berryteuthis magister, Eledone massyae, Sepia robsoni, Loligo reynaudii, Doryteuthis (Amerigo) pealeii, Doryteuthis (Amerigo) gahi, Sepiola rondeletii, , Adinaefiola ligulata, Sepia smithi, Sepia elliptica, Eledone palari, Eledone moschata, Rossia palpebrosa, Gonatus madokai, Gonatus kamtschaticus, Eledone cirrhosa, Sepia elegans, Rossia bipillata, Sepiola atlantica, Lolliguncula (Lolliguncula) panamensis, Octopus maya, Illex illecebrosus, Nototodarus gouldi, Gonatopsis octopedatus, Illex coindetii, Berryteuthis anonychus, Gonatus fabricii, Lusepiola* *birostrata, Octopus tetricus, Uroteuthis (Photololigo) sibogae, Doryteuthis (Doryteuthis) pleii, Doryteuthis sanpaulensis, Doryteuthis (Amerigo) surinamensis, Octopus hubbsorum, Macrotritopus defilippi, Octopus insularis, Loliolus (Nipponololigo) sumatrensis, Sepia recurvirostra, Sepia madokai, Sepia kobiensis, Amphioctopus aegina, Sepia officinalis, Sepioteuthis lessoniana, Todarodes pacificus, Octopus vulgaris, Heterololigo bleekeri, Octopus sinensis, Octopus americanus, Narrowteuthis nesisi, Ommastrephes bartramii, Sepiella japonica, Uroteuthis (Photololigo) edulis, Doryteuthis (Amerigo) opalescens, Architeuthis dux, Dosidicus gigas, Sepia esculenta, Amphioctopus fangsiao, Loligo vulgaris, Sepiola* spp.*, Octopus mimus, Octopus* spp.*, Octopus bimaculoides, Uroteuthis (Photololigo) chinensis, Uroteuthis (Photololigo) duvaucelii, Illex argentinus, Sepia aculeata, Sepiella inermis, Sepia lycidas, Sepia latimanus, Sepia apama, Sepia pharaonis, Loliolus (Nipponololigo) beka, Alloteuthis subulata, Nototodarus hawaiiensis, Sepia orbignyana, Sepia papuensis, Rossia macrosoma, Lolliguncula (Lolliguncula) brevis, Lolliguncula (Loliolopsis) diomedeae, Afrololigo mercatoris, Octopus bimaculatus, Octopus cyanea, Callistoctopus ornatus, Enteroctopus megalocyathus,, Sasakiopus salebrosus, Octopus berrima, Amphioctopus marginatus, Octopus maorum, Octopus fitchi, Amphioctopus neglectus, Loliolus (Nipponololigo) uyii, Loliolus (Nipponololigo) japonica, Bathyteuthis abyssicola, Semirossia patagonica, Cistopus taiwanicus, Sthenoteuthis oualaniensis, Watasenia scintillans, Gonatopsis okutanii, Uroteuthis (Aestuariolus) noctiluca, Sepioteuthis australis, Sepioteuthis sepioidea, Amphioctopus kagoshimensis, Amphioctopus membranaceus, Amphioctopus exannulatus, Amphioctopus rex* and *Sepia peterseni* (Group 2).

Specifically, the identified species of the family of Gastropoda are selected from the following Group 3: *Helix pomatia, Achatina fulica*, *Helix aspersa*, *Helix aspersa maxima*, *Helix thessalica*, *Helix lucorum, Helix nicaeensis, Achatina reticulata, Helix aperta, Helix albescens, Tyrrhenaria ceratina, Helix vladika, Helix* spp.*, Pleurodonte discolor, Pleurodonte lychnuchus, Erctella mazzullii, Erctella cephalaeditana, Pleurodonte formosa, Helix christophi, Helix nordmanni, Pleurodonte nucleola, Pleurodonte parilis, Gonostomopsis auridens, Caracolus caracollus, Lacteoluna selenina, Cernuella cisalpine, Cochlicella acuta, Disculella maderensis, Dialeuca nemoraloides, Monadenia fidelis, Cepaea nemoralis, Sphincterochila candidissima, Microphysula ingersolli, Helicodonta obvoluta* and *Cernuella virgata* (Group 3).

