Establishment and comparison of molecular biological methods for seafood species authentication

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Work presented in this dissertation was performed in the laboratory of Dr. Ingrid Huber, laboratory supervisor of research and development at the Bavarian Health and Food Safety Authority, Oberschleißheim. Germany.

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1. Introduction

Species identification of food has gained increasing interest due to scandals such as the 'horse meat scandal' in Europe, where food products labelled as beef contained up to 100% horse meat (Iwobi et al., 2017). According to a report of the European Parliament the recent food fraud cases, such as the mislabelling of seafood products, have damaged the consumer trust in the agro-food sector (2013/2091(INI)). Reliable species identification of seafood is necessary due to the high commercial interest of this group. In recent years, household consumption in Germany has remained stable at around 400.000 tonnes, with a per capita consumption between 13 and 15 kg per year (FIZ, 2018). The major part (74%) of sold seafood products was processed fish (e.g., preserves and marinades, frozen and smoked). This makes it difficult for the consumer to verify, whether the purchased seafood is the declared species or not. Hence, the adulteration of processed fish is appealing to the food retailer. Crustaceans and molluscs (fresh, frozen and pre-treated) represented only 14% of the sold seafood products in Germany (FIZ, 2018). The consumption of the three major seafood groups in Germany is comparable to the whole world trade. For instance, the percentage sold worldwide in 2016 was 65% fish, 23% crustaceans and 11% molluscs (FAO, 2018a). Among crustaceans, species of the order Decapoda (e.g., prawn, shrimps, lobsters, crabs and crayfish) are the most consumed (Cawthorn and Hoffman, 2017; Fernandes et al., 2017; Zagon et al., 2017). Consequently, this order is a potential target for food fraud.

As a response to the increasing phenomenon of adulterated and substandard food products Interpol and Europol initiated the 'Operation Opson' in 2011. A key aim of these operations is the identification of organized criminal networks behind illicit trade of counterfeit food (Europol, 2014). The 'Opson' operations are carried out annually and are assisted by the food control authorities of several countries around the world. Over 400 tons of seafood products were seized for incorrect labelling or food safety concerns in 2017 based on the 'Operation Opson VI' (Interpol, 2017). Seafood was seized in several countries, including Italy, France, the USA, and Iraq. Like in previous operations, seafood was one of the most afflicted products. When Germany fully participated for the first time in the 'Operation Opson' in 2015, the focus was on trade of Asian fish and the risk of species substitution (Interpol, 2016). Especially the two high priced fish species red snapper [*Lutjanus malabaricus (*Bloch & Schneider, 1801)] and Japanese eel (*Anguilla japonica* Temminck & Schlegel, 1846) were targets of the operation. These species are known to be replaced by lower priced fish, for instance rockfish (*Sebastes* sp.) or European eel [*Anguilla anguilla* (Linnaeus, 1758)] (Interpol, 2016).

Frequently, the declared fish or crustacean species did not match the determined species (Table 1 and Table 2). There are several reasons for wrong declaration by the food business operator, such as lack of knowledge about the species or prospect of economic gain. When species are similar in morphology and caught together, mislabelling can also occur unintentionally. For instance, Guardone et al. (2017) revealed labelling *Merluccius paradoxus* Franca, 1960 as *Merluccius capensis* Castelnau, 1861 and *Metapenaeus monoceros* (Fabricius, 1798) as *Metapenaeus affinis* (H. Milne Edwards, 1837 [in Milne Edwards, 1834-1840]). However, mislabelling is unlikely to be accidental if the species do not occur in the same area and/or the substitution happens with a lower value species. For instance, when the lower-priced freshwater fish *Pangasianodon hypophthalmus* [(Sauvage, 1878), pangasius] is labelled as the marine flatfish *Solea solea* [(Linnaeus, 1758), sole]. This common case of substitution was uncovered, inter alia, during market surveys in Italy and Germany (Filonzi et al., 2010; Kappel and Schröder, 2016; Gerdes et al., 2017). Furthermore, the whiteleg shrimp *Litopenaeus vannamei* (Boone, 1931) was replaced by the blue shrimp *Litopenaeus stylirostris* (Stimpson, 1871). *Litopenaeus stylirostris* has a different smell and taste, but looks similar to the consumer (Wilwet et al., 2018). Further reported cases are the declaration of lower priced sturgeon species (*Acipenser* spp.) as the more expensive beluga [*Huso huso* (Linnaeus, 1758)] in caviar (Fain et al., 2013; Ludwig et al., 2015; Harris and Shiraishi, 2018). Moreover, the false declaration of the less valued Greenland halibut [*Reinhardtius hippoglossoides* (Walbaum, 1792)] as Atlantic halibut [(*Hippoglossus hippoglossus* (Linnaeus, 1758)] was revealed in Germany and Italy by Filonzi et al. (2010) and Günther et al. (2017).

A further concern is the mislabelling of endangered species, such as the labelling of European eel (*Anguilla anguilla*) as Japanese eel (*Anguilla japonica*) (Pfund et al., 2018). Besides, occurrences of unintentional mislabelling of eels caught in European waters cannot be excluded due to reported cases of using American eel [*Anguilla rostrata* (Lesueur, 1817)] instead of European eel as stocking material in European aquaculture (Trautner, 2006). In contrast to Japanese eel and American eel that are listed as endangered, the European eel is classified as critically endangered in the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (Jacoby and Gollock, 2014b; Jacoby and Gollock, 2014a; Jacoby et al., 2017). Therefore there is an import and export ban for the European eel since the end of 2010 in the EU (Dekker, 2019).

Furthermore, Marín et al. (2018) detected labelling of the vulnerable hammerhead shark *Sphyrna zygaena* (Linnaeus, 1758) and the near threatened blue shark *Prionace glauca* (Linnaeus, 1758) as smoothhound (*Mustelus* sp.). Besides, Feitosa et al. (2018) revealed illegal trade of the shark species *Ginglymostoma cirratum* (Bonnaterre, 1788) in Brazil, where harvesting of this species is prohibited.

In addition, mislabelling can lead to health risk when toxic fish species are substituted for non-toxic species, for instance declaration of species assigned to the puffer fish family Tetraodontidae as monkfish (*Lophius* sp.), as revealed by Cohen et al. (2009) as well as Gerdes et al. (2017). This family can contain the neurotoxin tetrodotoxin, leading to paralysis and potential death. Furthermore, as uncovered by Lowenstein et al. (2009) and Staffen et al. (2017) escolar [*Lepidocybium flavobrunneum* (Smith, 1843)] was sold as tuna (*Thunnus* sp.). Escolars contain high levels of wax esters that can cause oily diarrhoea (keriorrhoea) and other acute gastrointestinal symptoms after consumption (Ling et al., 2009).

Due to the multitude of reported case of mislabelling of seafood products, reliable methods to combat food fraud are badly needed to protect the consumer from deceit and health risk as well as to prevent species from extinction.

Table 1 Exemplary recorded cases of mislabelled fish and detection methods.

Labelled species (English name)	Detected species (English name)	Detection method (Gene)	Investigated country	Reference
Epinephelus marginatus (Grouper)	*Lates niloticus* (Nile perch)	DNA sequencing (16S rDNA[1])	Spain	Horreo et al. (2019)
Eusphyra blochii (Hammerhead shark)	*Sphyrna lewini* (Scalloped hammerhead)	DNA sequencing (*COI*[2])	Indonesia	Abdullah and Rehbein (2017)
Gadus morhua (Cod)	*Gadus macrocephalus* (Pacific cod), *Melanogrammus aeglefinus* (Haddock), *Pollachius virens* (Pollock)	DNA sequencing (*COI*)	France, Canada	Bénard-Capelle et al. (2015); Shehata et al. (2018); (Tinacci et al., 2018)
Hippoglossus hippoglossus (Atlantic halibut)	*Reinhardtius hippoglossoides* (Greenland halibut)	DNA sequencing (*COI*, *cytb*[3])	Germany Italy	Filonzi et al. (2010); Günther et al. (2017)
Huso huso (Beluga)	*Acipenser baerii* (Siberian sturgeon), *Acipenser gueldenstaedtii* (Russian sturgeon), *Acipenser schrenckii* (Amur sturgeon)	DNA sequencing (*cytb*)	Austria, USA, Russia	Fain et al. (2013); Ludwig et al. (2015); Harris and Shiraishi (2018)
Lophius sp. (Monkfish)	Tetraodontidae (Puffer fish), *Ephippion guttifer* (Puffer fish)	DNA sequencing (*COI*, *cytb*)	Germany, USA	Cohen et al. (2009); Gerdes et al. (2017)
Merluccius capensis (Whiting)	*Merluccius paradoxus* (Whiting)	DNA sequencing (*COI*)	Italy	Guardone et al. (2017)
Mustelus sp. (Shark)	*Prionace glauca (*Blue shark), *Sphyrna zygaena* (Hammerhead shark)	DNA sequencing (*COI*)	Peru	Marín et al. (2018)
Solea solea (Sole)	*Arnoglossus laterna* (Scaldfish), *Cynoglossus senegalensis* (Senegalese tonguesole), *Limanda aspera* (Yellowfin sole), *Lepidopsetta polyxystra* (Northern rock sole), *Pangasianodon hypophthalmus* (Pangasius), *Synaptura lusitanica* (Portuguese sole)	DNA sequencing (*COI*, *cytb*), PCR-RFLP[4] (*COI*)	Belgium, France, Germany, Italy	Bénard-Capelle et al. (2015); Pappalardo and Ferrito (2015); Kappel and Schröder (2016); Christiansen et al. (2018); Pappalardo et al. (2018)
Thunnus sp. (Tuna)	*Lepidocybium flavobrunneum* (Escolar)	DNA sequencing (*COI*)	Brazil, North America	Lowenstein et al. (2009); Staffen et al. (2017)

[1]16S rDNA: 16S ribosomal DNA, [2]*COI*: Cytochrome *c* oxidase subunit 1, [3]*cytb*: Cytochrome b, [4]PCR-RFLP: Polymerase chain reaction-restriction fragment length polymorphism

Table 2 Exemplary recorded cases of mislabelled crustacean and detection methods.

Labelled species (English name)	Detected species (English name)	Detection method (Gene)	Investigated country	Reference
Chionoecetes sp. (Snow crab)	*Cancer irroratus* (Atlantic rock crab)	DNA sequencing (*COI*[1])	Canada	Shehata et al. (2018)
Fenneropenaeus indicus (Whiteleg shrimp)	*Litopenaeus vannamei* (Whiteleg shrimp)	DNA sequencing (*COI*, 16S rDNA[2])	South Africa	Cawthorn and Hoffman (2017)
Litopenaeus vannamei (Whiteleg shrimp)	*Litopenaeus stylirostris* (Blue shrimp), *Parapenaeopsis* sp. (Shrimp), *Sicyonia brevirostris* (Rock shrimp)	DNA sequencing (*COI*) PCR-RFLP[3] (16S rRNA/tRNA Val[4])	India, Italy, USA	Guardone et al. (2017); Stern et al. (2017); Wilwet et al. (2018)
Metapenaeus affinis (Shrimp)	*Metapenaeopsis* sp. (Shrimp), *Litopenaeus vannamei* (Whiteleg shrimp)	DNA sequencing (*COI*, 16S rDNA)	Italy, South Africa	Cawthorn and Hoffman (2017); Guardone et al. (2017)
Metapenaeus dobsoni (Shrimp)	*Parapenaeopsis cornuta* (Shrimp)	DNA sequencing (*COI*)	Italy	Guardone et al. (2017)
Metapenaeus ensis (Shrimp)	*Penaeus monodon* (Shrimp)	HRM[5] (*COI*)	Portugal	Fernandes et al. (2017)
Metapenaeus monoceros (Shrimp)	*Metapenaeus affinis* (Shrimp)	DNA sequencing (*COI*)	Italy	Guardone et al. (2017)
Nephrops norvegicus (Norway lobster)	*Metanephrops australiensis* (Lobsterette), *Metanephrops rubellus* (Lobsterette), *Metanephrops challengeri* (Lobsterette)	Morphological analysis	Italy	Meloni et al. (2015)
Penaeus monodon (Shrimp)	*Litopenaeus vannamei* (Whiteleg shrimp)	DNA sequencing (*COI*, 16S rDNA)	Germany, South Africa	Cawthorn and Hoffman (2017); Günther et al. (2017)
Penaeus indicus (Shrimp)	*Metapenaeus affinis* (Shrimp)	HRM (*COI*)	Portugal	Fernandes et al. (2017)
Solenocera melantho (Shrimp)	*Parapenaues* sp. (Shrimp), *Pleoticus robustus* (Royal red shrimp)	DNA sequencing (*COI*, 16S rDNA)	Italy, South Africa	Cawthorn and Hoffman (2017); Guardone et al. (2017)

[1] *COI*: Cytochrome *c* oxidase subunit, [2] 16S rDNA: 16S ribosomal DNA, [3] PCR-RFLP: Polymerase chain reaction-restriction fragment length polymorphism, [4] 16S rRNA/tRNA Val: 16S ribosomal RNA/transfer RNA Val, [5] HRM: High resolution melting

2. Review of molecular biological methods for fish and crustacean species authentication

According to the Council Regulation (EC) No 1379/2013 on the common organization of the markets in fishery and aquaculture, fishery and aquaculture products have to be labelled, inter alia, with the commercial designation of the species and its scientific name. The member states of the European Union are enforced to draw up and publish a list of the commercial designations accepted in their territory, together with their scientific names. Only the listed species are allowed to be traded in the respective country. The German list contains over 600 fish species, 160 molluscs and 80 crustacean species (BLE, 2018). Due to the large number of listed seafood species with different degrees of processing and possibly closely related species not authorised for sale, reliable methods of species authentication are required, covering as many species as possible.

Most morphological characteristics, for instance heads, tails and fins in case of fish, or external carapace in case of shrimps, are lost during processing (Teletchea, 2009; Fernandes et al., 2017; FAO, 2018b). Consequently, morphological species identification of smoked, canned or filleted seafood often reaches its limits. In addition, even if the morphological characteristics have not been removed, discrimination of closely related species is difficult and often needs specialized taxonomists for correct identification (Mafra et al., 2008; Tizard et al., 2019). Therefore, it is almost impossible for the consumer as well as for the control authorities to ascertain if the purchased seafood is the declared species by morphological characteristics. Consequently, control authorities need reliable analytical methods for unambiguous species authentication. The European Parliament and Council recommend to the national authorities, responsible for monitoring of fishery and aquaculture products, to use available technologies, such as DNA-testing, to prevent operators from mislabelling catches (Council Regulation (EC) No 1379/2013).

Standardised methods as basis for a reliable assessment of analytical data and for a nationwide standardized quality of examination to ensure a uniform enforcement of existing laws are offered by the Official Collection of Methods of Analysis and Sampling (ASU) according to § 64 of the German Food and Feed Code (BVL, 2014). The Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (Federal Office of Consumer Protection and Food Safety) in Germany is responsible for publication of the ASU. It proposed to consider new modern analytical methods, such as DNA barcoding and matrix-assisted laser desorption ionization time of-flight mass spectrometry (MALDI-TOF MS), to provide the control authorities with powerful and standardised methods for their monitoring tasks (Szabo et al., 2017).

The most common methods for seafood species authentication are protein- or DNA-based methods (Horstkotte and Rehbein, 2003; Ortea et al., 2012; Verrez-Bagnis et al., 2018). Indeed, all official analytical methods recommended for animal species authentication in the ASU are protein- or DNA-based (BVL, 2014). Only one of the four methods concerning seafood authentication is protein-based and uses isoelectric focusing (IEF) for fish species detection: L 11.00-6 Detection of fish species of native muscle by means of isoelectric focusing. A further method recommended for the authentication of fish is the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP): L 11.00-7 Identification of fish species in raw and heat-processed fish products. The two other methods use DNA sequencing for identification of fish and crustacean, respectively: L 10.00-12 Fish species identification in raw fish and fish products by means of sequence analysis of cytochrome b sequences and L 12.01-03 Crustacean species identification in raw and processed crustacean products by means of sequence analysis of 16S rRNA sequences.

Aside from the content of a respective reference library the suitability of these methods depends on the focus of analysis (e.g., species identification, delimitation or detection) and the processing grade and storage temperature (e.g., fresh, frozen, smoked). Hereinafter, application, advantages as well as disadvantages of existing molecular biological methods in the context of the official control of foodstuff as well as recent approaches for fish and crustacean species authentication are evaluated.

2.1. Protein-based methods

Although DNA-based methods are mostly applied for seafood species authentication, some long-established methods such as IEF are still used due to their simplicity and cost efficiency. In addition, MALDI-TOF MS, for which suitability for species identification has been proven for several organisms, is increasingly used for seafood species.

2.1.1. Isoelectric focusing (IEF)

IEF is based on separating proteins in a polyacrylamide gel using a pH gradient (Rehbein et al., 1995; Ortea et al., 2012). After visualization of the proteins by staining, verification is performed by comparing the band patterns with those of reference species running on the same gel (Verrez-Bagnis et al., 2018).

Among the protein-based methods, IEF is a cost-effective and simple technique used for fish and crustacean species identification (Ortea et al., 2012; Verrez-Bagnis et al., 2018). Ortea et al. (2010) showed the potential of species identification using IEF for 14 commercially important shrimp species of the order Decapoda. Moreover, a wide variety of fish species (e.g., fish of the families Gadidae, Clupeidae and Pleuronectidae) was identified by the application of IEF (Rehbein, 1990; Abdullah and Rehbein, 2015; Böhme et al., 2015). Although IEF is one of the three official analytical methods recommended for fish species identification in the ASU, it is less suitable for processed seafood due to the lack of stability of some proteins during thermal processing (Rehbein, 1990; Verrez-Bagnis et al., 2018). In addition, it needs to be examined whether closely related species, of which at least one is the target of the analysis, produce identical or highly similar band patterns (Rehbein, 1990; Kappel and Schröder, 2016). Therefore, the scope of IEF is limited (Kappel and Schröder, 2015). Nevertheless, it is still used as a screening method for fish species identification (Abdullah and Rehbein, 2015; Kappel and Schröder, 2016; Verrez-Bagnis et al., 2018). Since IEF is quite time-consuming, MALDI-TOF MS offers a faster protein-based approach for animal species identification that requires fewer preparation steps (Table 3).

2.1.2. Matrix-assisted laser desorption ionization time of-flight mass spectrometry (MALDI-TOF MS)

MALDI-TOF MS is a fast, non-targeted protein-based method being based on co-crystallization of the sample (whole cell or extracted proteins) on a target plate with an energy-absorbing matrix. By the assistance of the matrix, a pulsed laser desorbs and ionizes the sample. Subsequently, the generated charged ions of the sample are accelerated by a strong electric field, are discriminated according to their mass-to-charge ratio, and finally are measured with the time-of-flight mass spectrometer (Figure 1). The generated protein spectra are correlated to a database containing reference protein spectra for species identification (Pavlovic et al., 2011).

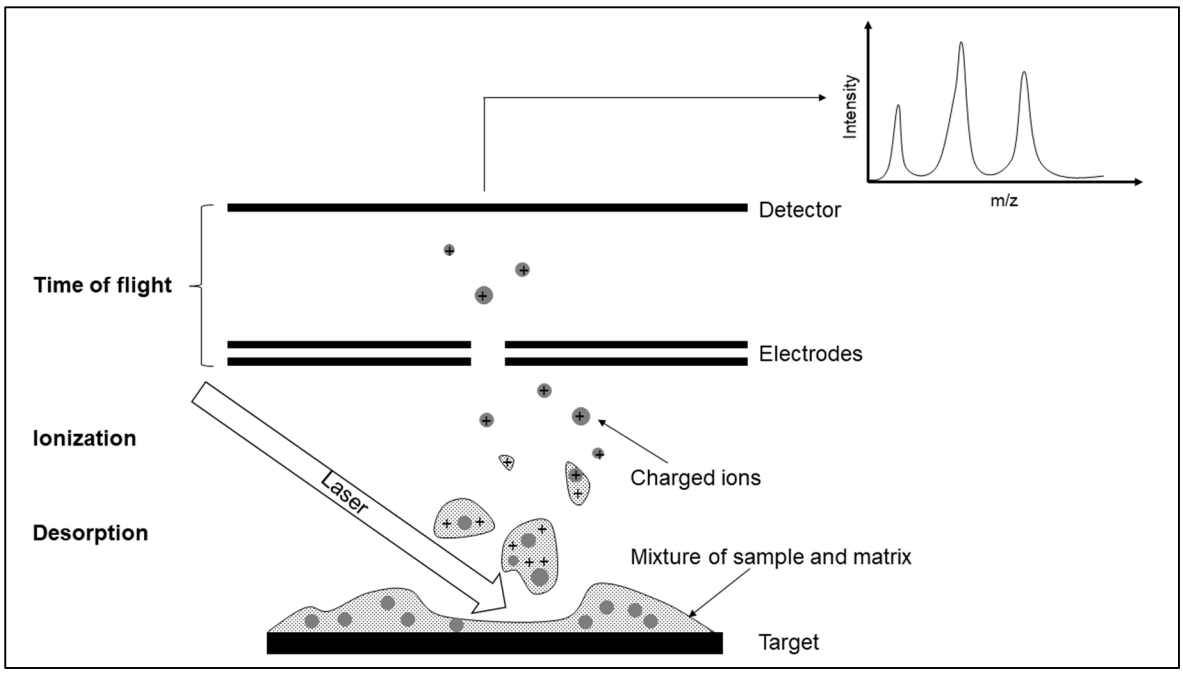

Figure 1 Schematic representation of matrix-assisted laser desorption ionization time of-flight mass spectrometry (MALDI-TOF MS). First, the sample is co-crystallized on the target plate with an energy-absorbing matrix. This matrix assists the desorption and ionisation of the samples via a pulsed laser (MALDI). The generated charged ions of the sample are subsequently accelerated using a strong electric field. These ions of various sizes are discriminated according to the mass-to-charge ratio and measured with the time of flight detector mass spectrometer (TOF MS). The generated protein spectra are subsequently evaluated against a database containing reference protein spectra for species identification.