Specifically, the identified species of the family of Veneridae are selected from the following Group 4: *Tridacna mbalavuana***,** *Siliqua alta***,** *Megangulus zyonoensis, Megangulus venulosus, Donax faba, Donax cuneatus, Donax kiusiuensis, Mactra quadrangularis, Ensis ensis, Chamelea gallina, Spisula subtruncata, Polititapes rhomboides, Callista chione, Venerupis corrugata, Polititapes aureus, Venus crebrisulca, Mercenaria campechiensis, Antigona lamellaris, Ameghinomya antiqua, Ameghinomya* spp.*, Callista erycina, Venerupis aspera, Paphia philippiana, Venus casina, Ensis* spp.*, Mactra stultorum, Ensis macha, Siliqua minima, Ensis leei, Polititapes durus, Cerastoderma glaucum, Tridacna* spp.*, Donax longissimus, Solen vaginoides, Venus verrucosa, Ezocallista brevisiphonata,, Procardium indicum, Cardium maxicostatum, Cardium costatum, Acanthocardia paucicostata, Acanthocardia echinata, Acanthocardia aculeata, Solen* spp.*, Ruditapes philippinarum, Corculum cardissa, Spisula solida, Scrobicularia plana, Mactra spp., Chamelea striatula, Ensis siliqua, Serripes groenlandicus, Tridacna elongatissima, Tridacna rosewateri, Meretrix lamarckii, Meretrix lusoria, Paphia euglypta, Meretrix* spp.*, Acanthocardia tuberculata, Tridacna maxima, Lutraria rhynchaena, Meretrix lyrata, Arctica islandica, Solen strictus, Paratapes undulatus, Paratapes textilis, Paphia amabilis, Solen grandis, Lutraria maxima, Donax vittatus, Donax variegatus, Donax trunculus, Donax semistriatus, Ruditapes decussatus, Cerastoderma edule, Tridacna squamosa, Mactra chinensis* and *Mercenaria mercenaria* (Group 4)**.**

Specifically, the identified species of the family of Ostreidae are selected from the following Group 5: *Magallana bilineata, Magallana gigas, Crassostrea virginica, Magallana* spp.*, Magallana angulata, Magallana sikamea, Magallana ariakensis, Ostrea denselamellosa, Magallana nippona, Ostrea edulis, Crassostrea* spp., *Crassostrea tulipa, Ostrea angasi, Magallana belcheri, Crassostrea rhizophorae,, Talonostrea talonata, Crassostrea corteziensis, Ostrea* spp.*, Ostrea chilensis, Ostrea algoensis, Ostrea megodon, Saccostrea cuccullata, Saccostrea palmula, Saccostrea malabonensis, Saccostrea scyphophilla, Saccostrea kegaki,* and *Saccostrea* spp*.* (Group 5)*.*

Specifically, the identified species of the family of Pectinidae are selected from the following Group 6: *Euvola* spp.*, Mimachlamys crassicostata, Gloripallium pallium, Flexopecten glaber,, Aequipecten opercularis, Nodipecten nodosus, Scaeochlamys livida, Pecten* spp.*, Talochlamys multistriata, Patinopecten caurinus, Chlamys behringiana, Placopecten septemradiatus, Pecten maximus, Zygochlamys delicatula,* *Chlamys hastata, Ylistrum japonicum, Talochlamys gemmulata, Zygochlamys patagonica, Argopecten purpuratus, Argopecten irradians, Azumapecten farreri, Mizuhopecten yessoensi, Placopecten magellanicus, Chlamys islandica, Argopecten ventricosus, Mimachlamys varia, Amusium pleuronectes, Mimachlamys sanguinea, Talochlamys dichroa, Mimachlamys gloriosa, Mimachlamys cloacata, Mimachlamys asperrima, Annachlamys striatula, Decatopecten radula, Bractechlamys vexillum, Aequipecten glyptus, Scaeochlamys lemniscata, Chlamys rubida, Karnekampia sulcata, Crassadoma gigantea,* and *Ylistrum balloti* (Group 6).

Specifically, the identified species of the family of Mytilidae are selected from the following Group 7: *Mytilus* spp., *Perna perna, Mytilus unguiculatus, Perna viridis, Mytilus californianus, Mytilus trossulus, Mytilus galloprovincialis, Mytilus edulis and Perna canaliculus* (Group 7).