MALDI-TOF MS has become a well-established technique for identification of bacteria and fungi (see Seng et al., 2009; Welker, 2011; Wieser et al., 2012; Bader, 2017). In recent years MALDI-TOF MS was applied for authentication of several metazoan species such as insects (e.g., Perera, 2005; Ulrich et al., 2017), mammals (e.g., Hiller et al., 2017), molluscs (e.g., Stephan et al., 2014), fish (e.g., Mazzeo et al., 2008; Volta et al., 2012; Stahl and Schröder, 2017) as well as crustacean (e.g., Laakmann et al., 2013; Salla and Murray, 2013; Kaiser et al., 2018). However, for the various groups of animals no standardised protocol is applied, and up to now the influence of fat-content, storage temperature and level of food processing has been studied only to a limited extent.

In case of molluscs, Stephan et al. (2014) used formic acid including chloroform–methanol defatting for protein preparation of fresh and frozen tissues to uncover mislabeling of *Placopecten magellanicus* (Gmelin, 1791) as *Pecten maximus* (Linnaeus, 1758). Salla and Murray (2013) identified skeletal muscles from six shrimps to species level irrespective of their storage condition (fresh and frozen) using trifluoroacetic acid for protein preparation. Furthermore, Laakmann et al. (2013) showed the suitability of MALDI-TOF MS for the discrimination of 11 calanoid copepod species (Crustacea) from tissue fixed in ethanol on different developmental stages without preceding protein preparation. Concerning fish species identification, Volta et al. (2012) compared protein spectra, yielded from frozen muscle and liver prepared with formic acid, of three fish species [*Alosa agone* (Scopoli, 1786), *Coregonus macrophthalmus* Nüsslin, 1882 and *Rutilus rutilus* (Linnaeus, 1758)] and showed that both tissues are suitable for discrimination. Mazzeo et al. (2008) examined proteins prepared with trifluoroacetic acid of frozen muscle tissue from 25 fish species of the orders Perciformes, Gadiformes and Pleuronectiformes. By applying this protein preparation protocol, it was possible, besides genera discrimination, to differentiate species within the genus *Merluccius*. Stahl and Schröder (2017) developed a MALDI-TOF MS database of 54 fish species (belonging to 14 orders) using protein spectra prepared with trifluoroacetic acid of frozen filets and also assessed the impact of contamination of the fillets with bacterial proteins on identification of the fish species. They demonstrated that a cell content of about 1% of *Escherichia coli*, which may be expected on fresh fish filets, does not have an impact on fish species identification.

As for all protein-based techniques, species identification using MALDI-TOF MS has the limitation that proteins are less thermostable than DNA. Furthermore, like DNA sequencing a comprehensive available background database (reference library) is required. However, MALDI-TOF MS possesses great advantages over IEF and several DNA-based methods as it requires only few and simple preparation steps along with short analysis times (Wang et al., 2012; Stephan et al., 2014; Stahl and Schröder, 2017).

2.2. DNA-based methods

DNA-based methods offer several advantages over protein-based: i) independence of sample origin (all cells of an organism contain DNA) and developmental stages (from egg to adults or rests), ii) higher information content (down even to populations), and iii) the suitability for processed samples (e.g., heated) due to the higher thermostability of DNA in contrast to proteins (Wolf et al., 1999; Teletchea, 2009; Ward et al., 2009; Wilwet et al., 2018). Therefore, three of the four official analytical methods for seafood species authentication in the ASU are DNA-based methods, in particular PCR-RFLP and DNA sequencing (BVL, 2014). Additionally, the loop-mediated isothermal amplification (LAMP) technique is becoming an alternative approach for animal species detection.

2.2.1. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

PCR-RFLP is based on endonucleases that recognize specific restriction sites of an amplified fragment and digest them into smaller fragments of different sizes. These fragments can be separated by gel electrophoresis and visualized (Teletchea, 2009; Hellberg and Morrissey, 2011).

PCR-RFLP is a much easier to perform and less expensive method than DNA sequencing for species identification of seafood (Teletchea, 2009; Pappalardo et al., 2018; Verrez-Bagnis et al., 2018). For instance, Pascoal et al. (2008) developed a PCR-RFLP method for 17 prawn and shrimp species targeting a gene region of 16S ribosomal RNA/transfer RNA Val, also used by Wilwet et al. (2018) for authentication of commercially important shrimp species in India. In addition, several working groups applied PCR-RFLP for fish species identification. Sanjuan and Comesaña (2002) as well as Pappalardo et al. (2018) differentiated flatfish species while Sivaraman et al. (2018) used PCR-RFLP for authentication of snappers. Furthermore, Frankowski et al. (2009), Rehbein et al. (2002) and Gagnaire et al. (2007) used PCR-RFLP for identification of (in sum eight) eel species based on the cytochrome b (*cytb*) or the 16S ribosomal RNA (16S rRNA) gene.

However, PCR-RFLP is vulnerable to errors due to intraspecific variability as well as incomplete enzyme digestion and does not provide the high level of information acquired with DNA sequencing (Lockley and Bardsley, 2000; Hellberg and Morrissey, 2011). One of the three official analytical methods recommended for fish species identification in the ASU uses PCR-RFLP analysis of amplified mitochondrial cytb-DNA fragments (BVL, 2014). However, this method is used only to a limited extent in the official control of foodstuff since there are other methods available offering more advantages, for instance DNA sequencing (Kappel and Schröder, 2015).

2.2.2. DNA sequencing

The DNA sequencing technique includes several steps. First, amplification of a specific DNA fragment using PCR and purification steps. Subsequently, the DNA sequence is determined using the didesoxy terminator DNA sequencing method developed by Sanger et al. (1977). For species identification, the generated sequence is matched to reference sequences in a DNA database through phylogenetic analysis (FINS: Forensically informative nucleotide sequencing) or similarity searches.

The most commonly used DNA-based techniques for fish species identification are based on amplification of mitochondrial DNA (Mafra et al., 2008; Verrez-Bagnis et al., 2018). The advantages of mitochondrial DNA in contrast to nuclear DNA are i) much faster evolution, ii) higher copy number (approximately 100-1000 times higher) and iii) for many aquatic organisms complete mitochondrial DNA sequences are known (Chow et al., 1997; Mackie et al., 1999; Teletchea et al., 2005; Rasmussen and Morrissey, 2008; Wilwet et al., 2018).

The choice of the most suitable DNA gene region (marker) is essential for reliable species identification of the target group. This marker needs high interspecific and low intraspecific sequence variation for discrimination between the selected species, conserved regions as binding sites for universal primers to ensure amplification across a wide taxonomic range, as well as enough available reference sequences in the database (Dawnay et al., 2007; Hellberg and Morrissey, 2011; Ferrito and Pappalardo, 2017). In cases of species identification of marine animals, the three mitochondrial genes *cytb*, 16S rRNA and the 'DNA-barcode', cytochrome *c* oxidase subunit 1 (*COI*) are the most frequently used (see Verrez-Bagnis et al., 2018). For instance, Jamandre et al. (2007) analysed three *Anguilla* species via sequencing of *cytb* and 16S rDNA and Lago et al. (2012) used FINS of *cytb* for differentiation of twelve eel species. Furthermore, Shehata et al. (2018) analysed over 300 seafood species in the regulatory context in Canada by *COI* sequencing. Comparing the applicability of *cytb*, *COI* and 16S rDNA using 50 European marine fish species (of 20 different families) Kochzius et al. (2010) revealed discrimination failure of 16S rDNA for closely related flatfish and gurnard species.

Most control authorities in Germany still use DNA-sequencing of a *cytb* fragment for fish species identification (BVL, 2014; Kappel and Schröder, 2015). Nevertheless, *COI* sequencing is currently discussed for implementation in the ASU for fish species identification (Kappel and Schröder, 2015). Furthermore, it is applied in many other countries (e.g., Brazil, Canada and the United States) as a regulatory tool for seafood species identification in terms of combating food fraud (Shehata et al., 2018).

Hebert et al. (2003) proposed a region of *COI* of about 650 base pairs (bp) at its 5'-end as the standard 'DNA-barcoding' gene for metazoan species identification. The main advantages of *COI* over other mitochondrial genes are the potentially higher phylogenetic signal and the availability of robust universal primer-sets for this region, covering most of the animal phyla (Folmer et al., 1994; Hebert et al., 2003; Hebert et al., 2016a). A huge variety of studies has shown the potential of unambiguous species identification of animals via *COI* sequencing, such as mammals (e.g., Bitanyi et al., 2011; Kumar et al., 2018; Kundu et al., 2019), birds (e.g., Hebert et al., 2004; Huang and Ruan, 2018; Tizard et al., 2019), and insects (e.g., Hausmann et al., 2011; Hebert et al., 2016b; Ashfaq et al., 2018).

Furthermore, the potential of *COI* sequencing for fish and crustacean species identification was shown by several working groups (e.g., Radulovici et al., 2009; Haye et al., 2012; Nicolè et al., 2012; Raupach et al., 2015; Muñoz-Colmenero et al., 2016; Shen et al., 2016; Staffen et al., 2017; Stern et al., 2017; Mantelatto et al., 2018; Sarmiento-Camacho and Valdez-Moreno, 2018). Besides *COI*, 16S rDNA is the most used gene region for crustacean species identification (Pascoal et al., 2011; Ortea et al., 2012; Cawthorn and Hoffman, 2017; Lee et al., 2017; Mantelatto et al., 2018; Verrez-Bagnis et al., 2018). In the ASU DNA sequencing of a 16S rDNA fragment is recommended for crustacean species identification (BVL, 2014). However, this method has some limitations. For instance, it is not suitable for analysing species of the shrimp genus *Crangon* because no suitable amplicons are obtained (BVL, 2014).

With DNA sequencing the largest amount of information from PCR fragments is produced directly (Lockley and Bardsley, 2000; Civera, 2003; Rasmussen and Morrissey, 2008). The possibility of using 'universal' primers enables obtaining DNA sequences of a wide range of animals without prior knowledge of the animal species. Therefore this technique is suitable for seafood species that are often sold processed (e.g., eviscerated, beheaded, skinned, filleted) and thus lose their morphological characteristics (Mafra et al., 2008; Tizard et al., 2019). DNA sequencing is one of the most commonly applied DNA based techniques for seafood species identification (see Teletchea, 2009; Verrez-Bagnis et al., 2018). In recent years, it was widely used for revealing food fraud (Table 1 and Table 2). However, DNA sequencing is comparatively time-consuming and needs several preparation steps (Gil, 2007; Laakmann et al., 2013; Fernandes et al., 2017). Furthermore, a reference database is required for data analysis, as in case of MALDI-TOF MS (Table 3).

One of the most frequently used DNA database for species identification is GenBank (of the National Institute of Health (NIH), built and distributed by the National Center for Biotechnology Information (NCBI): http://www.ncbi.nlm.nih.gov). GenBank contains over 5 million mitochondrial animal sequences (March 2019). The sequences of GenBank can be searched and aligned using BLAST (Basic Local Alignment Search Tool) whereby similarities between a query sequence and GenBank sequences can be detected (Benson et al., 2017). However, species identification of sequences in GenBank often is highly doubtful and usually cannot be checked (Vilgalys, 2003; Federhen, 2015).

Another often used database is the Barcode of Life Data System (BOLD: http://www.boldsystems.org) from the Consortium for the Barcode of Life (CBOL). This database mainly uses the *COI* gene for animal identification and contains over 8 million public animal *COI* sequences (March 2019). BOLD has the aim to build a barcode library for all eukaryotic life by using standard protocols (Ratnasingham and Hebert, 2007). Sequences in BOLD which gained the 'barcode status' include voucher specimen with taxonomic identifications and further data (Ratnasingham and Hebert, 2007). *COI* sequences and data, which are publicly accessible in BOLD, are regularly migrated to GenBank (Ratnasingham and Hebert, 2007). Furthermore, BOLD imports *COI* sequences and data fulfilling the requirements of BOLD (Raupach and Radulovici, 2015).

2.2.3. Loop-mediated isothermal amplification (LAMP)

The LAMP developed by Notomi et al. (2000) is a highly specific and rapid technique running under isothermal conditions. For this technique a set of two inner and two outer primers (each consist of one forward and one reverse primer), as well as a polymerase with high strand-displacement activity is used (Figure 2). The outer primers are only used in the initial steps of the reaction, in combination with the inner primers. One of the resulting DNA structures of the initial steps is a stem-loop. The yielded stem-loop is the starting material for the subsequent cycling reaction and only requires the inner primers. The inner primers consist of two sequences (one corresponds to the sense and the other to the antisense sequence of the target DNA) and a spacer between them. The antisense sequence binds in the first steps for elongation whereas the sense sequence is for self-priming in the later steps. The final products after the elongation and recycling step are different stem-loop DNA and cauliflower-like structures with several loops (Notomi et al., 2000; Notomi et al., 2015).

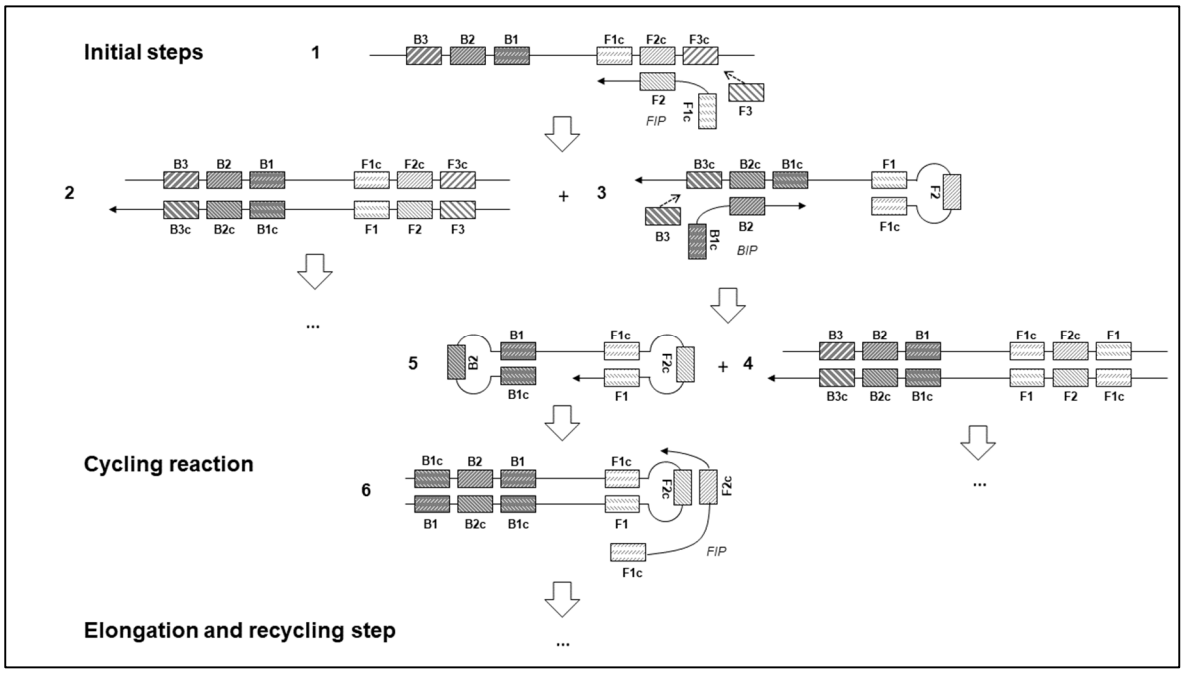

Figure 2 Schematic representation of the first stages of loop-mediated isothermal amplification (LAMP) analogous to Notomi et al. (2000). The forward inner primer (FIP) consists of the F2 sequence (corresponding to the sense sequence of the target DNA F2c) a spacer, and the F1c sequence (corresponding to the antisense sequence of the target DNA F1). The reverse inner primer (BIP) consists of the B2 sequence, a spacer and the B1c sequence. The outer primers are F3 (forward) corresponding to the sense sequence of the target DNA F3c and B3 (reverse), respectively. In the initial steps, the inner and outer primers are used. DNA synthesis could start with FIP or BIP primer. Here starting with the FIP primer is described. FIP binds to the sequence F2c of the target DNA and initiates synthesis of the complementary strand. When F3 binds to the sequence F3c of the target DNA, it initiates DNA synthesis via strand displacement (1). One of the resulting DNA is a double strand (2) and the other is a single strand with a loop structure at one end (3). Now the BIP primer binds to the other end of the single strand as well as the B3 primer, leading again to DNA synthesis via strand displacement as described above. One of the resulting DNA is a double strand (4). The resulting dumb-bell structure is converted to a stem-loop due to self-priming DNA synthesis (5). This stem-loop serves as starting material for the second stages of the LAMP reaction (cycling reaction) whose first step is illustrated in the figure above (6) where again FIP binds to the DNA. For further steps of the cycling reaction as well as for elongation and recycling step, leading to the final products of different stem-loop DNA and cauliflower-like structures with several loops, see Notomi et al. (2000).

In contrast to PCR, which uses heat, strand separation of the DNA within LAMP is performed using enzymatic activity (Tanner et al., 2012). For detection of positive LAMP reactions, techniques such as visual detection using DNA-binding dyes, turbidity detection of precipitated magnesium or gel electrophoresis are applied subsequently to the reaction (see Tanner et al., 2012; Zhang et al., 2014; Wong et al., 2018). Furthermore, real-time fluorescence detection and subsequent melting curve analysis can be performed on instruments such as real-time cyclers or real-time fluorimeters [e.g., the portable Genie instrument from OptiGene (Horsham, United Kingdom), specially designed for isothermal amplification].

LAMP has already been successfully applied for detection of bacteria and viruses (Notomi et al., 2000; Fu et al., 2011; Dhama et al., 2014) as well as for plant species in herbal medicine and food (Focke et al., 2013; Li et al., 2016). Due to its high specificity and short analysis time identification of meat species using LAMP is becoming an alternative approach to PCR-based methods, such as for detection of poultry and mammalian species (Ahmed et al., 2010; Abdulmawjood et al., 2014; Cho et al., 2014; Sul et al., 2019). In case of seafood species detection Ye et al. (2017) developed a LAMP assay, based on *COI* as target gene, for identification of the jumbo flying squid *Dosidicus gigas* (D'Orbigny, 1835). Furthermore, Saull et al. (2016) developed a LAMP assay for discrimination of Atlantic cod (*Gadus morhua* Linnaeus, 1758) from Pacific cod (*Gadus macrocephalus*; Tilesius, 1810) and pollock (*Gadus chalcogrammus* Pallas, 1814), based on *cytb* as target gene, for the analysis of frozen and smoked fish fillets.

The main advantages of the LAMP technique are that it is less prone to inhibitors and more sensitive than PCR (Notomi et al., 2000; Keremane et al., 2015; Wong et al., 2018). Furthermore, it needs only easy to handle equipment (no thermal cycling equipment is required in contrast to PCR) making it suitable for on-site analysis (e.g., field studies). However, for development of LAMP assays prior sequence information of the target DNA sequence is required and it is limited to a defined species spectrum, as for all species-specific techniques (Lockley and Bardsley, 2000).

Table 3 Comparison of existing molecular biological methods for marine species authentication in the context of official control of foodstuff and recent approaches for fish and crustacean species.

	Simple protocol	Analysis time[1]	Main advantage[2]	Main disadvantage(s)[3]	ASU[4]-method
Protein-based					
IEF[5]	-	+++	Easy to perform	Time-consuming	x (Fish)
MALDI-TOF MS	x	+	Fast	Database required	-
DNA-based					
PCR-RFLP[7]	-	+++	Cost-effective	Incomplete enzyme digestion	x (Fish & Crustacean)
DNA sequencing	-	++++	Quantity of information	Time-consuming, database required	x (Fish & Crustacean)
LAMP[8]	x	++	Fast	Limited to a defined species spectrum	-

Methods marked with an 'x' indicate that they exhibit the corresponding feature.

[1]Analysis time: time required from the extraction of DNA/protein to result, [2]Main advantage compared to the other listed methods, [3]Main disadvantage compared to the other listed methods, [4]ASU: Official Collection of Methods of Analysis and Sampling according to § 64 of the German Food and Feed Code (Lebensmittel-und Futtermittelgesetzbuch; LFGB) in case of food monitoring, [5]IEF: Isoelectric focusing, [6]MALDI-TOF MS: Matrix-assisted laser desorption ionization time of-flight mass spectrometry, [7]PCR-RFLP: Polymerase chain reaction-restriction fragment length polymorphism, [8]LAMP: Loop-mediated isothermal amplification

3. Aims of the thesis

Due to the many reported cases concerning mislabelling of seafood products, food control authorities require reliable methods to protect the consumer against deceit and health risks or to uncover trade or consumption of protected species.