Further provided herein is a kit for identifying seafood species in a sample, comprising one or more primer sets selected from the group of

i. ps 1 for identifying seafood species of the family of Crustacean comprising one or more primer pairs of one forward primer selected from any one of SEQ ID NOs: 1 and 2, and the reverse primer SEQ ID NO: 15 and/or the reverse complement sequences of the primer sequences;

ii. ps 2 for identifying seafood species of the family of Cephalopods comprising one or more primer pairs of one forward primer selected from any one of SEQ ID NOs: 3 to 5, and the reverse primer SEQ ID NO: 16 and/or reverse complement sequences of the primer sequences;

iii. ps 3 for identifying seafood species of the family of Gastropoda comprising one or more primer pairs of the forward primer SEQ ID NO: 6, and one reverse primer selected from any one of SEQ ID NOs: 17 to 18 and/or reverse complement sequences of the primer sequences;

iv. ps 4 for identifying seafood species of the family of Veneridae comprising one or more primer pairs of one forward primer selected from any one of SEQ ID NOs: 7 to 11, and one reverse primer selected from any one of SEQ ID NOs: 19 to 21 and/or reverse complement sequences of the primer sequences;

v. ps 5 for identifying seafood species of the family of Ostreidae comprising the forward primer SEQ ID NO: 12 and the reverse primer SEQ ID NO: 22 and/or reverse complement sequences of the primer sequences;

vi. ps 6 for identifying seafood species of the family of Pectinidae comprising the forward primer SEQ ID NO: 13 and the reverse primer SEQ ID NO: 23 and/or reverse complement sequences of the primer sequences; and

vii. ps 7 for identifying seafood species of the family of Mytilidae comprising one or more primer pairs of the forward primer SEQ ID NO: 14, and one reverse primer selected from any one of SEQ ID NOs: 24 to 25 and/or reverse complement sequences of the primer sequences,

optionally further comprising PCR components, buffers, reagents and/or an instruction manual.

Further provided herein is a library of primer sequences comprising any one of SEQ ID NOs: 1 to 25.

CLAIMS

1. A method for identifying seafood species in a sample, comprising the steps of

a) isolating DNA from the sample,

b) amplifying fragments of said DNA with one or more primer sets (ps) selected from the group of

i. ps 1 for identifying seafood species of the family of Crustacean comprising one or more primer pairs of one forward primer selected from any one of SEQ ID NOs: 1 and 2, and the reverse primer SEQ ID NO: 15 and/or the reverse complement sequences of the primer sequences;

ii. ps 2 for identifying seafood species of the family of Cephalopods comprising one or more primer pairs of one forward primer selected from any one of SEQ ID NOs: 3 to 5, and the reverse primer SEQ ID NO: 16 and/or reverse complement sequences of the primer sequences;

iii. ps 3 for identifying seafood species of the family of Gastropoda comprising one or more primer pairs of the forward primer SEQ ID NO: 6, and one reverse primer selected from any one of SEQ ID NOs: 17 to 18 and/or reverse complement sequences of the primer sequences;

iv. ps 4 for identifying seafood species of the family of Veneridae comprising one or more primer pairs of one forward primer selected from any one of SEQ ID NOs: 7 to 11, and one reverse primer selected from any one of SEQ ID NOs: 19 to 21 and/or reverse complement sequences of the primer sequences;

v. ps 5 for identifying seafood species of the family of Ostreidae comprising the forward primer SEQ ID NO: 12 and the reverse primer SEQ ID NO: 22 and/or reverse complement sequences of the primer sequences;

vi. ps 6 for identifying seafood species of the family of Pectinidae comprising the forward primer SEQ ID NO: 13 and the reverse primer SEQ ID NO: 23 and/or reverse complement sequences of the primer sequences; and

vii. ps 7 for identifying seafood species of the family of Mytilidae comprising one or more primer pairs of the forward primer SEQ ID NO: 14, and one reverse primer selected from any one of SEQ ID NOs: 24 to 25 and/or reverse complement sequences of the primer sequences,

c) sequencing the amplified DNA fragments of step b), and

d) identifying the seafood species by comparison of the sequences obtained by steps a) to c) with reference sequences of seafood species.