Consequently, the first aim of this thesis was to establish DNA- and protein-based molecular biological methods for seafood species authentication, in particular fishes and crustaceans. The second aim was to compare these methods for suitability to authenticate seafood species enabling the control authorities to select appropriate methods to combat food fraud. MALDI-TOF MS as protein-based and DNA sequencing as well as LAMP as DNA-based methods were evaluated for their suitability for seafood species authentication. In particular, it was ascertained which molecular biological method should be preferred for a particular focus of analysis (species identification, delimitation or detection), as well as for which processing grade and storage temperature (fresh, refrigerated, frozen, cooked or smoked).

DNA sequencing as well as MALDI-TOF MS show some limitations, such as the need for expensive and bulky equipment as well as DNA sequence- or protein spectra-databases. The LAMP technique offers an alternative when the focus of analysis is the delimitation of two species or specific detection of known species. This technique can be easily performed with portable equipment for a fast on-site analysis. Suitability of LAMP for fish was already shown for identification of frozen and smoked Atlantic cod (*Gadus morhua* Linnaeus, 1758) filets. However, applicability of LAMP had not yet been shown for fish eggs. Besides, cases of mislabelling of the critical endangered European eel [(*Anguilla anguilla* (Linnaeus, 1758)] as Japanese eel (*Anguilla japonica* Temminck & Schlegel, 1846) as well as wrong stocking of American eel [*Anguilla rostrata* (Lesueur, 1817)] instead of European eel in European waters were reported. Consequently, delimitation of the critical endangered European eel from the other eel species of the genus *Anguilla* is of high relevance. Therefore, one part of the thesis was to examine the suitability of LAMP assays for species delimitation on frozen and smoked fish as well as fish eggs. This was performed by developing assays for specific detection of DNA of *Anguilla anguilla* and of the genus *Anguilla* respectively.

For analysis of unknown species MALDI-TOF MS, a much faster approach than DNA sequencing due to fewer preparation steps, can be applied. Suitability of animal species identification was shown by several working groups (e.g., for crustacean, fish, molluscs, mammals as well as insects). However, the influences of the preparation protocol, fat-content, storage temperature and level of food processing have been studied only to a limited extent. Therefore, using fish as an example, the suitability of five preparation methods for proteins were compared for subsequent MALDI-TOF MS analysis. Furthermore, influence of different fat-content, storage temperature, and level of food processing (fresh, refrigerated, frozen, cooked and smoked) on identification suitability and reproducibility were examined in this thesis by using high-fat Atlantic mackerel (*Scomber scombrus* Linnaeus, 1758) and low-fat rainbow trout [*Oncorhynchus mykiss* (Walbaum, 1792)] as representatives.

In case of DNA sequencing, the available official control method in the food monitoring sector for fish species identification based on *cytb* is currently discussed to be supplemented with *COI* sequencing. However, the official method for crustacean species identification via DNA sequencing of a 16S rDNA fragment has some limitations. Therefore, suitability of another 16S rDNA marker region and the *COI* barcoding region were examined for species identification of crustacean of the most consumed order Decapoda in comparison with the official control method.

4. Publication I Using loop-mediated isothermal amplification for fast species delimitation in eels (genus *Anguilla*), with special reference to the European eel (*Anguilla anguilla*)

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Using loop-mediated isothermal amplification for fast species delimitation in eels (genus *Anguilla*), with special reference to the European eel (*Anguilla anguilla*)

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ABSTRACT

Fast, reliable and easy to handle methods are required to survey the introduction of foreign eel species into German waters, to detect incorrect labelling of food as well as for ecological field studies e.g. on board a ship. Several DNA-based methods are available for identification of the European eel (*Anguilla anguilla*). However, the need for expensive and bulky equipment is not convenient for all applications, particularly those related to field work. Therefore, loop-mediated isothermal amplification (LAMP) assays were developed for identification of *A. anguilla* and the genus *Anguilla*, respectively. The *Anguilla* genus LAMP assay was able to detect at least 500 pg DNA per reaction of all four tested *Anguilla* species, the European eel (*A. anguilla*), its closest relatives (*A. rostrata* and *A. australis*), and the Japanese eel (*A. japonica*). The detection limit for the *A. anguilla* specific assay was also 500 pg DNA per reaction and, in combination with a rapid simplified DNA extraction method, both assays were able to detect one single egg of the species *A. anguilla* per LAMP reaction. None of the two LAMP assays showed false positive results among 112 non-*Anguilla* species tested. The *Anguilla* genus-specific LAMP system detected all 34 *Anguilla* specimens examined, while the second LAMP only detected the 18 *A. anguilla* specimens, but not the 16 specimens classified as other *Anguilla* species. The total analysis time, including DNA extraction and data evaluation, was no more than 90 min per experiment. A further time reduction to 70 min is possible by shortening the amplification time and omitting the melting curve analysis without lose of critical information.

1. Introduction

Of the 19 species currently classified in genus *Anguilla* (for actual phylogeny see Inoue et al., 2010), *A. anguilla* (Linnaeus, 1758; European eel), the closely related *A. rostrata* (Lesueur, 1817; American eel) and *A. australis* J. Richardson, 1841 (Australian eel), and the more distantly related *A. japonica* Temminck & Schlegel, 1846 (Japanese eel) are the most consumed (Lago, Vieites, & Espiñeira, 2012; Pérez, Barrera, Asturiano, & Jover, 2004). *A. anguilla* is the most sold eel species in Europe and is usually consumed smoked, canned or as glass eel (Rehbein et al., 2002; Ringuet, Muto, & Raymakers, 2002).

A. anguilla and the *A. rostrata* partly share their spawning area in the Sargasso Sea in the North Atlantic Ocean (Ringuet et al., 2002; Trautner, 2013; van Ginneken & Maes, 2005). Furthermore, *A. rostrata* also occurs in natural German and other European inland waters, being probably introduced by stockings of glass eels or elvers of *A. rostrata* (Frankowski et al., 2009). A possible reason for the wrong stocking is, that *A. rostrata* is cheaper than *A. anguilla*, and therefore is increasingly used as stocking material in aquaculture (Trautner, 2006).

A. rostrata is listed in the IUCN (International Union for Conservation of Nature) Red List of Threatened Species as endangered, whereas *A. anguilla* is currently classified as critically endangered. Therefore, the Council of the European Union has published a regulation to establish measures for the recovery of the stock of *A. anguilla* (Council Regulation (EC) No 1100/2007). Thus, identification techniques are required to prove the introduction of foreign eel species into European river systems (Trautner, 2013). These identification methods would also have the benefit of protecting consumers against mislabelling of eel species. Application of an on-site analysis would facilitate ecological studies on board ships, which offers an additional advantage.

The morphological differentiation of the *Anguilla* species is difficult, as their external morphology is very similar and also varies depending

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Table 1
Summary of DNA-based methods applied for identification of *Anguilla* species.

Technique[a]	Target genes[b]	Species	Reference
Sequencing	*cytb*, 16S rRNA	*Anguilla bicolor bicolor* *Anguilla bicolor pacifica* *Anguilla marmorata*	Jamandre et al. (2007)
FINS	*cytb*	*Anguilla anguilla* *Anguilla australis* *Anguilla bicolor* *Anguilla celebesensis* *Anguilla dieffenbachia* *Anguilla japonica* *Anguilla malgumora* *Anguilla marmorata* *Anguilla megastoma* *Anguilla nebulosa* *Anguilla reinhardtii* *Anguilla rostrata*	Lago et al. (2012)
PCR-RFLP	*cytb*	*Anguilla anguilla* *Anguilla australis* *Anguilla japonica* *Anguilla rostrata*	Rehbein et al. (2002)
	16S rRNA	*Anguilla bicolor bicolor* *Anguilla marmorata* *Anguilla mossambica* *Anguilla nebulosi labiata*	Gagnaire et al. (2007)
	cytb, 16S rRNA	*Anguilla rostrata*	Frankowski et al. (2009)
real-time PCR	16S rRNA	*Anguilla anguilla*	Espiñeira and Vieites (2016)
	16S rRNA	*Anguilla japonica*	Watanabe et al. (2004)
real-time PCR + melting curve analysis	*cytb*	*Anguilla anguilla* *Anguilla japonica* *Anguilla rostrata*	Trautner (2013)

[a] Technique abbreviations: FINS, Forensically informative nucleotide sequencing; PCR-RFLP, restriction fragment length polymorphism.
[b] Target genes abbreviations: *cytb*, cytochrome b; 16S rRNA, 16S ribosomal RNA.

on the respective development stage (Jamandre, Yambot, Shen, & Tzeng, 2007; Lago et al., 2012). Moreover, it is nearly impossible to determine the species of processed fish (smoked, canned) due to the loss of external characteristics. Several methods based on analysis of mitochondrial DNA, which offer an alternative approach for identification of *Anguilla* species, have been described (Table 1): Jamandre et al. (2007) delimitate *A. marmorata* (Quoy & Gaimard, 1824), *A. bicolor bicolor* (McClelland, 1844) and *A. bicolor pacifica* (Schmidt, 1928) by DNA sequencing. Lago et al. (2012) proposed a FINS (Forensically informative nucleotide sequencing) technique to discriminate 12 different *Anguilla* species. In addition, several PCR-RFLP (restriction fragment length polymorphism) methods have been published, dealing with the identification of *Anguilla* species (Frankowski et al., 2009; Gagnaire et al., 2007; Rehbein et al., 2002). A disadvantage of these methods is the requirement for time-consuming and labour-intensive RFLP follow-up analysis after PCR. Real-time PCR, which does not require subsequent sequencing or gel electrophoresis, is described as a rapid and specific alternative for distinction between several *Anguilla* species. Trautner (2013) developed a real-time PCR with subsequent melting curve analysis for differentiating between *A. rostrata*, *A. japonica* and *A. anguilla*. Another real-time PCR traces *A. anguilla* in aquaculture and seafood products (Espiñeira & Vieites, 2016). Watanabe, Minegishi, Yoshinaga, Aoyama, and Tsukamoto (2004) established a real-time PCR assay for the authentication of *A. japonica* eggs on board ships.

The loop-mediated isothermal amplification (LAMP) developed by Notomi et al. (2000) is a new approach, which has already been successfully used in various fields like the detection of pathogens, including bacteria and viruses (Dhama et al., 2014; Fu et al., 2011), GM targets (Fraiture et al., 2015), and the detection of plant species in herbal medicine (Li, Xiong, Liu, Liang, & Zhou, 2016).

LAMP is less prone to inhibitors (Keremane et al., 2015), requires easy to handle equipment, and is therefore also suitable for field studies. Saull, Duggan, Hobbs, and Edwards (2016) demonstrated the suitability of LAMP for seafood authentication by verifying the identification of the Atlantic cod (*Gadus morhua* (Linnaeus, 1758)). LAMP is highly specific and rapid under isothermal conditions (Notomi et al., 2000). For LAMP, a polymerase with high strand-displacement activity and a set of two inner (FIP: forward inner primer, BIP: backward inner primer) and two outer primers (F3: forward primer, B3: backward primer) is used. Depending on the task, two additional loop primers may be used to increase sensitivity. There are many options available for detection of positive LAMP reactions, for example gel electrophoresis or visual detection using pH sensitive dyes (Notomi et al., 2000; Saull et al., 2016). The Genie instrument (OptiGene, Horsham, United Kingdom) combines isothermal amplification, fluorescence detection and melting curve (anneal derivative) analysis for higher specificity. A further advantage of this instrument is that it is portable and battery-powered, thus allowing on-site detection.

In this study, two LAMP assays were developed. One assay was designed to detect all *Anguilla* species, while the other assay should only detect the critically endangered species *A. anguilla*. Furthermore, since not all laboratories have the Genie instrument, and also because real-time PCR cyclers offer the advantage to analyse more samples by accommodating up to 384 wells, the suitability of a real-time cycler for the developed LAMP assays was determined, in addition to the validation of both developed LAMP assays on the Genie instrument.

The developed LAMP assays, combined with a simplified DNA extraction method, can be applied by researchers for identification of individual eggs for ecological studies (e.g. on board a ship), or by customs (e.g. detection of illegal import). Additionally the method can be applied by relevant food control authorities for detection of food

Table 2
Threshold times (Tt) and melting temperatures (Tm) of the developed LAMP assays for detection of the genus eel (*Anguilla* spp.) and the European eel (*A. anguilla*) using different *Anguilla* species.

Species	Common name	State	DNA extraction	No. of samples	*Anguilla* spp.				*Anguilla anguilla*			
					Tt [mm:ss]		Tm [°C]		Tt [mm:ss]		Tm [°C]	
					Min	Max	Min	Max	Min	Max	Min	Max
Anguilla anguilla	European eel	Smoked	CTAB	7	15:30	18:35	84.99	85.38	26:15	33:00	81.26	81.84
		Raw	CTAB	3	16:15	17:00	85.24	85.53	27:15	29:15	81.26	81.50
		1 egg	Chelex	4	17:45	23:00	85.26	85.60	25:30	28:30	81.12	81.42
		Raw	Chelex	4	18:00	18:45	85.49	85.64	29:30	30:30	81.28	81.61
Anguilla japonica	Japanese eel	Smoked	CTAB	9	17:30	23:30	85.35	85.85	-[a]	–	–	–
		Raw	CTAB	2	19:15	23:45	85.64	85.96	–	–	–	–
Anguilla rostrata	American eel	Smoked	CTAB	1	18:00	18:00	85.19	85.20	–	–	–	–
		Raw	CTAB	3	17:15	21:30	84.53	85.47	–	–	–	–
Anguilla australis	Australian eel	Raw	CTAB	1	18:30	19:00	85.1	85.61	–	–	–	–

[a] -: no amplification.

fraud, as well as in the context of fishery control.

2. Material and methods

2.1. Samples

Determination of the specificity of both LAMP assays was carried out with 146 specimens classified to 116 fish species of 50 families, including clupeids, cods, sturgeons, salmonids and cyprinids (Supplementary Material Table S1). These samples were obtained from the official food control authorities, from local markets or were provided by various German institutes. The identity of all fish samples was previously verified using a suitable method, in particular DNA sequencing (of either the cytochrome b, the 16S ribosomal RNA or the cytochrome oxidase *c* subunit 1 gene (Spielmann et al., 2018)). To test whether both LAMP assays are suitable for processed fish, muscle tissue of seven smoked *A. anguilla*, nine smoked *A. japonica* as well as one smoked *A. rostrata* was analysed. Furthermore, single eggs from *A. anguilla* were applied to test a rapid DNA extraction procedure for use in on-site analyses (Table 2).

2.2. DNA extraction

DNA from all 146 samples was extracted using a modified cetyltrimethylammonium bromide (CTAB) protocol (ISO 21571:2005).

To test a rapid simplified DNA extraction procedure, either a single egg or 5 mg muscle tissue of *A. anguilla* were homogenized in 100 µl of 5% Chelex 100 resin (Bio-Rad Laboratories GmbH, Munich, Germany) using a micro pistil. After incubation at 95 °C for 20 min followed by 10 min at room temperature, 5 µl of the supernatant was applied for LAMP reactions.

The concentration of the nucleic acids obtained by the aforementioned protocol was determined either by using the Nanodrop 1000 (Peqlab, Darmstadt, Germany) or the Qubit 3.0 Fluorimeter with the Qubit high sensitivity assay reagent (Invitrogen, Carlsbad, USA).

2.3. Primer design

In this study, two LAMP primer sets were designed using the PrimerExplorer V5 software. Each primer set consisted of two outer (F3 and B3) and two inner (FIP and BIP) primers (Table 3). The *Anguilla* genus-specific LAMP assay targets the nuclear C-type lectin gene. The following sequences were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) for primer design: *A. anguilla* (Acc. no. LC049084.1), *A. australis* (LC049085.1 and LC049086.1), *A. bengalensis bengalensis* (LC049087.1), *A. bicolor pacifica* (LC049090.1), *A. celebesensis* (LC049092.1), *A dieffenbachii* (LC049093.1), *A. interioris* (LC049094.1), *A. japonica* (LC049095.1), *A. luzonensis* (LC049096.1), *A. malgumora* (LC049091.1), *A. marmorata* (LC049097.1), *A megastoma* (LC049098.1), *A. mossambica* (LC049099.1), *A. obscura* (LC049100.1), *A. reinhardtii* (LC049101.1) and *A. rostrata* (LC049102.1).

A. anguilla species-specific primers targeting the mitochondrial d-Loop region were designed using the following sequences retrieved from GenBank: *A. anguilla* (Acc. no. KJ564253.1; KJ564263.1; KJ564270.1), and *A. rostrata* (KJ564208.1). Specificity of both primer sets was verified *in silico* by using the Primer BLAST tool from NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/).

2.4. LAMP assay

For both LAMP assays, reactions were carried out in a total of 25 µl reaction volume containing 15 µl of the GspSSD Isothermal Master Mix ISO 001 Kit (OptiGene, Horsham, United Kingdom), 5 pmol (each) of the respective primers F3 and B3, 20 pmol (each) of the respective primers FIP and BIP as well as 5 µl DNA adjusted to 20 ng/µl.

Reactions were carried out on the Genie II instrument (OptiGene) as well as in the CFX96 real-time PCR cycler (Bio-Rad Laboratories GmbH). Cycling conditions were 45 min at 65 °C with continuous fluorescence detection, followed by annealing from 90 to 80 °C (*Anguilla* spp.) or 85 to 75 °C (*A. anguilla*) with a ramping rate of 0.05 °C/min. Reactions were considered positive when amplification (fluorescence

Table 3
Sequences and length of LAMP primers for the detection of *Anguilla* spp. and *A. anguilla*.

Assay	Target gene	Primer	Sequence 5′-3′	Length (bp)
Anguilla spp.	C-type lectin	AngSpp_F3	GTA AAA TGA ATA TGA TCC ACA AGG	24
		AngSpp_B3	ACA TGC AAG AAG CAT CGG	18
		AngSpp_FIP (F1c + F2)	TAA CAC TGT TGG ATT GCC GGT CCA TTG ATC CTT ATT GTG GGT TTG	45
		AngSpp_BIP (B1c + B2)	ATG TGC TCC TCA GTA TCT CAC GAT TAT GTT TTG CCC AAC CTT	42
Anguilla anguilla	Mitochondrial d-Loop	AngAng_F3	CCA TAT TCC TAT GTT CAA ATC AAC A	25
		AngAng_B3	GTA ACG AGT CTA ATG TAY TAT ACC A	25
		AngAng_FIP (F1c + F2)	GCA ACT CTA CAA TTA CTG TCC TTG ACA GCT AAA TGT AAT AAG AAA TCA CC	50
		AngAng_BIP (B1c + B2)	ATG AAC TAT TAC TGG CAT TTG GCT CCA GAT ATA TTC AAA TTA TGG GGA A	49

signal above a given threshold) was detected and the melting temperature of the amplicon corresponded to that of the positive control.

2.5. Sensitivity of the developed LAMP assays

The limit of detection (LOD) for both assays was determined on the Genie instrument by measuring DNA three times in decimal dilutions (ranging from 1 ng/µl to 10 pg/µl) in sextuplicates. For the genus-specific LAMP assay, all four available *Anguilla* species (*A. anguilla, A. japonica*, *A. rostrata* and *A. australis*) were tested. For the *A. anguilla* LAMP assay only this species was used.

2.6. Specificity of the developed LAMP assays

All 112 fish specimens not classified to the genus *Anguilla* as well as the 9 raw *Anguilla* specimens (Supplementary Material Table S1) extracted with the CTAB method were adjusted to 20 ng/µl and measured on the Genie instrument in duplicates with both LAMP assays to determine false positive or negative LAMP reactions.

2.7. Suitability of the LAMP assays for DNA extracted with the simplified method and smoked eels

DNA was extracted in fourfold determination from single eggs and fish muscle of *A. anguilla* with 5% Chelex 100 resin solution. DNA was measured on the Genie instrument in duplicates to evaluate the performance of the simplified DNA extraction method (Table 2).

Furthermore, in addition to the DNA from raw eels, DNA adjusted to 20 ng/µl from smoked eels of the species *A. anguilla*, *A. japonica* and *A. rostrata* were used as template for the LAMP reactions to determine the suitability of the LAMP assays for processed food (Table 2).

2.8. Visual detection of LAMP reactions

In order to use the method for on-site analysis without special detection devices, SYBR Gold nucleic acid gel stain was used as an alternative detection reagent. 1 µl of SYBR Gold nucleic acid gel stain (1:10 dilution of a 10.000 stock solution, Invitrogen) was added to a positive reaction, containing *A. anguilla*, and one negative reaction, containing water. Visual detection was performed in the absence and presence of UV light.

2.9. Suitability of a real-time PCR cycler for the developed LAMP assays

Suitability of the CFX96 real-time PCR cycler (Bio-Rad Laboratories GmbH) for the two developed LAMP assays was determined with *A. anguilla*, *A. japonica*, *A. rostrata* and *A. australis* at concentrations of 100 pg/µl DNA as well as DNA extracted from single eggs and 5 mg of fish muscle with the simplified DNA extraction method. Reactions of the two developed LAMP methods were carried out at 65 °C for 1 min for 45 cycles followed by annealing from 90 to 75 °C (both assays) with temperature increment steps of 0.5 °C every 5 s.

3. Results and discussion

3.1. Sensitivity of the LAMP assays

To determine the LOD of the LAMP assays, decimal dilutions (1 ng/µl to 10 pg/µl) of DNA extracted from fish muscle tissue were tested. Each concentration was analysed in sextuplicates on the Genie instrument.

When coupled with the CTAB DNA extraction method, the *A. anguilla* specific LAMP assay was able to detect all six replicates of 500 pg total DNA (100 pg/µl) of *A. anguilla* DNA in all three runs (Table 4). The sensitivity of the genus-specific LAMP assay for DNA from *A. anguilla, A. japonica, A. rostrata* as well as *A. australis* was also 500 pg total DNA

Table 4
Threshold times (Tt) and melting temperatures (Tm) of the developed LAMP assays for determination of the limit of detection (LOD).

Species	Common name	Concentration	*Anguilla* spp.						*Anguilla anguilla*								
			No. of amplified replicates			Tt [mm:ss]		Tm [°C]		No. of amplified replicates			Tt [mm:ss]		Tm [°C]		
			Run 1	Run 2	Run 3	Min	Max	Min	Max	Run 1	Run 2	Run 3	Min	Max	Min	Max	
Anguilla anguilla	European eel	1 ng/µl	6/6	6/6	6/6	17:15	20:30	85.18	85.41	6/6	6/6	6/6	31:15	41:45	81.02	81.45	
		100 pg/µl [a]	**6/6**	**6/6**	**6/6**	19:30	23:00	85.13	85.41	**6/6**	**6/6**	**6/6**	33:15	44:30	80.92	81.23	
		10 pg/µl	3/6	3/6	2/6	21:30	35:00	85.17	85.53	1/6	0/6	2/6	42:45	44:30	81.05	81.15	
Anguilla japonica	Japanese eel	1 ng/µl	6/6	6/6	6/6	16:30	19:15	85.41	86.36	n.d.	–	–	–	–	–	–	
		100 pg/µl	**6/6**	**6/6**	**6/6**	19:00	25:00	85.61	85.98	–	–	–	–	–	–	–	
		10 pg/µl	2/6	3/6	4/6	19:00	31:30	85.60	86.18	–	–	–	–	–	–	–	
Anguilla rostrata	American eel	1 ng/µl	6/6	6/6	6/6	17:15	19:15	85.09	85.56	–	–	–	–	–	–	–	
		100 pg/µl	**6/6**	**6/6**	**6/6**	19:30	21:00	85.32	85.62	–	–	–	–	–	–	–	
		10 pg/µl	6/6	4/6	5/6	19:45	44:30	85.21	85.64	–	–	–	–	–	–	–	
Anguilla australis	Australian eel	1 ng/µl	6/6	6/6	6/6	19:30	21:30	85.32	85.70	–	–	–	–	–	–	–	
		100 pg/µl	**6/6**	**6/6**	**6/6**	19:45	33:00	85.49	85.80	–	–	–	–	–	–	–	
		10 pg/µl	3/6	5/6	2/6	23:30	43:00	85.14	85.58	–	–	–	–	–	–	–	

n.d.: no data.
[a] Concentration in which 6 out of 6 replicates in all three runs were amplified are in bold.

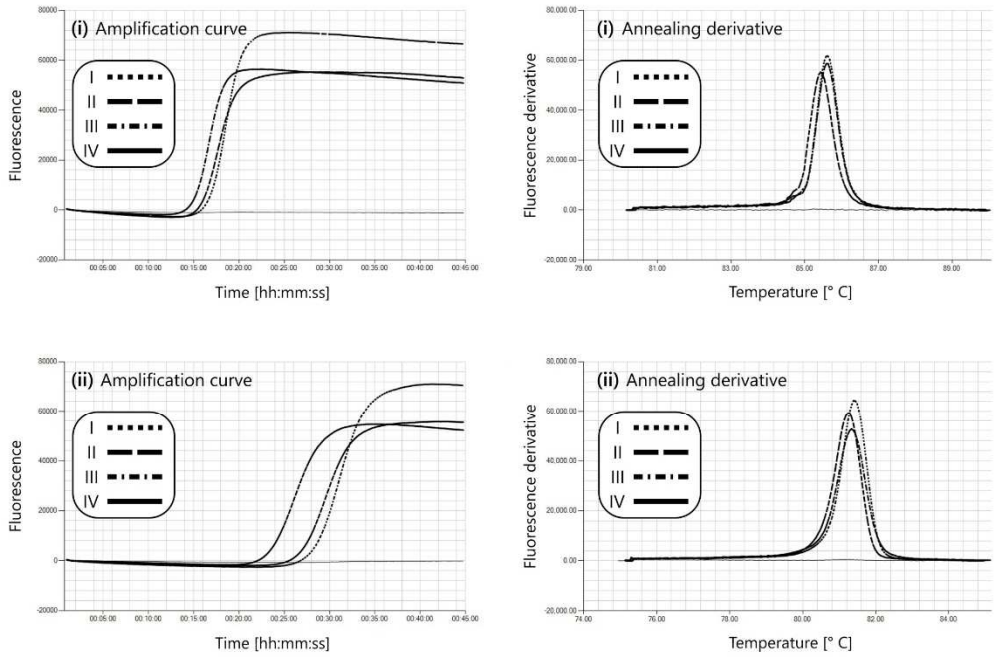

Fig. 1. Amplification and annealing derivative for LAMP assays on the Genie instrument (i) *Anguilla* spp. and (ii) *Anguilla anguilla*. (I) 5 mg fish muscle of *A. anguilla* extracted with the simplified DNA extraction method, (II) one egg of *A. anguilla* extracted with the simplified DNA extraction method, (III) positive control DNA of *A. anguilla* and (IV) negative control (water).

(100 pg/µl) for each of the four species. The fast real-time PCR developed by Espiñeira and Vieites (2016) detects 0.2 pg DNA of *A. anguilla* per reaction. The LOD with 500 pg (100 pg/µl) for both LAMP assays developed in this study (*Anguilla* spp. and *Anguilla anguilla*) was clearly far above the LOD derived by Espiñeira and Vieites (2016), but had the advantage to be performed on a portable battery-powered instrument which can be applied in field studies.

3.2. Specificity of the developed LAMP assays

To test the specificity of the LAMP primers, DNA extracted from 116 fish species of 50 different families was measured with the Genie instrument. The 112 fish specimens not classified to the genus *Anguilla* were not detected with either assay, i.e. no false positive was observed (Supplementary Material Table S1). Furthermore, the four mostly consumed eel species *A. anguilla, A. japonica*, *A. rostrata* and *A. australis* were tested using both LAMP assays. All 34 specimens classified to the four aforementioned *Anguilla* species were detected with the genus-specific LAMP assay. These results show that both developed LAMP assays are highly specific for detection of the genus *Anguilla* and for the *A. anguilla* species respectively. However, when using the *A. anguilla* specific assay, only the *A. anguilla* samples yielded positive results, while the 16 samples of the other *Anguilla* species were negative.

The observed melting temperatures of the four examined eel species were between 84.10 and 85.96 °C (*A. anguilla*: 84.99–85.64 °C, *A. japonica*: 85.35–85.96 °C, *A. rostrata*: 84.53–85.47 °C and *A. australis* 84.10–85.61 °C) using the *Anguilla* genus-specific LAMP assay. Because of the overlapping melting temperature ranges, the analysis of the melting temperature was proved as unsuitable for differentiation between the four applied *Anguilla* species with the *Anguilla* genus-specific LAMP assay *Anguilla* spp. and can therefore be omitted to reduce analysis time. Regarding the species-specific assay, the melting temperature was observed at 81.12–81.84 °C for DNAs from *A. anguilla*.

3.3. Suitability of the LAMP assays for DNA extracted with the simplified method and smoked eels

DNA extracted with the rapid method from individual *A. anguilla* eggs in fourfold repetition yielded an average of 0.8 ± 0.3 ng/µl, while 1.4 ± 0.2 ng/µl where yielded from 5 mg of fish muscle tissue. These extracts produced positive results with both LAMP assays (Fig. 1). DNA extracted from one egg and from 5 mg fish muscle were detected no later than 23 min and 19 min with the genus-specific LAMP assay respectively, while application of the *A. anguilla* specific LAMP assay resulted in threshold times between 28.5 min and 30.5 min. This was probably due to better binding efficiency of the primer set for the detection of the genus *Anguilla* compared to primer set for the detection of the *A. anguilla* species.

Furthermore, it was examined whether the LAMP assays are also suitable for the analysis of processed fish samples. Here, DNA from 17 smoked specimens of *A. anguilla* (n = 7) *A. japonica* (n = 9) and A. *rostrata* (n = 1) were extracted using the CTAB method and compared to the results from raw frozen specimens.

There were no differences for all four analysed species concerning melting temperature between smoked and raw frozen specimens (Table 2). Additionally, the threshold times for smoked eel specimens were not higher than for the raw frozen ones. Therefore, the developed methods are suitable for low amounts of sample material or for food

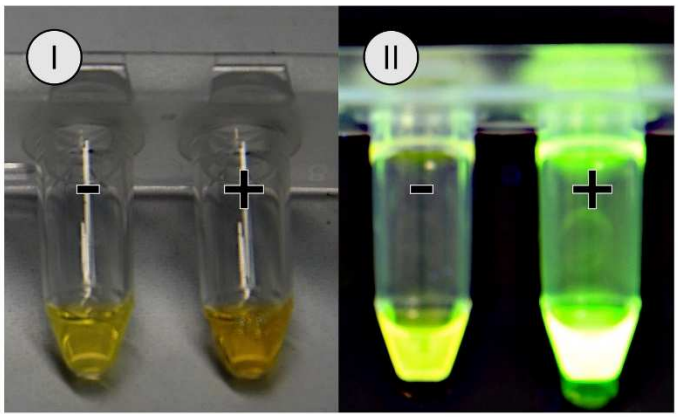

Fig. 2. Visualization of LAMP reactions using SYBR Gold. (I) Colour changes from orange seen in the negative reaction (−) to slight yellow seen in the positive reaction (+). (II) With UV transillumination: bright fluorescence occurred only in the positive reaction (+). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

control authorities, which often only get processed eel specimens for analysis.

3.4. Visual detection of LAMP reactions

Visual detection using SYBR Gold nucleic acid gel stain was possible in the absence of UV light. Negative reactions showed an orange coloration, while positive reactions turned slightly yellow. However, interpretation was much easier using UV light (Fig. 2) as bright fluorescence signal occurred only in the positive reactions. Due to the possibility to detect positive LAMP reaction with dyes like propidium iodide (Hill et al., 2008) without special equipment, LAMP is appropriate for on-site analyses-only with simple heating. In this study SYBR Gold nucleic acid gel stain was used for visual detection. However, when using alternative detection methods, the use of a less expensive LAMP master mix without intercalating dyes should be considered. Furthermore, the specificity of the reaction cannot be confirmed by a melting curve analysis.

3.5. Suitability of a real-time PCR cycler for the developed LAMP assays

To explore the possibility of using an alternative device to a specific LAMP instrument like the Genie (OptiGene), the CFX96 real-time PCR cycler (Bio-Rad Laboratories GmbH) was trialled exemplarily. DNA of *A. anguilla*, A. *japonica, A. rostrata* and *A. australis* was adjusted to 100 pg/µl. Results of both LAMP assays were comparable to those achieved using the Genie II instrument (OptiGene). Furthermore, DNA extracted from individual eggs and 5 mg of fish muscle tissues using the simplified method were also detected using the CFX96 real-time PCR cycler (data not shown). This shows that the assay can also be carried out on other platforms. Nonetheless, the Genie instrument has the great advantage to be portable and therefore can be used for analysis directly on board ships or in field studies. Depending on the application, attention should be paid to the availability of a melting curve analysis for the alternative device. Furthermore, LAMP reactions can be carried out on a heating block as shown by Lee (2017) when using visualization of the DNA amplification products by addition of pH sensitive dyes.

4. Conclusions

In this study, two specific, sensitive, rapid and robust LAMP assays were developed and validated. The species-specific assay was capable of detecting *A. anguilla* in DNA extracted from individual eggs. This enables the control of authenticity of the species *A. anguilla* among the whole commercial chain from stocking to table. Currently, the detection of eel species with the LAMP assays developed in this study takes 85 min only (35 min DNA extraction using the rapid simplified method followed by 45 min of amplification and 5 min for melting curve analysis). Depending on the required sensitivity and the type of sample material, the LAMP can be shortened even to 35 min ensuring detection of DNA extracted from single eel eggs and 5 mg of fish muscle with the simplified DNA extraction method. Therefore, these methods are suitable for fast detection of the endangered species *A. anguilla*, even on board a research vessel. Furthermore, due to the potential of visual detection via addition of pH sensitive dyes or alternative nucleic acid stains and isothermal amplification (with the developed LAMP assays at 65 °C), only a heating block is necessary. Moreover, the *Anguilla* genus-specific LAMP assay could serve as a control for successful DNA extraction as well as an inhibition control. In the future, developing a LAMP assay for the selective detection of *A. rostrata* would facilitate controlling of stocking eel species. This is due to the fact that currently, negative results of the presented selective *A. anguilla* LAMP assay need to undergo further examination with other methods, like DNA-sequencing, in order to determine if the analysed species is *A. rostrata* or one of the other *Anguilla* species.

Conflicts of interest

None.

Informed consent

Not applicable.

Compliance with ethics requirements

This article does not contain any studies with human participants by any of the authors. All institutional and national guidelines for the care and use of animals were followed.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2019.02.022.

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Table S1 Fish samples used for specificity testing of the developed LAMP assays for detection of the genus eel (*Anguilla* spp.) and the European eel (*A. anguilla*). The mean of duplicate measurement of threshold time (Tt) and melting temperature (Tm) is shown.

					***Anguilla* spp.**		***Anguilla anguilla***	
Order	**Family**	**Subfamily**	**Species**	**Common name**	**Tt [mm:ss]**	**Tm [°C]**	**Tt [mm:ss]**	**Tm [°C]**
Acipenseriformes	Acipenseridae		*Acipenser baerii*	Sturgeon	-[a]	-	-	-
			Acipenser brevirostrum	Sturgeon	-	-	-	-
			Acipenser gueldenstaedtii	Danube sturgeon	-	-	-	-
			Acipenser nudiventris	Sturgeon	-	-	-	-
			Acipenser ruthenus	Sterlet sturgeon	-	-	-	-
			Acipenser stellatus	Starry sturgeon	-	-	-	-
			Acipenser sturio	Sturgeon	-	-	-	-
			Acipenser transmontanus	Sturgeon	-	-	-	-
			Huso huso	Beluga	-	-	-	-
Anabantiformes	Osphronemidae	Luciocephalinae	*Trichogaster pectoralis*	Snakeskin gourami	-	-	-	-
Anguilliformes	Anguillidae		*Anguilla anguilla* S1 (smoked)	European eel	18:35	85.26	26:15	81.26
			Anguilla anguilla S2 (smoked)	European eel	15:30	85.34	28:45	81.34
			Anguilla anguilla S3 (smoked)	European eel	16:52	85.02	32:45	81.72
			Anguilla anguilla S4 (smoked)	European eel	16:37	85.24	28:45	81.81
			Anguilla anguilla S5 (smoked)	European eel	16:15	85.27	27:15	81.32
			Anguilla anguilla S6 (smoked)	European eel	16:07	85.19	31:45	81.52
			Anguilla anguilla S7 (smoked)	European eel	16:00	85.13	26:45	81.55
			Anguilla anguilla S8 (raw)	European eel	16:22	85.29	28:15	81.35
			Anguilla anguilla S9 (raw)	European eel	16:52	85.51	27:30	81.38
			Anguilla anguilla S10 (raw)	European eel	16:22	85.27	29:15	81.49
			Anguilla anguilla S11/1 (1 egg)	European eel	20:07	85.29	28:30	81.33
			Anguilla anguilla S11/2 (1 egg)	European eel	18:37	85.30	28:30	81.31

		Anguilla anguilla S11/3 (1 egg)	European eel	23:00	85.49	28:15	81.15	
		Anguilla anguilla S11/4 (1 egg)	European eel	18:00	85.58	28:15	81.33	
		Anguilla anguilla S12/1 (5 mg)	European eel	18:15	85.59	30:30	81.34	
		Anguilla anguilla S12/2 (5 mg)	European eel	18:07	85.63	30:30	81.50	
		Anguilla anguilla S12/3 (5 mg)	European eel	18:22	85.61	30:15	81.43	
		Anguilla anguilla S12/4 (5 mg)	European eel	18:37	85.54	30:30	81.53	
		Anguilla japonica S1 (smoked)	Japanese eel	17:52	85.61	-	-	
		Anguilla japonica S2 (smoked)	Japanese eel	20:00	85.62	-	-	
		Anguilla japonica S3 (smoked)	Japanese eel	21:30	85.54	-	-	
		Anguilla japonica S4 (smoked)	Japanese eel	22:07	85.62	-	-	
		Anguilla japonica S5 (smoked)	Japanese eel	17:45	85.71	-	-	
		Anguilla japonica S6 (smoked)	Japanese eel	18:45	85.65	-	-	
		Anguilla japonica S7 (smoked)	Japanese eel	18:00	85.83	-	-	
		Anguilla japonica S8 (smoked)	Japanese eel	18:00	85.39	-	-	
		Anguilla japonica S9 (smoked)	Japanese eel	20:52	85.73	-	-	
		Anguilla japonica S10 (raw)	Japanese eel	23:37	85.65	-	-	
		Anguilla japonica S11 (raw)	Japanese eel	19:22	85.91	-	-	
		Anguilla rostrata S1 (smoked)	American eel	18:00	85.20	-	-	
		Anguilla rostrata S2 (raw)	American eel	21:00	84.57	-	-	
		Anguilla rostrata S3 (raw)	American eel	19:15	85.19	-	-	
		Anguilla rostrata S4 (raw)	American eel	17:22	85.42	-	-	
		Anguilla australis (raw)	Australian eel	18:45	85.36	-	-	
	Beloniformes	Belonidae	*Belone belone*	Garfish	-	-	-	-
		Scomberesocidae	*Cololabis saira*	Pacific saury	-	-	-	-
	Carangiformes	Carangidae	Trachinotinae	*Trachinotus carolinus*	Florida pompano	-	-	-
	Carcharhiniformes	Carcharhinidae		*Prionace glauca*	Blue shark	-	-	-

Chimaeriformes	Callorhinchidae		*Callorhinchus callorynchus*	Plownose chimaera	-	-	-	-
Cichliformes	Cichlidae	Pseudocrenilabrinae	*Oreochromis niloticus*	Tilapia	-	-	-	-
Clupeiformes	Clupeidae		*Clupea harengus*	Herring	-	-	-	-
		Clupeinae	*Sardina pilchardus*	Pilchard	-	-	-	-
		Clupeinae	*Sprattus sprattus*	Sprat	-	-	-	-
Clupeiformes	Engraulidae		*Engraulis anchoita*	Anchovy	-	-	-	-
			Engraulis encrasicolus	Anchovy	-	-	-	-
Cypriniformes	Cyprinidae	Alburninae	*Alburnus chalcoides*	Carp	-	-	-	-
		Barbinae	*Barbus barbus*	Barbel	-	-	-	-
		Cyprininae	*Cyprinus carpio*	Carp	-	-	-	-
		Leuciscinae	*Blicca bjoerkna*	Carp	-	-	-	-
		Leuciscinae	*Abramis brama*	Bream	-	-	-	-
		Leuciscinae	*Blicca bjoerkna*	Carp	-	-	-	-
		Leuciscinae	*Leuciscus cephalus*	Carp	-	-	-	-
		Leuciscinae	*Leuciscus idus*	Carp	-	-	-	-
		Leuciscinae	*Leuciscus leuciscus*	Carp	-	-	-	-
		Tincinae	*Tinca tinca*	Tench	-	-	-	-
Esociformes	Esocidae		*Esox lucius*	Northern pike	-	-	-	-
Gadiformes	Lotidae		*Molva molva*	Ling	-	-	-	-
	Gadidae		*Gadus macrocephalus*	Cod	-	-	-	-
			Gadus morhua	Cod	-	-	-	-
		Lotinae	*Lota lota*	Burbot	-	-	-	-
		Gadinae	*Melanogrammus aeglefinus*	Haddock	-	-	-	-
		Gadinae	*Merlangius merlangus*	Whiting	-	-	-	-
		Gadinae	*Pollachius pollachius*	Pollack	-	-	-	-
		Gadinae	*Pollachius virens*	Saithe	-	-	-	-

		Gadinae	*Gadus chalcogrammus*	Alaska pollack	-	-	-	
		Gadinae	*Trisopterus luscus*	Pout	-	-	-	
	Merlucciidae	Merlucciinae	*Merluccius merluccius*	European hake	-	-	-	
		Merlucciinae	*Micromesistius poutassou*	Blue whiting	-	-	-	
	Chanidae		*Chanos chanos*	Milkfish	-	-	-	
	Xiphiidae		*Xiphias gladius*	Swordfish	-	-	-	
	Scaridae		*Hipposcarus longiceps*	Parrotfish	-	-	-	
			Scarus ghobban	Parrotfish	-	-	-	
	Lamnidae		*Lamna nasus*	Shark	-	-	-	
	Lophiidae		*Lophius budegassa*	Monkfish	-	-	-	
			Lophius litulon	Monkfish	-	-	-	
			Lophius piscatorius	Monkfish	-	-	-	
			Lophius vomerinus	Monkfish	-	-	-	
	Moronidae		*Dicentrarchus labrax*	Sea bass	-	-	-	
			Dicentrarchus punctatus	Spotted sea bass	-	-	-	
	Mugilidae		*Liza ramada*	Grey mullet	-	-	-	
	Osmeridae		*Mallotus villosus*	Capelin	-	-	-	
	Anarhichadidae		*Anarhichas denticulatus*	Wolffish	-	-	-	
			Anarhichas lupus	Wolffish	-	-	-	
	Serranidae	Epinephelinae	*Cephalopholis taeniops*	Bluespotted seabass	-	-	-	
		Epinephelinae	*Epinephelus aeneus*	Grouper	-	-	-	
		Epinephelinae	*Epinephelus bleekeri*	Grouper	-	-	-	
	Latidae		*Lates calcarifer*	Barramundi	-	-	-	
			Lates niloticus	Nile perch	-	-	-	
	Gempylidae		*Lepidocybium flavobrunneum*	Escolar	-	-	-	
	Lutjanidae		*Lutjanus argentimaculatus*	Snapper	-	-	-	