2. The method of claim 1, wherein amplifying of fragments of DNA in step b) is performed by a polymerase chain reaction (PCR), preferably by a PCR comprising 25-30 cycles, more preferably by 25 cycles, and preferably at an annealing temperature of 60-65 °C, more preferably at an annealing temperature of 62°C.

3. The method of claim 1 or 2, wherein amplifying fragments of the DNA in step b) is performed with at least 1, 2, 3, 4, 5, 6, or 7 primer sets.

4. The method of any one of claims 1 to 3, wherein the amplified DNA fragments of step b) are 16S rDNA fragments, preferably the amplified DNA fragments comprise 120bp-220bp of the 16S rDNA.

5. The method of any one of claims 1 to 4, wherein the reference sequences of seafood species comprise the DNA sequence of the 16S rDNA of said seafood species.

6. The method of claim 5, wherein the reference sequences are SEQ ID NOs: 28 to 1153.

7. The method of any one of claims 1 to 6, wherein the identified species of the family of Crustacean are selected from Group 1.

8. The method of any one of claims 1 to 6, wherein the identified species of the family of Cephalopods are selected from Group 2.

9. The method of any one of claims 1 to 6, wherein the identified species of the family of Gastropoda are selected from Group 3.

10. The method of any one of claims 1 to 6, wherein the identified species of the family of Veneridae are selected from Group 4.

11. The method of any one of claims 1 to 6, wherein the identified species of the family of Ostreidae are selected from Group 5.

12. The method of any one of claims 1 to 6, wherein the identified species of the family of Pectinidae are selected from Group 6.

13. The method of any one of claims 1 to 6, wherein the identified species of the family of Mytilidae are selected from Group 7.

14. A kit for identifying seafood species in a sample, comprising one or more primer sets (ps) selected from the group of

i. ps 1 for identifying seafood species of the family of Crustacean comprising one or more primer pairs of one forward primer selected from any one of SEQ ID NOs:

1 and 2, and the reverse primer SEQ ID NO: 15 and/or the reverse complement sequences of the primer sequences;

ii. ps 2 for identifying seafood species of the family of Cephalopods comprising one or more primer pairs of one forward primer selected from any one of SEQ ID NOs: 3 to 5, and the reverse primer SEQ ID NO: 16 and/or reverse complement sequences of the primer sequences;

iii. ps 3 for identifying seafood species of the family of Gastropoda comprising one or more primer pairs of the forward primer SEQ ID NO: 6, and one reverse primer selected from any one of SEQ ID NOs: 17 to 18 and/or reverse complement sequences of the primer sequences;

iv. ps 4 for identifying seafood species of the family of Veneridae comprising one or more primer pairs of one forward primer selected from any one of SEQ ID NOs: 7 to 11, and one reverse primer selected from any one of SEQ ID NOs: 19 to 21 and/or reverse complement sequences of the primer sequences;

v. ps 5 for identifying seafood species of the family of Ostreidae comprising the forward primer SEQ ID NO: 12 and the reverse primer SEQ ID NO: 22 and/or reverse complement sequences of the primer sequences;

vi. ps 6 for identifying seafood species of the family of Pectinidae comprising the forward primer SEQ ID NO: 13 and the reverse primer SEQ ID NO: 23 and/or reverse complement sequences of the primer sequences; and

vii. ps 7 for identifying seafood species of the family of Mytilidae comprising one or more primer pairs of the forward primer SEQ ID NO: 14, and one reverse primer selected from any one of SEQ ID NOs: 24 to 25 and/or reverse complement sequences of the primer sequences,

optionally further comprising PCR components, buffers, reagents and/or an instruction manual.

15. A library of primer sequences comprising any one of SEQ ID NOs: 1 to 25.

ABSTRACT

A method for identifying seafood species in a sample, comprising the steps of a) isolating DNA from the sample, b) amplifying fragments of the isolated DNA with one or more primer sets, selected from the group of ps 1 for identifying seafood species of the family of Crustacean, ps 2 for identifying seafood species of the family of Cephalopods, ps 3 for identifying seafood species of the family of Gastropoda, ps 4 for identifying seafood species of the family of Veneridae, ps 5 for identifying seafood species of the family of Ostreidae, ps 6 for identifying seafood species of the family of Pectinidae, and ps 7 for identifying seafood species of the family of Mytilidae, c) sequencing the amplified DNA fragments of step b), and d) identifying the seafood species by comparison of the sequences obtained by steps a) to c) with reference sequences of seafood species. Further provided is a primer library and a kit.

6. Official work presentation

9th October 2018	Presentation for evaluation 30. KM FFoQSI at IFA Tulln
7-9th November 2018	First FFoQSI Annual Assembly
15th January 2019	Progress report at the Department of Analytical Chemistry, Faculty of Chemistry, University of Vienna
9th September 2019	Presentation for evaluation, 34. KM FFoQSI at LC OÖ
10-11th October 2019	Second FFoQSI Annual Assembly
05th November 2019	ecoplus Clusterland Award 2019
10th December 2019	Presentation at LVA
08th October 2020	Presentation at LVA
12th November 2020	Third FFoQSI Annual Assembly (Zoom videoconference)
Once a semester	Presentation at the Vet Med University of Vienna, Institute for food safety, food technology and veterinary public health, unit for food microbiology

Like so many things it is not what is on the outside, but what inside that counts

- Disney's Aladdin



SUCCESS STORY

Forschung wirkt.

FFoQSI
Austrian Competence Centre for Feed and Food Quality, Safety and Innovation

Programm: COMET – Competence Centers for Excellent Technologies

Förderlinie: COMET-Zentrum (K1)

Projekt: Animal Species
Laufzeit: 01.03.2018-30.03.2021
Strategisches Projekt

Was ist das auf meinem Teller?

© Kristina Gense

GEHEIME SPEZIES IM ESSEN UND WIE SIE ZU FINDEN SIND

EIN NEUES METABARCODING SYSTEM ZEIGT FALSCHDEKLARATIONEN VON MEERESFRÜCHTEN AUF UND SORGT FÜR MEHR SICHERHEIT UND TRANSPARENZ.

Sie kennen sicher die Situation, wenn sie sich fragen „Was befindet sich hier auf meinem Teller?“ Sie schauen es sich von allen Seiten an, nehmen einen Bissen, können es aber immer noch nicht zuordnen.

Optisch ist es sehr schwierig eine Aussage darüber zu treffen, welche Bestandteile in einem verarbeiteten Lebensmittel enthalten sind. Können wir darauf vertrauen, dass die Tintenfischringe wirklich Tintenfische enthalten, oder ist es vielleicht Schweinedarm in Panade? Authentische Lebensmittel sind wichtig für Menschen mit Allergien, Intoleranzen, bestimmten Ernährungsweisen oder zur Einhaltung religiöser Ernährungsvorschriften. Eine ausreichende und korrekte Kennzeichnung ermöglicht zudem Rückverfolgbarkeit, die Eindämmung illegaler Fischerei und den Schutz bedrohter Arten. Eine unzureichende bzw. falsche Deklaration kann am Ende auch dazu führen, dass wir viel Geld für ein minderwertiges Produkt bezahlen.

Es ist Fakt, dass Meeresfrüchten sehr oft falsch deklariert werden. Mögliche Ursachen hierfür sind Fehler bei der Spezies-Zuordnung oder Übersetzung, mangelhafte Kenntnis der Deklarationsvorgaben, aber auch bewusste Lebensmittelverfälschung. Die Kontrolle gestaltet sich schwierig, da die meisten Nachweismethoden zielgerichtet sind und nur nach einer bestimmten Familie oder Gattung suchen, das heißt es wird die Richtung der Suche vorausgesetzt. Zusätzlich werden nur einzelne Spezies erfasst aber keine Mischungen von mehreren Spezies gleichzeitig.

SUCCESS STORY

Daher entwickelte FFoQSI gemeinsam mit den Kooperationspartnern AGES und LVA eine DNA-Metabarcoding Methode, die eine Identifikation roher und verarbeiteter handelsüblicher Meeresfrüchte erlaubt. Kurze für eine Tier-Spezies spezifische DNA-Abschnitte werden dazu mit Next Generation Sequencing (NGS) sequenziert und mit einer Datenbank abgeglichen. Die Neuheit besteht in einer nicht zielgerichteten („non-targeted") Analyse mehrerer Tierarten gleichzeitig und der Kombination verschiedener Einzeluntersuchungen zu einer einzigen Methode.