			Lutjanus bohar	Snapper	-	-	-	-	-	-
			Lutjanus erythropterus	Snapper	-	-	-	-	-	-
		Lutjaninae	*Pinjalo lewisi*	Snapper	-	-	-	-	-	-
		Lutjaninae	*Pinjalo pinjalo*	Snapper	-	-	-	-	-	-
	Moronidae		*Morone chrysops*	White bass	-	-	-	-	-	-
	Percidae	Luciopercinae	*Sander lucioperca*	Zander	-	-	-	-	-	-
		Percinae	*Perca fluviatilis*	European perch	-	-	-	-	-	-
	Pomatomidae		*Pomatomus saltatrix*	Bluefish	-	-	-	-	-	-
	Mullidae		*Pseudupeneus prayensis*	Goatfish	-	-	-	-	-	-
	Scombridae	Scombrinae	*Thunnus*	Tuna	-	-	-	-	-	-
		Scombrinae	*Scomber scombrus*	Mackerel	-	-	-	-	-	-
	Carangidae		*Seriola quinqueradiata*	Yellowtail	-	-	-	-	-	-
			Trachurus trachurus	Horse mackerel	-	-	-	-	-	-
Pleuronectiformes	Pleuronectidae	Pleuronectinae	*Atheresthes stomias*	Arrowtooth flounder	-	-	-	-	-	-
		Pleuronectinae	*Glyptocephalus cynoglossus*	Torbay sole	-	-	-	-	-	-
		Pleuronectinae	*Lepidopsetta bilineata*	Rock sole	-	-	-	-	-	-
		Pleuronectinae	*Lepidopsetta polyxystra*	Northern rock sole	-	-	-	-	-	-
		Pleuronectinae	*Limanda limanda*	Dab	-	-	-	-	-	-
		Pleuronectinae	*Platichthys stellatus*	Starry flounder	-	-	-	-	-	-
		Pleuronectinae	*Pleuronectes platessa*	European plaice	-	-	-	-	-	-
		Pleuronectinae	*Reinhardtius hippoglossoides*	Greenland halibut	-	-	-	-	-	-
	Cynoglossidae	Cynoglossinae	*Cynoglossus senegalensis*	Senegalese tonguesole	-	-	-	-	-	-
	Scophthalmidae		*Psetta maxima*	Turbot	-	-	-	-	-	-
	Soleidae		*Solea senegalensis*	African sole	-	-	-	-	-	-
			Solea solea	Sole	-	-	-	-	-	-
Salmoniformes	Salmonidae	Salmoninae	*Oncorhynchus gorbuscha*	Pink salmon	-	-	-	-	-	-

		Salmoninae	*Oncorhynchus keta*	Keta salmon	-	-	-	-
		Salmoninae	*Oncorhynchus mykiss*	Rainbow trout	-	-	-	-
		Salmoninae	*Salmo salar*	Salmon	-	-	-	-
		Salmoninae	*Salmo trutta*	Brown trout	-	-	-	-
		Salmoninae	*Salvelinus fontinalis*	Char	-	-	-	-
		Thymallinae	*Thymallus thymallus*	Grayling	-	-	-	-
	Scombriformes	Scombridae	Scombrinae	*Sarda sarda*	Bonito	-	-	-
	Siluriformes	Clariidae		*Clarias gariepinus*	Catfish	-	-	-
				Clarias macrocephalus	Catfish	-	-	-
				Heterobranchus longifilis	Catfish	-	-	-
		Ictaluridae		*Ictalurus punctatus*	American catfish	-	-	-
		Pangasiidae		*Pangasius hypophthalmus*	Pangasius	-	-	-
		Siluridae		*Silurus glanis*	Catfish	-	-	-
	Spariformes	Sparidae		*Pagrus caeruleostictus*	Sea bream	-	-	-
				Pagrus pagrus	Sea bream	-	-	-
				Parupeneus heptacanthus	Sea bream	-	-	-
				Pagellus bellottii	Sea bream	-	-	-
				Sparus aurata	Gilt-head bram	-	-	-
	Squaliformes	Squalidae		*Squalus acanthias*	Dogfish	-	-	-
	Tetraodontiformes	Tetraodontidae	Tetraodontinae	*Ephippion guttifer*	Prickly puffer	-	-	-

[a]-: no amplification.

5. Publication II Comparison of five preparatory protocols for fish species identification using MALDI-TOF MS

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Comparison of five preparatory protocols for fish species identification using MALDI- TOF MS

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Abstract

Rapid and reliable methods for fish authentication are required in order to protect consumers against food fraud. Matrix-assisted laser desorption ionization time of-flight mass spectrometry (MALDI-TOF MS) is known as a fast and accurate method for microorganisms. In this study the effect of five preparation protocols for fish samples on the quality and reproducibility of spectra using the MALDI Biotyper platform were evaluated. The suitability of the protocols for the identification of high-fat Atlantic mackerel (*Scomber scombrus*) and low-fat rainbow trout (*Oncorhynchus mykiss*) was examined in dependence on different storage temperatures and levels of food processing (fresh, refrigerated, frozen, cooked and smoked).

The results of the present study showed that acquisition of reproducible and high quality main spectra projections for high-fat and low-fat fishes in fresh and frozen states was only possible by sample preparation with 25% formic acid followed by chloroform-methanol defatting. MALDI-TOF MS based identification was also possible after treating samples at 99 °C for 5 min but not for smoked fish. Furthermore, log score values for identification of frozen fish remained stable even after fourteen months of storage at -20 °C.

Introduction

Fish is one of the most common food products susceptible to fraudulent labelling. A current example is the mislabeling of less expensive tonguefish (*Cynoglossus* spp.) as common sole (*Solea solea*) [1] or the replacement of European plaice *(Pleuronectes platessa)* by dab (*Limanda limanda*) and flounder (*Platichthys flesus*) [2]. Moreover, consumer deception can also cause health risks. The sale of specimens assigned to the family *Tetraodontidae* (puffer fish) as monkfish (*Lophius* spp.) is one of these cases. Puffer fish may contain lethal amounts of the toxic neurotoxin tetrodotoxin, which may lead to paralysis and potential death [3]. Rapid and reliable methods for fish authentication are required in order to protect consumers against food fraud.

Identification of fish species within the context of official control of foodstuffs in Germany is currently performed via sequencing of a fragment of the mitochondrial cytochrome b (*cytb*) gene [4]. But sequencing is a time consuming process including several steps like DNA extraction, PCR, purification of the PCR product, sequencing, and evaluation of the results. Therefore, several working groups studied the potential of matrix-assisted laser desorption ionization time of-flight mass spectrometry (MALDI-TOF MS) for fish and, more broadly seafood [5–9]. MALDI-TOF MS has emerged as a rapid and accurate method for the identification of microorganisms, but has already been successfully employed for species identification of eukaryotic cell lines or insects [10, 11]. This technology is used to identify genera and species via the generation of fingerprints of highly abundant proteins followed by correlation to reference spectra in a database. As shown by Mazzeo et al. MALDI-TOF MS can discriminate between *Merluccius* species [5]. Due to short turnaround times, low sample volume requirements and low reagent costs, MALDI-TOF MS has recently emerged as a powerful tool for the routine identification of microorganisms [12, 13].

For identification of bacteria by MALDI-TOF MS universal sample preparation protocols are generally available: the standard extraction protocol with 70% formic acid (FA) and a protocol typically used for spore-formers with 80% trifluoroacetic acid (TFA) [14, 15]. A similar universal protocol for fish has yet to be established. Various procedures have been published so far. Mazzeo et al. [5] used 0.1% TFA for sample preparation of fish specimen, while Stephan and co-workers [8] published a sample preparation protocol in which the seafood specimens are treated with 25% FA and chloroform-methanol. A third protocol was performed by Rau and colleagues (CVUA Stuttgart, Germany, personal communication). Here, zirconia beads are used for additional mechanical disruption. So far, this protocol has successfully been used for identification of land animals by MALDI-TOF MS, but has not been tested for seafood so far.

The aim of this study was to evaluate the potential of each protocol to generate high-quality and reproducible mass spectra for high- and low-fat fish, dependent on different storage conditions and levels of food processing (fresh, frozen, refrigerated, cooked and smoked). Moreover, it was determined whether the protein fingerprints of frozen fish specimen were stable enough to enable accurate species identification over a longer storage period.

Material and methods

Fish sampling and processing

A low-fat and a high-fat fish were chosen as sample materials: the rainbow trout (*Oncorhynchus mykiss*) which contains about 2% fat [16] and the Atlantic mackerel (*Scomber scombrus*) with about 12% fat content [17]. In total, two fresh and two smoked specimens of both species were purchased from the local market. For further analysis muscle tissue of the fish was used. The smoked fish were stored at +4 °C until use within three days. Each of the fresh fish specimens was cut into smaller parts. One part of approximately 20 cm^3 was frozen (-20 °C), one part of approximately 20 cm^3 was stored in a refrigerator for a maximum of seven days at +4 °C and one part of approximately 1 cm^3 was treated at 99 °C for 5 min before protein extraction. A further part of the fish was subjected immediately to MALDI-TOF MS analysis.

DNA Extraction and *cytb* sequencing

DNA was extracted from four grams of fish muscle using a modified cetyltrimethylammonium bromide (CTAB) protocol [18]. For molecular species identification, a fragment of mitochondrial *cytb* gene was chosen. Amplification by PCR and sequencing was performed as described in conformity with the official collection of analytical methods according to ASU § 64 of the German Food and Feed Code [4]. The *cytb* fragment was amplified in 50 µl reactions in a Mastercycler® gradient cycler (Eppendorf, Hamburg, Germany). The reaction mixtures contained 0.5 µM of each primer (L14735 5'-AAAAACCACCGTTGTTATTCAACTA-3' and H15149ad 5'-GCICCTCARAATGAYATTTGTCCTCA-3'), 1.5 mM $MgCl_2$, 200 µM each dNTP as well as 0.05 U µl-1 HotStarTaq-DNA-polymerase. All PCR reagents were purchased from Qiagen (Hilden, Germany). The PCR program consisted of an initial activation step for 15 min at 95 °C followed by 35 cycles of denaturation for 40 s at 95 °C, annealing for 80 s at 50 °C, extension for 80 s at 72 °C and a final extension step for 10 min at 72 °C. Amplicons were sequenced using the ABI Prism® 3130 Genetic Analyzer (Applied Biosystems, USA) and queried against the Basic Local Alignment Search Tool (BLAST).

MALDI-TOF MS

Sample preparation

Five different sample preparation protocols for MALDI-TOF MS analyses of fish muscle tissue were compared (Table 1).

Independent of the protocol applied, 1.0 µl of the supernatant was spotted on a polished steel target (Bruker Daltonics, Bremen, Germany). Immediately after drying 1.0 µl of the matrix solution was added to each spot and dried at room temperature. A saturated a-cyano-4-hydroxy-cinnamic acid (4-HCCA, Bruker Daltonics) solution dissolved in 50% (*v*/*v*) acetonitrile with 2.5% (*v*/*v*) TFA was added with subsequent air drying at room temperature.

Protocol A: 70% FA

FA extraction was performed according to standard sample preparation procedure for bacteria [14]. 10 mg fish sample were mixed with 200 µl 70% FA (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany). After vortexing, 200 µl of acetonitrile (Carl Roth GmbH, Karlsruhe, Germany) were added and mixed thoroughly. The homogenate was centrifuged at 20,000 x g for 2 min.

Protocol B: 0.1% TFA

100 mg fish sample were homogenized in 500 µl of 0.1% TFA (abcr GmbH, Karlsruhe, Germany) according to Mazzeo et al. [5]. Subsequently, the sample was vortexed for 30 sec and centrifuged for 2 min at 20,000 x g.

Protocol C: 80% TFA

10 mg fish sample were homogenized in 50 µl of TFA (80%) using micropistils. The sample was incubated at room temperature for 10 min. Subsequently, 150 µl of Diethylpyrocarbonate (DEPC) water (Pyrogen-free, Invitrogen, Carlsbad, USA) and 200 µl acetonitrile were added. The sample was vortexed for 10 sec and centrifuged for 2 min at 20,000 x g [15].

Protocol D: Zirconia beads in 50% acetonitrile and 2.5% TFA

5 mg zirconia beads (0.1 mm, Biospec Product, Carl Roth GmbH,) and 100 µl organic solvent (50% (*v*/*v*) acetonitrile with 2.5% (*v*/*v*) TFA) were added to 5 mg fish muscle tissue. The sample was homogenized using a micropistil, vortexed for 15 sec and centrifuged for 2 min at 20,000 x g according to the protocol performed by Rau for meat sample preparation (CVUA Stuttgart, Germany, personal communication).

Protocol E: 25% FA including chloroform-methanol defatting

5 mg fish sample were homogenized in 100 µl of FA (25%) using a micropistil and vortexed for 5 sec. After adding 100 µl of chloroform (Carl Roth GmbH) and 100 µl of methanol (Fisher Scientific GmbH, Schwerte, Germany) the sample was vortexed for 30 sec and centrifuged for 2 min at 20,000 x g [8].

Sample measurement

The MALDI-TOF MS measurements were performed on a microflex LT mass spectrometer (Bruker Daltonics) equipped with a 60-Hz nitrogen laser. All spectra were recorded in a linear positive detection mode within a mass range from 2,000 to 20,000 Da using FlexControl Version 3.4 Software (Bruker Daltonics) at the minimum laser power necessary for ionization of samples. Bacterial Test Standard (Bruker Daltonics) was used for mass calibration before a set of sum spectra was acquired and used as reference standard in every run.

For generation of a main spectra projection (MSP) 24 sum spectra were performed. A MSP is a reduced reference spectrum calculated from sum spectra by considering only a predefined number of reproducible peaks (here 100 peaks that had to occur in at least 25% of the spectra) with high intensities and high signal-to noise ratios. Each sample was spotted on eight positions of the target and sum spectra of each position were acquired in triplicate by collecting 240 laser shots for each sum spectra. For verification of a MSP samples were measured in duplicates.

Quality control of acquired raw spectra

All 24 sum spectra were imported into flexAnalysis Version 3.4 software (Bruker Daltonics). Baseline correction, smoothing and quality control were performed according to the criteria previously published by Zeller-Péronnet et al. [19]. The measurement deviation was examined in a mass range of 3.000 to 10.000 Da. Single spectra with more than 500 ppm variance from the average mass to charge ratio were removed.

MSP creation and in-house fish species database

Sum spectra that passed the manual quality check were uploaded using the Biotyper OC 3.1 software. MSPs were created with the standard Biotyper algorithm. The software setting for MSP creation was: maximal mass error of each single spectrum: 2,000; desired mass error for the MSP: 200; desired peak frequency minimum (%): 25; maximal desired peak number of the MSP: 70. MSPs were added to the main spectra in-house fish species database.

Verification of the MSP

The validity of the MSP was verified by repeating all sample preparations with a second specimen of both species in duplicates and two technical replicates in MALDI-TOF MS analysis. Acquired spectra were compared to the in-house fish species database using the Biotyper OC software (version 3.1). The resulting log (score) value represents the probability that the match is correct.

Stability test for mass spectra of frozen fish

Furthermore a fourteen-month long stability test of mass spectra was performed for the frozen *O. mykiss* and *S. scombrus* specimen. Samples were prepared in duplicate according to protocol *E* (25% FA including chloroform-methanol defatting). Once a month, two technical replicates of two independent sample preparations were analysed and the resulting mass spectra were searched against the in-house fish species database.

Results

Cytb gene sequencing

Sequence analysis of a *cytb* fragment was used as reference method for the identification of the eight fish specimen used in this study. The generated *cytb* sequences of the four rainbow trouts were identified with homologies between 99% and 100% as *O. mykiss*, while the generated *cytb* sequences of all four Atlantic mackerels showed 100% homology to *S. scombrus* (Table 2).

Choice of sample preparation protocol

24 separate sum spectra (eight spots with three technical replicates) of frozen and fresh aliquots of fish specimen S1_1 and S3_1 (Table 2) were collected for each protocol. Preliminary MSPs were calculated from the respective successfully acquired sum spectra. Spectra reproducibility was tested by using a second specimen (S1_2 and S3_2) (Table 2). Here, two independent sample preparations with two technical replicates were performed for each protocol. Further criteria for the selection of the optimal protocol were the average number of peaks with a signal to noise ratio (S/N) higher than three and an average (S/N) value for the fifteen most intense peaks. The results are summarized in Table 3.

Considering the common criteria of at least 20 sum spectra required for MSP calculation [19] only protocol *E* was successful for both species and both processing grades. Furthermore, only processing of fresh and frozen *S. scombrus* with protocol *B* complied with the above-mentioned criteria. Application of all other protocols resulted in yields between one and thirteen of 24 sum spectra.

For evaluation of spectra, log score values resulting from analysis of a second specimen, are meaningful. Criteria for the identification of microorganisms via MALDI Biotyper are as follows: scores values equal or greater than 2.300 indicate highly probable species identification, values between 2.000 and 2.299 indicate a secure genus and probable species identification, values between 1.700 and 1.999 indicate probable genus identification and score less than 1.700 are considered as unreliable identification. However, these criteria may vary for specific phylogenetic groups of fish. It is necessary to carry out further studies with closely related fish species to set limits for their MALDI-TOF MS based classification. Log scores between 1.94 and 2.38 were obtained for both fish species using protocol *E*. Protocol *D* showed good reproducibility for *O. mykiss* obtaining log scores of 2.36 and 2.45. However, protocol *D* failed for *S. scombrus*. As only two and accordingly seven of 24 sum spectra were generated for preliminary MSP generation, no spectra acquisition for identification was possible. The lowest reproducibility with log scores of 1.21 and 1.63 was found for fresh *O. mykiss* using protocols *A* and *B*. However, for fresh *S. scombrus* spectra, high reproducibility and satisfactory yield of sum spectra were obtained using protocol *B*. For frozen *S. scombrus* in turn protocol *B* resulted in the lowest reproducibility of all five protocols with an average log score of 1.74.

When looking at the number of peaks with S/N ratio higher than three, the maximum for frozen and fresh *S. scombrus* of 100 and accordingly 92 was obtained using protocol *E*. Protocol *E* also resulted in the maximum number of peaks for fresh *O. mykiss* (n=99). Taking into account the ranges of values peak number obtained with protocols *E* and *D* were comparable for frozen *O. mykiss* (n=74 ± 15 and n= 82 ± 12). The lowest number of peaks for *O. mykiss* was observed by using protocol *C* (n=57 ± 7 and n= 31). In case of *S. scombrus* protocol *A* resulted in the lowest number of peaks, which in turn also had the largest relative standard deviation (n=45 ± 14 and n= 59 ± 18).

In conclusion, protocol *E* was chosen as standard protocol for generation of an in-house MSP library for fish species. All in all, spectra reproducibility, yield of sum spectra and the resulting number of peaks were superior to the other protocols.

Influence of the storage period on the quality of spectra

Since fresh fish is very perishable and was possibly already longer frozen at the time of purchase, the influence of the storage period on the quality of spectra was examined for fresh and frozen fish. For this purpose, an aliquot of approximately 20 cm^3 was stored at -20 °C for up to fourteen months, while another aliquot of approximately 20 cm^3 was stored at +4 °C for seven days.

After a defined storage time, samples S1_2 and S3_2 were analyzed twice using protocol *E* and matched against the MSPs of samples S1_1 and S3_1 that were generated after 1 day storage at the respective temperature using protocol *E*. For fresh fish, log scores decreased from 1.94 and 2.31 to 1.83 and 1.78 already after one week of storage at +4 °C (Table 4). In contrast, spectra were stable even after fourteen months of storage at -20 °C for both fish species (Figure 1 and Figure 2): the respective log scores still had values of 2.44 (*O. mykiss*) and 2.39 (*S. scombrus*) (Table 4).

Influence of processing and sample preparation on identification results

In the further course of the study it was determined whether MSPs generated from frozen fish by protocol *E* were suitable for accurate identification i) of fresh, refrigerated, and processed fish (cooked and smoked) and ii) of fish samples that were treated with an alternative sample preparation protocol. The results are summarized in Table 5. It could be shown that MSPs generated from frozen fish by protocol *E* provide reliable identifications of fresh and cooked fish samples. Since the corresponding log scores are generally higher than 2.0. For *O. mykiss* no significant differences in the probability of correct identification where observed between frozen and cooked samples (log score 2.38 vs. 2.42), while log scores for *S. scombrus* decreased from 2.31 to 2.01 after cooking. However, acquisition of reproducible spectra from smoked fishes as well as identification via MSPs from frozen samples both failed, being reflected by log scores of 1.40 for O. mykiss and 0.35 for *S. scombrus*.

With regard to sample preparation using alternative protocols, clear differences in the effect on identification quality of both fish species were observed. Spectra from frozen *O. mykiss* treated with protocol *B* yielded a log score of 1.79 when matched against MSPs acquired by using protocol *E*. Apart from this, all spectra generated from fresh, frozen or cooked *O. mykiss* resulted in log scores higher than 2.0, independent of the protocol used. In contrast, species identification of *S. scombrus* failed when varying processing grade and sample preparation protocol. Even for frozen samples, preparation via protocol *B* resulted in a log score of 0.79, which does not permit species identification. Moreover, it was not possible to acquire sum spectra using protocol D for fresh, frozen and cooked *S. scombrus*.