Wirkungen und Effekte

Mit Hilfe dieses neuen Systems ist es nun möglich eine rasche und genaue Aussage zur Authentizität von Meeresfrüchten und Erzeugnissen daraus zu treffen. So stellte sich zum Beispiel bereits im Zuge der Entwicklungsarbeit heraus, dass von 21 rohen und verarbeiteten Lebensmittelproben aus der Gruppe der Kammmuscheln, zu denen auch die Jakobsmuschen zählen, nur 7 Produkte hinsichtlich der Auslobung der Tierspezies den rechtlichen Vorgaben entsprachen. Bei den restlichen 14 Proben reichten die Mängel von fehlenden Angaben der lateinischen Speziesnamen bis hin zur falschen Sachbezeichnung der Produkte (Irreführung, Täuschung). Was als Jakobsmuschel deklariert war, war also nicht immer eine Jakobsmuschel.

Sind das wirklich Jakobsmuscheln? © Kristina Gense

Es ist mit dieser neuen Methode für Lebensmittellabore und Kontrollbehörden wesentlich einfacher, die Authentizität von Meeresfrüchten zu überprüfen als bisher. Forschungen an neuen Methoden wie dem DNA Metabarcoding geben den Konsumenten Transparenz, Kontrolle und Sicherheit und sind zudem eine wichtige Voraussetzung für die Eindämmung illegaler Praktiken und den Artenschutz.

Beim Publikumsvoting für den *ecoplus Clusterland Award 2019* erreichte das von Kristina Gense (FFoQSI) und Verena Peterseil (AGES) präsentierte Projekt den herausragenden 2. Platz (von 11 Projekten).

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7. Summary

The appropriate declaration of seafood is stipulated by European regulations which also determine the responsibility of food producers for a correct authentication of their products. However, many pecuniary motivations do exist to manipulate food origin (e.g., food of regional versus global origin), food quality (e.g., organic vs. conventional production) or food composition (replacement of higher priced food components by other cheaper ones). Past food scandals show that ingredients in seafood products are not always what they claim to be. From a consumer`s perspective, seafood is seen as a luxury food and they are willing to accept higher prices than for more classical protein sources. Although accurate species assignment is already difficult when a seafood source is optically intact, assessment of the composition of ingredients in processed seafood is almost impossible. Traceable food authentication (sufficient and correct labelling) is important for a variety of reasons, personal ones (allergies, etc), food fraud related ones as well as for the sake of avoidance of illegal fishing and protection of endangered species.

Numerous analytical methods exist which are able to distinguish different seafood sources in processed conditions. Polymerase chain reaction (PCR) based assays are among the most widespread and PCR assays were developed either without or with specific probes (real time PCR). The disadvantage of these analytical methods is the limitation of a few species per assay.

As an advancement, a barcoding method was developed that sequences a specific part of the deoxyribonucleic acid (DNA) using Sanger technology. The disadvantage of this method is the limitation of analysing only one DNA target strand of a species. In the metabarcoding method, an extension of the barcoding approach, it is possible to sequence DNA containing different DNA target strands of multiple species simultaneously by using next generation sequencing.

In this presented work on the development of a metabarcoding system of bivalves, three common bivalve families (Mytilidae (mussels), Pectinidae (scallops), and Ostreidae (oysters)) were used. For this purpose, new universal primers for each bivalve´s family were designed and combined into a triplex system. The 150 bp long target gene is localised on the 16S rDNA and allows to distinguish between a number of different bivalve species. Sequencing was performed on the Illumina MiSeq and iSeq platforms. Seventy-five different foods were

analysed using the new method. It was demonstrated that it is possible to detect bivalves in even highly processed foods and to identify them at the species level. This allows a large number of food products to be analysed at the same time. This metabarcoding method is suitable for routine investigations and therefore supports fighting against food crime, strengthening consumer confidence in the industry, and keeping food authentic.

8. Zusammenfassung

Die Deklaration der Meeresfrüchte sowie die Haftung der Lebensmittelhersteller für die Authentizität ihrer Produkte werden mittels Verordnungen der Europäischen Union geregelt. Jedoch zeigen vergangene Lebensmittelskandale, dass nicht immer die Zutaten in den Lebensmittelprodukten enthalten sind, die auf den Produkten angegeben werden. Optisch ist es sehr schwierig eine Aussage darüber zu treffen, welche Bestandteile in einem verarbeiteten Lebensmittel enthalten sind. Authentische Lebensmittel (ausreichende und korrekte Kennzeichnung) sind aus verschiedenen persönlichen Gründen (Allergien, etc.) für Endkonsumenten, sowie zur Erfassung globaler Auswirkungen (illegale Fischerei, Rückverfolgbarkeit und Schutz bedrohter Arten) wichtig.

Es existieren zahlreiche Nachweismethoden, die verschiedene Meeresfrüchte in verarbeiteten Lebensmitteln identifizieren können, wie zum Beispiel die Polymerasekettenreaktion (PCR) und PCR mit spezifischen Sonden (real-time PCR). Der Nachteil dieser Analysemethoden ist die Beschränkung auf nur wenige Spezies pro Analyselauf.

Bei der Barcoding Methode wird ein spezifischer Teilbereich der Desoxyribonukleinsäure (DNA) mittels Sanger Sequenzierung sequenziert. Nachteilig ist hier, dass die Lebensmittel nur auf eine Tierspezies gleichzeitig untersucht werden können. Durch die Erweiterung dieser Methode, „Metabarcoding“, ist es möglich, Lebensmittel, die verschiede Spezies enthalten, mittels Next Generation Sequenzierung gleichzeitig zu sequenzieren.

In dieser vorgestellten Arbeit über die Entwicklung eines Metabarcodingsystems der Muscheln, wurden drei konventionelle Muschelfamilien (*Mytilidae* (Miesmuscheln), *Pectinidae* (Kammmuscheln) und *Ostreidae* (Austern)) genutzt. Dazu wurden neue, universelle Primer für jede Muschelfamilie entwickelt und zu einem Triplexsystem zusammengefügt. Das 150 bp lange Ziel-Gen ist auf der 16S rDNA lokalisiert und erlaubt es eine Reihe verschiedener Muschelspezies voneinander zu unterscheiden. Die Sequenzierung erfolgte auf der Illumina MiSeq© und iSeq© Plattform. Es wurden 75 verschiedene Lebensmittel (unterschiedlichen Verarbeitungsgrad) mit der neuen Analysemethode untersucht. Dabei zeigte sich, dass es möglich ist Muscheln in hochverarbeiteten Lebensmitteln zu detektieren und auf Spezieslevel zu unterscheiden. Eine Vielzahl an Lebensmittelprodukten kann so zur selben Zeit analysiert werden. Somit ist dieses Metabarcodingsystem für Routineuntersuchungen geeignet und trägt dazu bei Lebensmittelkriminalität zu bekämpfen, wodurch das Vertrauen der Verbraucher in die Industrie gestärkt wird und Nahrungsmittel authentisch bleiben.

9. Abbreviation

Abbreviation	English	Deutsch
DNA	deoxyribonucleic acid	Desoxyribonukleinsäure
MALDI-TOF MS	ionization time of flight mass spectrometry	Matrix Assistierte Laser Desorption Ionisierung Flugzeitanalyse
n DNA	nucleic DNA	Zellkern DNA
mt DNA	mitochondrial DNA	Mitochondriale DNA
COI	cytochrome c oxidase subunit I	Zytochrome c Oxydase Untereinheit I
cyt b	cytochrome b	Zytochrom b
12S rDNA	12S ribosomal DNA	12S ribosomale DNA
16S rDNA	16S ribosomal DNA	16S ribosomale DNA
PCR	polymerase chain reaction	Polymerasekettenreaktion
HRM Analysis	high resolution melt analysis	hochauflösende Schmelzanalyse
bp	base pair	Basenpaare
NGS	next generation sequencing	Sequenzierung der nächsten Generation
CruTin system	system for crustacean and squid	System für Krustentiere und Tintenfische
snail system	system for eatable land snail	System essbare Landschnecken

MOS system	system for the three bivalves: mussels, oysters and scallops	System für Muscheln: Miesmuscheln, Austern und Kammmuscheln
Ven system	system for Venus clams	System für Venusmuscheln
NCBI	National Centre for Biotechnology Information	Nationales Zentrum für biotechnologische Informationen

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12. References

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