Discussion

MALDI-TOF MS is accepted as a fast and reliable technology usually applied for bacterial species identification. Concerning bacteria MALDI-TOF MS has certain advantages over genotypic techniques including speed of analysis, wide applicability and the simple procedure of analysis [12]. Exactly such a method is needed in order to counteract against the not uncommon practice of fraudulent as well as unintentional mislabeling of fish and fishery products.

Several working groups have published first results of seafood species identification using MALDI-TOF MS. While a key benefit of MALDI-TOF MS based bacterial species identification is the availability of a universal sample preparation protocol, there are various protocols for seafood species identification [5–9].

The aim of this study was to evaluate various protocols for MALDI-TOF MS based identification of fish species to enable exchange of spectra between working groups as well as the set-up of a common database. The quality of the underlying database is a critical factor concerning the accuracy of the identification. The use of taxonomically verified fish specimens is a prerequisite for a correct database. Furthermore, the accuracy of species identification rises with the number of reference spectra present for each species [12, 20]. In case of bacterial species identification, at least five MSPs of each species are considered as necessary to ensure reliable identification [20].

Identification and subsequent delineation of fish species is only possible if a good number of reference spectra of all relevant species are available. Creation, maintenance and validation of a fish species database is time-consuming – particularly in view of the fact that until now there is no commercial MALDI-TOF MS database for animal species available. The collective work of several laboratories on a common, ideally global database is required to overcome this obstacle. Therefore, an appropriate standard protocol has to be selected first. All in all, of the five protocols compared in this study, protocol E using 25% FA for extraction followed by chloroform-methanol defatting showed the best results concerning spectra yield, reproducibility and number of peaks. This protocol has originally been published by Stephan and co-workers [8] for the identification of scallop species. By using sinapic acid instead of 4-HCCA the authors were able to prove the mislabeling of several *Placopecten magellanicus* specimen as *Pecten maximus*. They also showed a high resolution on species level through their ability to distinguish the two *Argopecten* species A. *irradians* and A. *purpuratus* by MALDI-TOF MS analysis.

Differences between the evaluated protocols were much more pronounced for the high-fat *S. scombrus* than for the low-fat *O. mykiss* (Table 3). The good performance of protocol E for *S. scombrus* may be explained by the defatting step.

Despite the universal sample preparation protocol for bacterial isolates, experience gained there shows that some groups need a special sample preparation procedure to improve spectra quality and significance [21]. That can, of course, also be the case for fish. Even though protocol *E* is now used as the protocol of choice in our laboratory and has already been used for successful generation of MSP from several fish species, it cannot be excluded that an alternative protocol will be required for certain fish species. Generally, metadata of MSP should provide details about the sample preparation protocol used. If an alternative protocol is used for MSP generation, information about the possibility to identify the respective species using the standard protocol should be available. In this context, parameters for fish species identification have to be discussed. It is questionable whether the MALDI Biotyper criteria for the identification of microorganisms can simply be adopted for fish classification. For more accurate conclusions about log (score) cut-off values recommended for fish genera and species identification, extensive expansions of the database and (genera specific) validation studies are still required.

The possibility to identify cooked fish via MSPs generated by protocol *E* from frozen samples is an essential information in terms of routine analysis (Table 5). As expected this was not the case for smoked fish as well as fish refrigerated for several days. Concerning refrigerated samples, log (score) values decreased below 2.0 for *O. mykiss* and S. *scombrus* after seven days. Here, sequence based identification should remain the protocol of choice in future. Anyhow, according to the results presented in Table 4 spectra of frozen fish remain stable even after fourteen months of storage at -20 °C. This test will be continued in order to determine the stability period for spectra generation.

Conclusions

In this study, it was shown that sample preparation with 25% FA followed by chloroform-methanol defatting resulted in spectra of highest quality and reproducibility. Frozen fish are well suited for MALDI-TOF MS based identification over a longer time of storage. Furthermore, substitution of the sample preparation protocol used for MSP acquisition when identifying samples must be treated with due caution. Taking this into account, MALDI-TOF MS has the potential to be used as an alternative to DNA sequencing as soon as a valid database with similar species resolution as DNA sequencing has been established.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Table 1 Brief overview of the characteristics of the five different sample preparation protocols used for MALDI-TOF MS analysis of fish muscle tissue

Protocol *A*	Protocol *B*	Protocol *C*	Protocol *D*	Protocol *E*
70% FA	0.1% TFA	80% TFA	Zirconia beads, 50% (v/v) acetonitrile with 2.5% (v/v) TFA	25% FA, Methanol, Chloroform

Table 2: Results of the *cytb*-sequence analysis of the eight fish specimen used in this study. Samples S1_1, S2_1, S3_1 and S4_1were used for MSP generation. Samples S1_2, S2_2, S3_2 and S4_2 were used for confirmation of the MSP

			***Cytb*-sequence**	
Sample	**Declaration**	**Species**	**Homology**	**Accession number**
S1_1	rainbow trout (fresh)	*O. mykiss*	99.0%	KP013084.1
S1_2	rainbow trout (fresh)	*O. mykiss*	99.0%	KP013084.1
S2_1	smoked rainbow trout	*O. mykiss*	100%	KP013084.1
S2_2	smoked rainbow trout	*O. mykiss*	100%	KP013084.1
S3_1	Atlantic mackerel (fresh)	*S. scombrus*	100%	AB120717.1
S3_2	Atlantic mackerel (fresh)	*S. scombrus*	100%	AB120717.1
S4_1	smoked Atlantic mackerel	*S. scombrus*	100%	AB120717.1
S4_2	smoked Atlantic mackerel	*S. scombrus*	100%	AB120717.1

Table 3: Comparison of the sum spectra for preliminary MSP, mean log score values, sucessfully aquired sum spectra for spectra identification, number of peaks with a signal-to-noise (S/N) ratio >3 and average S/N of the 15 most intense peaks obtained by the five sample preparation protocols (*A*: 70% FA, *B*: 0.1% TFA, *C*: 80% TFA, *D*: zirconia beads in 50% acetonitrile and 2.5% TFA, *E*: 25% FA followed by chloroform-methanol defatting) for *O. mykiss* and *S. scombrus.* Sum Spectra were matched against the corresponding MSP of each sample preparation protocol and processing grade. Log score values greater than 2.3 or the highest log score values, the highest yields for sum spectra, the highest no. of peaks S/N > 3 and the highest S/N average are in bold

			O. mykiss					***S. scombrus***			
Processing grade	**Protocol**	**Sum spectra for prelimary MSP**	**Mean log score value**	**Successfully acquired sum spectra for identification [1]**	**No. of peaks[2] S/N > 3**	**Average S/N of the 15 most intense peaks**	**Sum spectra for prelimary MSP**	**Mean log score value**	**Successfully acquired sum spectra for identification [1]**	**No. of peaks[2] S/N > 3**	**Average S/N of the 15 most intense peaks**
	A	08/24	1.21 ± 0.06	**4/4**	93 ± 5	16 ± 2	02/24	1.71 ± 0.05	2/4	45 ± 14	6 ± 2
	B	08/24	1.63 ± 0.11	**4/4**	75 ± 7	12 ± 1	23/24	2.24 ± 0.28	**4/4**	70 ± 8	**21** ± 2
Fresh	*C*	03/24	**2.32** ± 0.06	3/4	57 ± 7	13 ± 1	02/24	1.77	1/4	37	5
	D	13/24	**2.36** ± 0.03	2/4	86 ± 20	17 ± 3	07/24	no peaks found	0/4	no peaks found	no peaks found
	E	**24/24**	1.94 ± 0.10	**4/4**	**99** ± 3	**21** ± 2	**24/24**	**2.28** ± 0.10	**4/4**	**92** ± 6	9 ± 1
	A	03/24	2.27	1/4	66	12 ±	09/24	**2.32** ± 0.10	**4/4**	59.0 ± 18	12 ± 6
	B	07/24	**2.53** ± 0.03	3/4	56 ± 8	11 ± 1	21/24	1.74 ± 0.35	2/4	61 ± 16	16 ± 1
Frozen	*C*	01/24	2.04	1/4	31	6	01/24	**2.39** ± 0.2	2/4	75 ± 25	12 ± 2
	D	09/24	**2.45** ± 0.08	3/4	**82** ± 12	14 ± 7	02/24	no peaks found	0/4	no peaks found	no peaks found
	E	**24/24**	**2.38** ± 0.10	**4/4**	**74** ±15	13 ± 3	**23/24**	**2.31** ± 0.08	**4/4**	**100** ± 0	19 ± 1

[1]obtained from two sample preparations with two measured spots each, [2]calculated from successfully acquired sum spectra for identification

Table 4: Influence of the period of storage of fish samples at -20 °C and +4 °C on the quality of MALDI-TOF MS spectra. Mean log score values of at least three independent measurements are presented. Sum spectra were acquired by treating samples with protocol *E* and matched against MSP obtained by using protocol *E* and the respective storage temperature. Log score values greater than 2.3 are in bold

		Period of storage								
		1 day	**7 days**	**1 month**	**2 months**	**3 months**	**6 months**	**9 months**	**12 months**	**14 months**
At -20 °C	***O. mykiss***	**2.38** ± 0.10	n. d.[1]	**2.54** ± 0.03	**2.53** ± 0.02	**2.56** ± 0.03	**2.47 ±** 0.05	**2.46 ±** 0.03	**2.40** ± 0.05	**2.44** ± 0.07
	S. scombrus	**2.31** ± 0.08	n. d.	**2.47** ± 0.05	**2.38** ± 0.22	**2.40** ± 0.06	**2.45 ±** 0.02	2.24 ± 0.13	**2.34** ± 0.05	**2.39** ± 0.07
At +4 °C	***O. mykiss***	**1.94** ± 0.10	**1.83** ± 0.06	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.
	S. scombrus	**2.28** ± 0.10	**1.78** ± 0.06	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.	n. d.

[1]n. d. – not determined

Table 5: Influence of sample preparation on identification results. Fish samples S1_2 to S4_2 were treated with five different sample preparation protocols (*A*: 70% FA, *B*: 0.1% TFA, *C*: 80% TFA, *D*: zirconia beads in 50% acetonitrile and 2.5% TFA, *E*: 25% FA followed by chloroform-methanol defatting) and matched against MSP of frozen *O. mykiss* and *S. scombrus*, which were acquired by using protocol *E* and samples S1_1 and S3_1. Log score values greater than 2.3 are in bold.

		mean log score value	
Processing grade	**Protocol**	***O. mykiss***	***S. scombrus***
Fresh	*A*	**2.40** ± 0.08	1.63 ± 0.05
	B	**2.32** ± 0.05	0.30 ± 0.17
	C	**2.29** ± 0.04	1.60
	D	**2.29** ± 0.02	no spectra[1]
	E	**2.54** ± 0.06	**2.31** ± 0.10
Frozen	*A*	**2.33**	2.13 ± 0.06
	B	1.79 ± 0.10	0.79 ± 0.27
	C	2.09	**2.32** ± 0.18
	D	**2.34** ± 0.10	no spectra
	E	**2.38** ± 0.10	**2.31** ± 0.08
Cooked	*A*	**2.29** ± 0.08	1.75 ± 0.01
	B	2.07 ± 0.06	no spectra
	C	**2.24** ± 0.14	2.02
	D	**2.14** ± 0.18	no spectra
	E	**2.42** ± 0.09	2.01 ± 0.17
Refrigerated, 7d	*A*	2.02 ± 0.06	1.87 ± 0.09
	B	1.32 ± 0.09	0.97 ± 0.12
	C	2.15 ± 0.13	1.76 ± 0.06
	D	0.71 ± 0.16	1.44 ± 0.10
	E	1.96 ± 0.15	1.79 ± 0.06
Smoked	*A*	1.47 ± 0.30	0.262 ± 0.14
	B	no spectra	no spectra
	C	no spectra	0.46 ± 0.26
	D	no spectra	0.39
	E	1.40	0.35 ± 0.17

[1] Application of the respective protocol did not result in any spectra

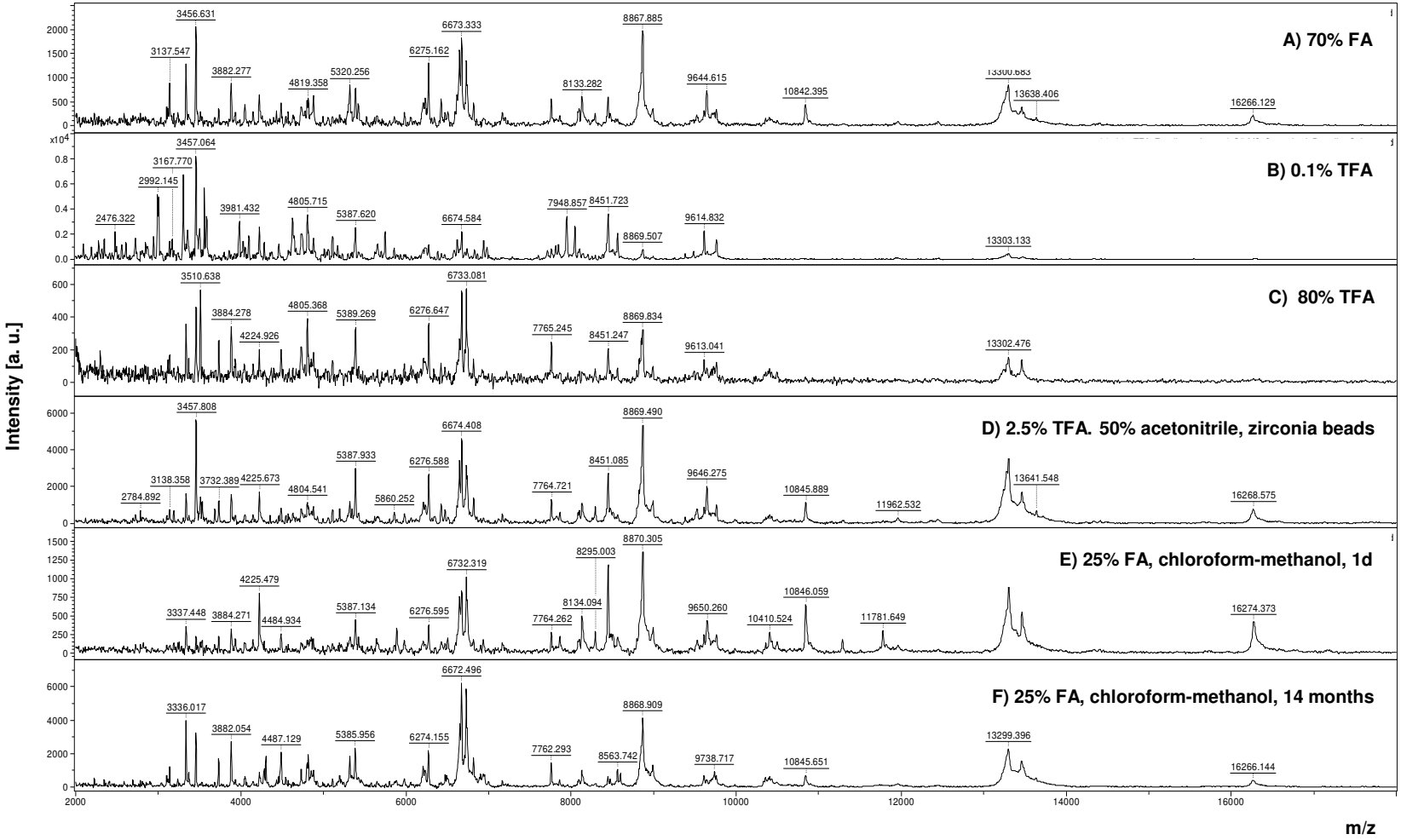

Fig. 1: Representative MALDI-TOF MS profiles of frozen *O. mykiss*. Sample S1_2 was treated with five different sample preparation protocols - *A*: 70% FA, *B*: 0.1% TFA, *C*: 80% TFA, *D*: zirconia beads in 50% acetonitrile and 2.5% TFA, *E*: 25% FA followed by chloroform-methanol defatting, *F*: Spectra acquisition with protocol *E* was repeated with sample S1_2 after fourteen months of storage at -20 °C

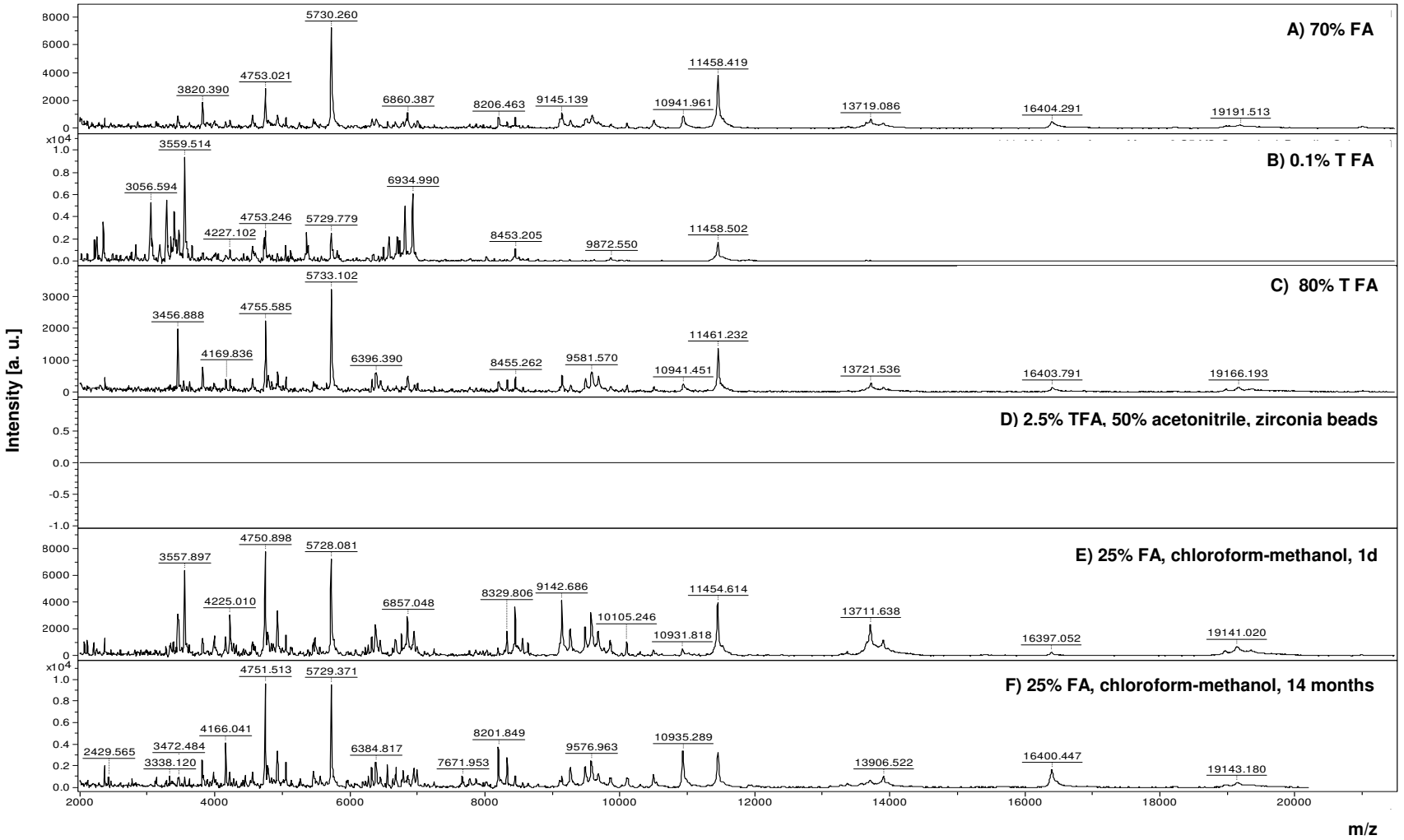

Fig. 2: Representative MALDI-TOF MS profiles of frozen *Scomber scombrus*. Sample M_3 was treated with five different sample preparation methods -*A*: 70 % FA, *B*: 0.1 % TFA, *C*: 80 % TFA, *D*: zirconia beads in 50 % acetonitrile and 2.5 % TFA, *E*: 25 % acetic acid followed by chloroform-methanol defatting, F: Spectra acquisition with E was repeated with sample S_3 after fourteen months of storage at -20°C.

6. Publication III Comparison of three DNA marker regions for identification of food relevant crustaceans of the order Decapoda

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Comparison of three DNA marker regions for identification of food relevant crustaceans of the order Decapoda

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Abstract

As a result of the commercial importance of food relevant crustaceans belonging to the order Decapoda, reliable methods for species identification are required to protect consumers against adulteration and mislabelling of crustacean products. The aim of this present study is therefore to compare three marker regions for their suitability to identify food relevant crustaceans of the order Decapoda: (*A*) the official control method in Germany for identification of crustacean species based on 16S rDNA sequences; (*B*) sequencing of another 16S rDNA fragment developed by Palumbi and colleagues; (*C*) 'DNA barcoding' by sequencing of the mitochondrial cytochrome *c* oxidase subunit 1 gene (*COI*).

Marker region *A* showed some disadvantages compared to marker region *C* because of inadequate amplification or resulting low-quality sequences for several decapods. Marker region *B* showed better species identification results than marker region *A* but offered no advantage compared to marker region *A* combined with marker region *C*. Marker region *A* is only to be preferred for species, of which the reference sequences of *COI* are not yet present in public sequence databases. The results of this study show that the most suitable marker region for the identification of food relevant decapods is *COI* and that this marker region has the potential to supplement or even replace the current official method of Germany in the official control of foodstuff.

Moreover, this study shows that for certain food relevant crustacean species there is still a lack of reference sequences.

Introduction

Crustaceans belonging to the order of Decapoda (prawns, shrimps, lobsters, crayfish, or crabs) have a significant commercial importance [1, 2]. Under the Council Regulation (EC) No 1379/2013 on the common organization of the markets in fishery and aquaculture, products have to be labelled among others with (i) the commercial designation of the species and its scientific name, (ii) the production method, and (iii) the area where the product was caught or farmed. This regulation has enforced the EU member states to draw up and publish a list of the commercial designations for fish and seafood species that are accepted in their territory, indicating the scientific name and the corresponding name in the official language. In Germany, the Federal Office for Agriculture and Food is responsible for this list.

Crustaceans are commonly consumed as food products and thus are exposed to the risk of adulteration [4-7]. In many cases a species of higher value is substituted by another similar species of lower value after being processed. Well-known replacements are *Fenneropenaeus indicus* (H. Milne Edwards, 1837) by *Metapenaeus* species (Wood-Mason, 1891), *Farfantepenaeus aztecus* (Ives, 1891) by *Farfantepenaeus brasiliensis* (Latreille, 1817) or *Nephrops norvegicus* (Linnaeus, 1758) by *Metanephrops* species [5, 8]. Therefore, reliable analytical methods for species identification are required in order to protect consumers against adulteration and mislabelling of products [1]. Because of phenotypic similarities among Decapoda and due to industrial processing, species identification based on morphological analysis underlies limitations and is almost impossible in many cases [1, 9-11]. Species identification of crustacean is performed with several molecular biology methods. Among the protein based methods isoelectric focusing electrophoresis (IEF) for species identification of crustacean [12-15] is commonly used [1]. A recent approach is MALDI-TOF MS whose suitability for crustacean species identification was shown by Salla and Murray [16]. But protein based techniques often reach their limits with processed seafood due to denaturation of the proteins [17]. Among DNA based methods PCR-RFLP is, besides DNA sequencing, the most commonly used method for species identification of crustacean [18, 19]. However, this method has the disadvantage that incomplete restriction digestion or depletion or creation of additional restriction sites due to intraspecific variation can lead to wrong species identification results [20]. In this context, DNA-sequencing offers an alternative approach, also enabling the identification of processed (e.g., cooked) seafood [9]. Mitochondrial DNA has, in contrast to nuclear DNA, the advantage of an elevated rate of mutation. Therefore, smaller gene segments can be used for reliable species identification [21, 22]. Moreover, due to higher copy numbers (approx. 1.000 times the copy of nuclear DNA in some cases) even processed samples have been successfully analysed by DNA-sequencing [22]. Although mitochondrial DNA has several advantages to nuclear DNA some aspects have to be considered [23]. The great disadvantage of sequencing mitochondrial DNA is the potential occurrence of nuclear mitochondrial pseudogenes (numts). Numts are non-functional nuclear sequences of mitochondrial origin [24]. Numts occur in a variety of metazoan among other in crustacean [e.g., 24-26]. By amplifying these nuclear sequences instead of or in addition to the mitochondrial sequence this can lead to wrong phylogenetic replacements, frameshift mutations, stop codons as well as ambiguous sequences [27]. By checking for the occurrence of these effects, numts can be detected.

Identification of crustacean species in raw specimens and crustacean products for official control of foodstuffs in Germany is performed with the official method for identification of crustacean species afforded by §64 of the German Food and Feed Code (LFGB) via sequencing 312 base pairs (bp) of a fragment of the mitochondrial 16S rRNA gene (12.01-03 Crustacean species identification in raw and processed crustacean products by means of sequence analysis of 16S rRNA sequences). However, sequencing this marker region is not suitable for all food relevant crustacean species: For example, it is not recommended for the analysis of species of the genus *Crangon* (Fabricius, 1798), because no suitable amplicons were obtained [28]. For this reason, we aimed to find a marker region able to identify food relevant crustacean species and to determine whether one marker region alone is sufficient - or if two or more should be combined - for an all-around identification. The combination of more than one marker region can lead to a higher success of species identification, like in case of hakes, tunas and decapod species [29-32]. Furthermore, analyzing more than one marker can prevent wrong species identification due to the presence of nuclear mitochondrial pseudogenes (numts) [33].

Mitochondrial cytochrome *c* oxidase subunit 1 gene (*COI*) is as barcode widely used for species identification in a variety of taxa [34] and is widely used for species identification such as fish [e.g., 35-42], molluscs [e.g., 43-45] and crustacean [e.g., 7, 9, 11, 23, 32, 46, 47]. The purpose of DNA barcoding is identification of known species and discovering undescribed species [48] even by non-experts [49] but in some taxa species identification reaches it limits, like in the case of tuna species [30, 50, 51]. For such species analysis of other markers instead or in addition are recommended. Another 16S rDNA marker region with the primers by Palumbi et al. [52] amplifying a 570 bp fragment of 16S rDNA enclosing the 312 bp fragment amplified by the official method primers (localisation of both 16S rDNA primer pairs is shown in supplementary material Fig. S1) is used successfully for species identification of commercially important penaeid shrimp [53] and other Decapoda [54, 55]. Thus, these marker regions are presenting promising alternatives for identification of crustacean species. Accordingly, 19 decapod species were analysed using three gene markers and compared for their suitability for species identification in this work.

Material and methods

Sample collection

The 19 crustacean species of commercial interest (see Table 1 for species) – all belonging to the order Decapoda - were samples of the routine analysis or purchased from local markets. The samples were frozen at -20 °C until further use. Of each species two specimens were analysed.

DNA extraction

DNA was extracted from two grams of decapod muscle using a modified cetyltrimethylammonium bromide (CTAB) protocol [56].

DNA-sequencing

The *COI* and 16S rRNA marker regions were amplified with approximately 100 ng DNA in 50 µl reactions in a Mastercycler® gradient cycler (Eppendorf, Germany). The quality of the amplified products was checked with the 2100 Bioanalyzer Instrument (Agilent Technologies, Santa Clara, CA, USA) and purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Amplicons were sequenced using the ABI Prism® BigDye™ Terminator V 1.1 Kit and the ABI Prism® 3130 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA).

16S rDNA

Marker region *A*

The reaction mixture of the official German method contained 0.5 µM of each Primer (16S 312F 5'-GRAGGCTTGTATGAATGGTTG-3' and 16S 312R-1 3'-AARWARATWACGCTGTTA-5'), 1.5 mM $MgCl_2$, 200 µM of each dNTP as well as 0.05 U $µl^{-1}$ HotStarTaq DNA Polymerase [28]. All PCR reagents were purchased from Qiagen. The used PCR program consisted of an initial activation step for 15 min at 95 °C followed by 35 cycles of denaturation for 60 s at 95 °C, annealing for 60 s at 50 °C, extension for 60 s at 72 °C and a final extension step for 10 min at 72 °C.

Marker region *B*

The reaction mixture of the 16S rDNA marker region by Palumbi et al. [52] contained 0.12 µM of each Primer (16Sar-L 5'-CGCCTGTTTATCAAAAACAT-3' and 16Sbr-H 5'-CCGGTCTGAACTCAGATCACGT-3'), 2 mM $MgCl_2$, 100 µM each dNTP as well as 0.04 U $µl^{-1}$ TaKaRa Ex Taq DNA Polymerase. All PCR reagents were purchased from Takara Bio Europe (Saint-Germain-en-Laye, France). The used PCR program consisted of an initial activation step for 2 min at 94 °C followed 30 cycles of denaturation for 30 s at 94 °C, annealing for 40 s at 55 °C, extension for 60 s at 72 °C and a final extension step for 5 min at 72 °C.

Cytochrome c oxidase subunit 1 (Marker region C)

The reaction mixture contained 0.3 µM of each Primer (LCO1490 5'-GGTCAACAAATCATAAAGATATTGG-3' and HCO2198 5'-TAAACTTCAGGGTGACCAAAAAATCA-3', [57]), 2 mM $MgCl_2$, 200 µM each dNTP as well as 0.025 U $µl^{-1}$ TaKaRa Ex Taq DNA Polymerase. All PCR reagents were purchased from Takara Bio Europe.

The used PCR program consisted of an initial activation step for 2 min at 94 °C followed by 5 cycles for 30 s at 94 °C, annealing for 40 s at 47° C, extension for 60 s at 72 °C, 35 cycles of denaturation for 30 s at 94 °C, annealing for 40 s at 52 °C, extension for 60 s at 72 °C and a final extension step for 10 min at 72 °C.

Data Analysis

Sequence chromatograms were checked visually and assembled in the Integrated Database Network System (IDNS®) Software (SmartGene Inc., Lausanne, Switzerland). Sequences were checked for the presence of nuclear copies of mitochondrial sequences [58] and subsequently queried against the publicly accessible databases Basic Local Alignment Search Tool (BLAST) and in case of *COI* also against the Barcode of Life Database (BOLD) [59] before the end of October 2018. All sequences derived from this study were deposited in GenBank (accession numbers: MH300622-MH300672 and MK000234-MK000286).

For sample S6 (*Heterocarpus reedi*, Bahamonde, 1955*), COI* sequences generated from all species from this study for marker region *C* with clear identification results for both genes, and further sequences from other decapod species extracted from GenBank (in total two sequences from each species) were assembled and aligned with BioEdit version 5 [60]. The alignment was trimmed to 432 nucleotides and used to construct a neighbour-joining tree using MEGA7 [61] utilizing the Kimura 2-parameter model [62]. Bootstrap values were calculated with 1000 pseudoreplicates [63].

Results

We analysed 38 specimens (19 species) of the order of Decapoda (Table 1). 32 specimens, for which the species and the scientific name were declared, belonged to nine different families (Aristeidae, Cambaridae, Cancridae, Crangonidae, Nephropidae, Pandalidae, Parastacidae, Penaeidae and Solenoceridae). In case of six specimens, no scientific name of the species (S8, S13 and S14) was declared. Identification by DNA-sequencing with at least one of the three marker regions revealed that they belonged to the families Penaeidae, Palaemonidae and Parastacidae. In total, ten families of the order Decapoda were analysed with all three marker regions (16S rDNA: marker region *A*_312 bp and marker region *B*_570 bp as well as *COI:* marker region *C*).

The three marker regions were compared for their suitability for the identification of decapods on the basis of these 19 species. Basis for comparison was the successful identification on species or genus level, identification failures due to inadequate amplification or low-quality sequences as well as lack of reference sequences in the databases.

By sequencing *COI* gene fragment (marker region *C*) all 38 specimens (19 species, 100%) were successfully sequenced. 34 specimens (17 species) of these were identified to species level (89%).

In case of 16S rDNA 28 (of 14 species, 74%, marker region *A*_312 bp) and 38 (of 19 species, 100%, marker region *B*_570 bp) sequences were successfully obtained. Of these, three species (six specimens) could not be identified to species level using either marker region (S5, S6 and S13). Identification of ten specimens (S1-1 and S1-2: *Nephrops norvegicus* (Linnaeus, 1758), S14-1 and S14-2: *Cherax destructor* (Clark, 1936), S15-1 and S15-2: *Cancer pagurus* (Linnaeus, 1758), S17-1 and S17-2: *Procambarus clarkii* (Girard, 1852) as well as S19-1 and S19-2: *Crangon crangon* (Linnaeus, 1758)) was hampered by insufficient sequences for marker region *A* (16S rDNA_312 bp), compared to marker region *B* (16S rRNA_570 bp) where all five species were successfully sequenced.

Samples S13-1 and S13-2 (‘Freshwater Shrimp’) showed, besides high sequence similarity to *Macrobrachium rosenbergii*, (de Man, 1879) also high sequence similarity to *Macrobrachium daqueti* (Sunier, 1925). With marker region *A* (16S rDNA_312 bp) sample S13-1 and S13-2 showed 100% sequence similarity to *Macrobrachium rosenbergii* and 99% sequence similarity (2 mismatches) to *Macrobrachium dacqueti*. With marker region *B* (16S rDNA *B*_570 bp) sample S13-1 and S13-2 showed 99% sequence similarity (1 mismatch) to *Macrobrachium rosenbergii* and *Macrobrachium dacqueti*.

With marker region *C* (*COI*) sample S13-1 showed 100% sequence similarity and sample S13-2 99% sequence similarity (1 mismatch) to *Macrobrachium rosenbergii* and both samples showed 99% sequence similarity (2 mismatches) to *Macrobrachium dacqueti.*

In case of one shrimp species (S5-1 and S5-2 declared as *Pandalus jordani* (Rathbun, 1902)), no 16S rDNA sequences of *Pandalus jordani* are deposited in GenBank. Sequencing of the 16S rRNA gene of sample S5-1 resulted in 96% (marker region *A*: 16S rDNA_312 bp) and 98% (marker region *B*: 16S rDNA_570 bp) sequence similarities to *Pandalus borealis* (Krøyer, 1838).

Samples S6-1 and S6-2 (declared as *Heterocarpus reedi*) were only matched on genus level with all three marker regions, because no database hits had sufficient identity for species identification and no sequence of *Heterocarpus reedi* was deposited in the sequence databases. The sequence similarities were for *COI*: 85% (S6-1) and 84% (S6-2) to *Heterocarpus laevigatus* (Bate, 1888, also with BOLD) and for 16S rDNA: 91-93% similarity to *Heterocarpus* species among other *Heterocarpus laevigatus*.

To identify the samples S6-1 and S6-2 at least on genus level, a neighbour-joining tree was constructed with all *COI* sequences from this study which had clear identification results with both genes (S1, S2, S3, S4, S7, S8, S9, S10, S11, S12, S14, S15, S16, S17, S18 and S19) and further sequences from diverse decapod species extracted from GenBank (altogether 20 species from nine families: Nephropidae, Cambaridae, Parastacidae, Pandalidae, Aristeidae, Solenoceridae, Cancridae, Penaeidae and Crangonidae; Figure 1).

The generated *COI* sequences of samples S6-1 and S6-2 was grouped in a cluster of species belonging to the genus *Heterocarpus* with a bootstrap value of 63% and showed closest relationship to the species *Heterocarpus laevigatus* with a bootstrap value of 88%. Further, samples S6-1 and S6-2 were grouped in a cluster with *Pandalus borealis* belonging to the family Penaeidae.

These results underline the presumption that samples S6-1 and S6-2 belong to the family Penaeidae and the genus *Heterocarpus* and that the species of the samples S6-1 and S6-2 has not yet been analysed with sequencing *COI* and 16S rDNA.

Discussion

The standard method for identification of crustacean species within the context of official control of foodstuffs in Germany (§64 LFGB) is the DNA-sequencing of a fragment of the mitochondrial 16S rDNA. Because this marker region is not suitable for every food relevant crustacean, the aim of this study was to establish a suitable DNA-sequencing-strategy by which food relevant crustacean species can be identified.

None of the three tested marker regions could identify all of the 38 analysed specimens at species level. However with marker region *C* (*COI*) most of the specimens (94%, in contrast to marker region *A* (16S rDNA_312 bp)*:* 58% and marker region *B* (16S rDNA_570 bp*:* 84%) were successfully identified on species level. Both 16S rDNA marker regions did not show an advantage to *COI* sequencing.

Moreover, the official German method (marker region *A:* 16S rDNA_312 bp) showed some deficiencies: Eight specimens were characterized by inadequate amplification or low-quality sequences such as *Crangon crangon* as already described in the official control method [28]. With marker region *B* (16S rDNA_570 bp) more specimens yielded sufficient sequences (38 in contrast to 28 with marker region *A*: 16S rDNA_312 bp), but for 28 specimens (14 species) DNA was amplified successfully with both 16S rDNA marker regions (*A* and *B*).

It was expected, that marker region *B* (16S rDNA_570 bp) would lead to a higher species resolution due to the generation of longer sequences. But in case of the 28 specimens (14 species, which were successfully sequenced with marker region *A* (16S rDNA_312 bp) and *B* (16S rDNA_570 bp), marker region *B* showed no higher species resolution than marker region *A*, probably because most of the regions which are additionally amplified with the primers for marker region B are conserved among the decapod species (supplementary material Fig. S1).

None of the three methods tested was able to differentiate between the two congeneric species *Macrobrachium rosenbergii* and *Macrobracium dacqueti* because of insufficient sequence divergence. Accordingly, in this case another marker might be more suitable. Furthermore, none of the three marker regions was able to identify samples S6-1 and S6-2 to species level. However, with a neighbour-joining tree analysis conducted with *COI* sequences, results indicate that the samples most probably belong to the genus *Heterocarpus*. If the samples were the species *Heterocarpus reedi* as declared, cannot be concluded with certainty because no *COI* or 16S rDNA sequence of this species is deposited in GenBank and BOLD.

In July 2018 public DNA barcodes of 4.306 decapod species were available in BOLD and the database is continuously growing. The main goal of DNA barcoding is to assign unidentified specimens to identified species. Most crustacean barcoding studies were found to build on existing reference libraries for identification purposes, and this trend will surely continue and probably increase in the future [64]. Indeed, it might be a matter of (probably short) time until a *COI* sequence of *Heterocarpus reedi* will be available in BOLD and GenBank.

The *COI* marker region showed the potential to supplement or even replace the official method, but there should be further studies like the analyses of more species and families of the order Decapoda as well as ring trials to confirm this.

The failure to identify samples S6-1 and S6-2 to species level showed the problem of still incomplete publicly accessible DNA-databases. For this reason, the collaborative project 'Development of DNA-based methods for the identification of fish and fishery products, as well as crustaceans and molluscs for practical use in the food and import control (MARINEFOOD)', covering also the present study, aims to fill these gaps for the most important species from the viewpoint of the official food control and surveillance authorities.

Conclusions

Because of inadequate amplification or low-quality sequences for several crustacean species, the official German method (marker region *A*) showed some disadvantages compared to marker region *C* (*COI)*. The most suitable method for identification of food relevant decapods is DNA-sequencing of *COI*. The official method is only to be preferred for species where reference sequences of *COI* are not yet available in databases. More crustacean species have to be sequenced, but in the future *COI* sequencing has the potential to replace the current official method in the official control of foodstuff. Although, except *Macrobrachium* spp., all analysed decapod species in this study were unambiguously identified by using the *COI* marker region, sequencing of more than one marker region can confirm the identification results or lead to species identification when sequences of *COI* are not yet available. The fact that there is still a lack of reference sequences shows the need for a publicly accessible database containing all food relevant seafood species for supporting the authorities to protect consumers against adulteration and mislabelling of products.

Table 1 Results of decapods identification by DNA-sequencing

Sample ID	Declared species		Species identified					
	Scientific name	Commercial name	Marker region *A* (16S rDNA_312 bp)		Marker region *B* (16S rDNA_570 bp)		Marker region *C* (*COI*)	
S1-1	*Nephrops norvegicus*	Norway Lobster	-[1]		100%	*Nephrops norvegicus*	99%	*Nephrops norvegicus*
S1-2	*Nephrops norvegicus*	Norway Lobster	-[1]		100%	*Nephrops norvegicus*	100%	*Nephrops norvegicus*
S2-1	*Homarus gammarus*	European Lobster	99%	*Homarus gammarus*	99%	*Homarus gammarus*	100%	*Homarus gammarus*
S2-2	*Homarus gammarus*	European Lobster	100%	*Homarus gammarus*	100%	*Homarus gammarus*	100%	*Homarus gammarus*
S3-1	*Homarus americanus*	American Lobster	100%	*Homarus americanus*	100%	*Homarus americanus*	100%	*Homarus americanus*
S3-2	*Homarus americanus*	American Lobster	100%	*Homarus americanus*	100%	*Homarus americanus*	100%	*Homarus americanus*
S4-1	*Pandalus borealis*	Shrimp	99%	*Pandalus borealis*	100%	*Pandalus borealis*	100%	*Pandalus borealis*
S4-2	*Pandalus borealis*	Shrimp	100%	*Pandalus borealis*	100%	*Pandalus borealis*	100%	*Pandalus borealis*
S5-1	*Pandalus jordani*	Shrimp	**96%**	***Pandalus borealis***	**98%**	***Pandalus borealis***	100%	*Pandalus jordani*
S5-2	*Pandalus jordani*	Shrimp	**97%**	***Pandalus borealis***	**98%**	***Pandalus borealis***	99%	*Pandalus jordani*
S6-1	*Heterocarpus reedi*	Shrimp	**91%**	***Heterocarpus laevigatus***	**93%**	***Heterocarpus laevigatus***	**85%**	***Heterocarpus laevigatus***
S6-2	*Heterocarpus reedi*	Shrimp	**91%**	***Heterocarpus laevigatus***	**93%**	***Heterocarpus laevigatus***	**84%**	***Heterocarpus laevigatus***

S7-1	*Penaeus monodon*	Shrimp	100%	*Penaeus monodon*	100%	*Penaeus monodon*	100%	*Penaeus monodon*
S7-2	*Penaeus monodon*	Shrimp	100%	*Penaeus monodon*	100%	*Penaeus monodon*	100%	*Penaeus monodon*
S8-1	n. a.	Shrimp	99%	*Metapenaeus ensis*	100%	*Metapenaeus ensis*	99%	*Metapenaeus ensis*
S8-2	n. a.	Shrimp	100%	*Metapenaeus ensis*	100%	*Metapenaeus ensis*	99%	*Metapenaeus ensis*
S7-1	*Penaeus monodon*	Shrimp	100%	*Penaeus monodon*	100%	*Penaeus monodon*	100%	*Penaeus monodon*
S7-2	*Penaeus monodon*	Shrimp	100%	*Penaeus monodon*	100%	*Penaeus monodon*	100%	*Penaeus monodon*
S8-1	n. a.	Shrimp	99%	*Metapenaeus ensis*	100%	*Metapenaeus ensis*	99%	*Metapenaeus ensis*
S8-2	n. a.	Shrimp	100%	*Metapenaeus ensis*	100%	*Metapenaeus ensis*	99%	*Metapenaeus ensis*
S9-1	*Xiphopenaeus kroyeri*	Seabob	100%	*Xiphopenaeus kroyeri*	100%	*Xiphopenaeus kroyeri*	99%	*Xiphopenaeus kroyeri*
S9-2	*Xiphopenaeus kroyeri*	Seabob	100%	*Xiphopenaeus kroyeri*	100%	*Xiphopenaeus kroyeri*	99%	*Xiphopenaeus kroyeri*
S10-1	*Penaeidae* sp.	Shrimp	100%	*Fenneropenaeus indicus*	99%	*Fenneropenaeus indicus*	99%	*Fenneropenaeus indicus*
S10-2	*Penaeidae* sp.	Shrimp	100%	*Fenneropenaeus indicus*	100%	*Fenneropenaeus indicus*	100%	*Fenneropenaeus indicus*
S11-1	*Penaeidae* sp.	Shrimp	100%	*Fenneropenaeus merguiensis*	100%	*Fenneropenaeus merguiensis*	99%	*Fenneropenaeus merguiensis*
S11-2	*Penaeidae* sp.	Shrimp	100%	*Fenneropenaeus merguiensis*	100%	*Fenneropenaeus merguiensis*	99%	*Fenneropenaeus merguiensis*

S12-1	*Litopenaeus vannamei*	Shrimp	99%	*Litopenaeus vannamei*	99%	*Litopenaeus vannamei*	100%	
S12-2	*Litopenaeus vannamei*	Shrimp	99%	*Litopenaeus vannamei*	100%	*Litopenaeus vannamei*	100%	
S13-1	n. a.	Freshwater Shrimp	100%	*Macrobrachium rosenbergii*	100%	*Macrobrachium rosenbergii*	100%	*Macrobrachium rosenbergii*
			100%	*Macrobrachium dacqueti*	99%	*Macrobrachium dacqueti*	99%	*Macrobrachium dacqueti*
S13-2	n. a.	Freshwater Shrimp	100%	*Macrobrachium rosenbergii*	100%	*Macrobrachium rosenbergii*	100%	*Macrobrachium rosenbergii*
			100%	*Macrobrachium dacqueti*	99%	*Macrobrachium dacqueti*	99%	*Macrobrachium dacqueti*
S14-1	n. a.	Crawfish		-[1]	99%	*Cherax destructor*	100%	*Cherax destructor*
S14-2	n. a.	Crawfish		-[1]	99%	*Cherax destructor*	100%	*Cherax destructor*
S15-1	*Cancer pagurus*	Rock Crab		-[1]	100%	*Cancer pagurus*	100%	*Cancer pagurus*
S15-2	*Cancer pagurus*	Rock Crab		-[1]	100%	*Cancer pagurus*	100%	*Cancer pagurus*
S18-1	*Aristeus alcocki*	Shrimp	99%	*Aristeus alcocki*	99%	*Aristeus alcocki*	99%	*Aristeus alcocki*
S18-2	*Aristeus alcocki*	Shrimp	99%	*Aristeus alcocki*	99%	*Aristeus alcocki*	100%	*Aristeus alcocki*
S19-1	*Crangon crangon*	Shrimp		-[1]	99%	*Crangon crangon*	100%	*Crangon crangon*
S19-2	*Crangon crangon*	Shrimp		-[1]	100%	*Crangon crangon*	99%	*Crangon crangon*

[1]: no amplification/low-quality sequences. n. a.: not available. *COI*: cytochrome *c* oxidase subunit I, 16S rDNA: 16S ribosomal DNA.
Species in bold have low match values

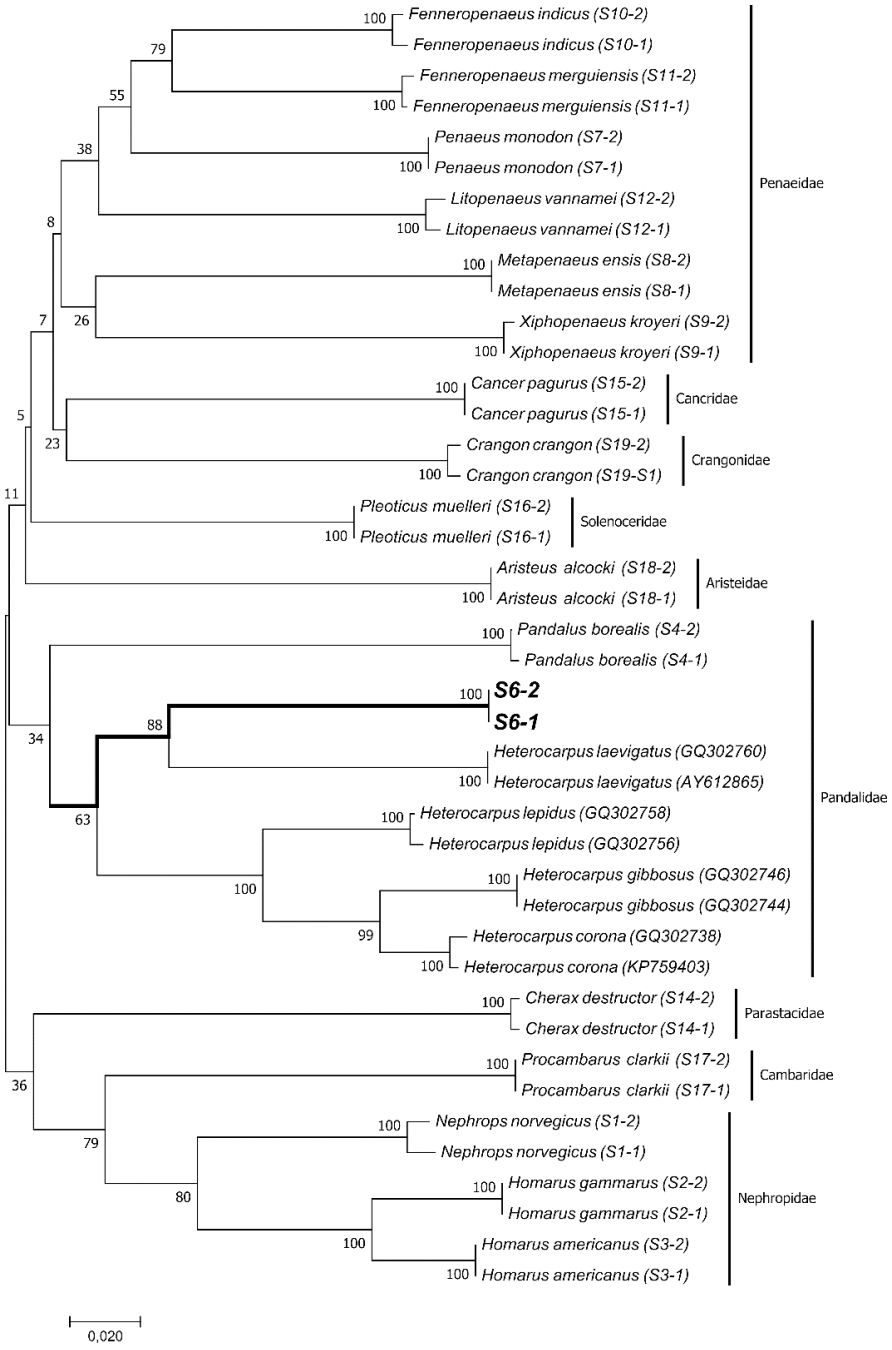


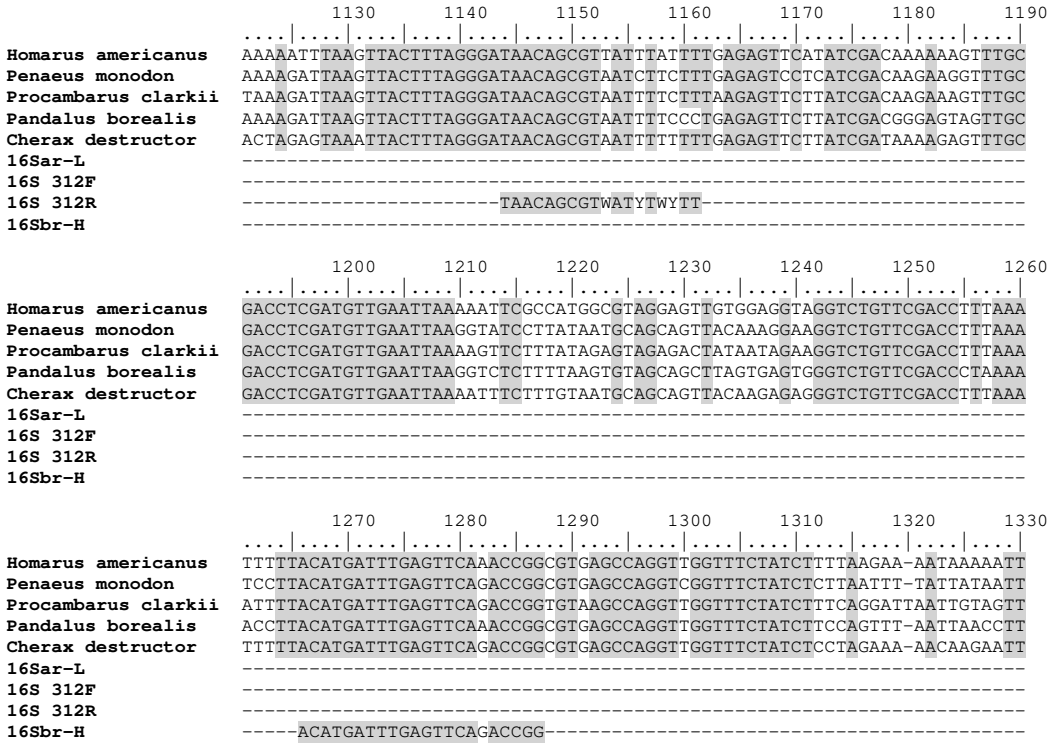
Fig. 1 Unrooted Neighbor-Joining tree based on partial sequences of *COI* gene obtained from this study and GenBank. GenBank sequences were indicated using their accession numbers. The percentage of replicate trees, in which the associated taxa clustered together in the bootstrap test (1000 replicates), are shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. The analysis involved 42 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 432 positions in the final dataset. Evolutionary analyses were conducted in MEGA7. The families to which the sequences belong to are indicated

Appendix

Supplementary material

Fig S1. Alignment of the used primers (16Sar-L and 16Sbr-H as well as 16S 312F and 16S 312R) with four 16S rDNA sequences from decapod species extracted from GenBank (*Homarus americanus* NC015607.1:c12603-11264, *Penaeus monodon* NC002184.1:c14066-12702, *Procambarus clarkii* NC016926.1:6951-8212, *Pandalus borealis* LC341266.1:c13978-12664 and *Cherax destructor* NC011243.1:c8668-7367). Conserved regions are highlighted in grey.

```
                    710       720       730       740       750       760       770
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
Homarus americanus  CTCGGCAAATA--AAGCTTCTGCCTGTTTATCAAAAACATGTCTATATGATG--GTT-TATAAAGTCTAA
Penaeus monodon     CTCGGCAAATA--CTACTTTTGCCTGTTTATCAAAAACATGTCTATATGATT--GTTATATAAAGTCTAG
Procambarus clarkii TTCGGCAAAAA--TTATTTCTGCCTGTTTAACAAAAACATGTCTTTATGGAG---GTTTATAAAGTCTAA
Pandalus borealis   CTCGGCAAATA--TTGCCTCTGCCTGTTTATCAAAAACATGTCTGTATGTTTTAATTATATGGAGTCTGG
Cherax destructor   TTCGGCAAAAAGTTTATTTCCGCCTGTTTATCAAAAACATGTCTGTATGGTA--GGTATATAAAGTCTGG
16Sar-L             --------------------TGCCTGTTTATCAAAAACAT----------------------------
16S 312F            --------------------------------------------------------------------
16S 312R            --------------------------------------------------------------------
16Sbr-H             --------------------------------------------------------------------

                    780       790       800       810       820       830       840
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
Homarus americanus  CCTGCCCACTGGGATCAAACTAAAGGGCCGCGGTATTTTAACCGTGCGAAGGTAGCATAGTCATTAGTCT
Penaeus monodon     CCTGCCCACTGAATTATTTTTAAAGGGCCGCGGTATACTGACCGTGCGAAGGTAGCATAATCATTAGTCT
Procambarus clarkii CCTGCCCATTGGGAAC----TAAAAGGCCGCGGTATTATGACCGTGCAAAGGTAGCATAATCATTAGTTT
Pandalus borealis   CCTGCCCACTGATGTT---TTTAAGGGCCGCGGTAATTTGACCGTGCGAAGGTAGCATAATCAGTAGTCT
Cherax destructor   CCTGCCCACTGAGGGT-ATTTAAAGG-CCGCTGTATTATGACCGTGCGAAGGTAGCATAATCATTAGTCT
16Sar-L             --------------------------------------------------------------------
16S 312F            --------------------------------------------------------------------
16S 312R            --------------------------------------------------------------------
16Sbr-H             --------------------------------------------------------------------

                    850       860       870       880       890       900       910
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
Homarus americanus  CTTAATTGGAGGCTTGTATGAATGGTTGGACAAGAAGCAAACTGTCTCAAAC-ACAAAAATTGAATTTGA
Penaeus monodon     TTTAATTGAAGGCTTGTATGAATGGTTGGACAAAAAGTAATCTGTCTCAGTT-ATAATAGTTGAACTTAA
Procambarus clarkii TTTAATTGAAGGCTAGAATGAATGGTTGAACAAGAAATAATCTGTCTTAAAT-TAATATATTGAATTTAA
Pandalus borealis   TTTAATTGGAGGCTGGAATGAATGGTTGGACAAGGGGGAAGCTGTCTCTTTTTACAATTTTTGAATTTTA
Cherax destructor   TTTAATTGGGGGCTGGTATGAAGGGTCGGACGAGAAATGAGCTGTCTTAAATTTTGAAAATTGAATTTAA
16Sar-L             --------------------------------------------------------------------
16S 312F            -------GRAGGCTTGTATGAATGGTTG----------------------------------------
16S 312R            --------------------------------------------------------------------
16Sbr-H             --------------------------------------------------------------------

                    920       930       940       950       960       970       980
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
Homarus americanus  CTTTAAGTGAAAAGGCTTAAATATTTAAAGGGACGATAAGACCCTATAAAGCTTAATAATTTAGTATA
Penaeus monodon     CTTTTAAGTGAAAAGGCTTAAATACTTTAAGGGGACGATAAGACCCTATAAAACTTAACAATAATTTGAT
Procambarus clarkii CTTTTAAGTGAAAAGGCTTAAATAATCTGGAGGGACGATAAGACCCTATAAAACTTTATATTTATAATAT
Pandalus borealis   CTTTTAAGTGAAAAGGCTTAAATAATTTGGGGGGACGATAAGACCCTATAAAATTTTACAAAATTGGGAT
Cherax destructor   CTTTTAAGTGAGAAGGCTTAAATAGGCTAGGGGGACGATAAGACCCTATAAAGTTTGACATTAAATTAAT
16Sar-L             --------------------------------------------------------------------
16S 312F            --------------------------------------------------------------------
16S 312R            --------------------------------------------------------------------
16Sbr-H             --------------------------------------------------------------------

                    990       1000      1010      1020      1030      1040      1050
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
Homarus americanus  TAATTAGATGAGTTGAAAGTTTAATATTATTTATATACTAAATTATTTCGTTGGGGCGACGATGATATAA
Penaeus monodon     TAAATT-ATAAATTGTTAGTATAACTTGATTTTAATTAATGTTTGTTGCGTTGGGGCGACGGGAATATAA
Procambarus clarkii AGTAGT--------TAGTTTTATTTAAGGGTATTATTTTAGAGTATTTGGTTGGGGTGACAAGGATAAAA
Pandalus borealis   TTTTTT-TTGAATTAA-GGTTTAA-ATTCTATAAGTTCTTATTTGTTTTGTTGGGGCGGCAAAGATAAAA
Cherax destructor   TAAGGG--TAATTTAGATTATAAAGTCTTAT-TATTATATAAGTGTTTAGTTGGGGCGACTAGGATATAA
16Sar-L             --------------------------------------------------------------------
16S 312F            --------------------------------------------------------------------
16S 312R            --------------------------------------------------------------------
16Sbr-H             --------------------------------------------------------------------

                    1060      1070      1080      1090      1100      1110      1120
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|
Homarus americanus  TTTGTAACTGTTTAAATTTAAAATACAGAGATAT-TTGTGTGTAATGA------TCCTTGTTGTTGATT
Penaeus monodon     TTAGTAACTGTTCTTAAATATTTTATTAACAAGT-ATAATTGAAGAATAATTGATCCTTTATTAAAGATT
Procambarus clarkii TATTAAATAACTGTCTTTTTTTTTTACAGTGATGTTTGGTTTAATGA----------TCCTAAAAGGGAT
Pandalus borealis   --ATTAGTAACTGTCATTTAATTAGAATAATTA---TAATTAGTTTA--ATTGATCCTTTATTAGAGATT
Cherax destructor   GTTATTTAACTGTTTCTTCATTCGAATCAAAAA---TTTTTGATTTTA---TGATCCTTTTT--AAGGGT
16Sar-L             --------------------------------------------------------------------
16S 312F            --------------------------------------------------------------------
16S 312R            --------------------------------------------------------------------
16Sbr-H             --------------------------------------------------------------------
```


1130 1140 1150 1160 1170 1180 1190
Homarus americanus AAAAATTTAAGTTACTTTAGGGATAACAGCGTTATTTATTTTGAGAGTTCATATCGACAAAAAAGTTTGC
Penaeus monodon AAAAGATTAAGTTACTTTAGGGATAACAGCGTAATCTTCTTTGAGAGTCCTCATCGACAAGAAGGTTTGC
Procambarus clarkii TAAAGATTAAGTTACTTTAGGGATAACAGCGTAATTTTCTTTAAGAGTTCTTATCGACAAGAAAGTTTGC
Pandalus borealis AAAAGATTAAGTTACTTTAGGGATAACAGCGTAATTTTCCCTGAGAGTTCTTATCGACGGGAGTAGTTGC
Cherax destructor ACTAGAGTAAATTACTTTAGGGATAACAGCGTAATTTTTTTTGAGAGTTCTTATCGATAAAAGAGTTTGC
16Sar-L
16S 312F
16S 312R TAACAGCGTWATYTWYTT
16Sbr-H
1200 1210 1220 1230 1240 1250 1260
Homarus americanus GACCTCGATGTTGAATTAAAAATTCGCCATGGCGTAGGAGTTGTGGAGGTAGGTCTGTTCGACCTTTAAA
Penaeus monodon GACCTCGATGTTGAATTAAGGTATCCTTATAATGCAGCAGTTACAAAGGAAGGTCTGTTCGACCTTTAAA
Procambarus clarkii GACCTCGATGTTGAATTAAAAGTTCTTTATAGAGTAGAGACTATAATAGAAGGTCTGTTCGACCTTTAAA
Pandalus borealis GACCTCGATGTTGAATTAAGGTCTCTTTTAAGTGTAGCAGCTTAGTGAGTGGGTCTGTTCGACCCTAAAA
Cherax destructor GACCTCGATGTTGAATTAAAATTTCTTTGTAATGCAGCAGTTACAAGAGAGGGTCTGTTCGACCTTTAAA
16Sar-L
16S 312F
16S 312R
16Sbr-H
1270 1280 1290 1300 1310 1320 1330
Homarus americanus TTTTTACATGATTTGAGTTCAAACCGGCGTGAGCCAGGTTGGTTTCTATCTTTTAAGAA-AATAAAAATT
Penaeus monodon TCCTTACATGATTTGAGTTCAGACCGGCGTGAGCCAGGTCGGTTTCTATCTCTTAATTT-TATTATAATT
Procambarus clarkii ATTTTACATGATTTGAGTTCAGACCGGTGTAAGCCAGGTTGGTTTCTATCTTTCAGGATTAATTGTAGTT
Pandalus borealis ACCTTACATGATTTGAGTTCAAACCGGCGTGAGCCAGGTTGGTTTCTATCTTCCAGTTT-AATTAACCTT
Cherax destructor TTTTTACATGATTTGAGTTCAGACCGGCGTGAGCCAGGTTGGTTTCTATCTCCTAGAAA-AACAAGAATT
16Sar-L
16S 312F
16S 312R
16Sbr-H ACATGATTTGAGTTCAGACCGG

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7. Results and discussion

Molecular biology is a constantly evolving research field and therefore the control authorities have to be up to date with using analytical methods for detection of food fraud. These methods should be fast, reliable and best applicable for all focus of analyses. Therefore, in this thesis one protein-based (MALDI-TOF MS) and two DNA-based (DNA sequencing and LAMP) methods were evaluated and compared for application suitability in the context of the official control of foodstuff for fish and crustacean species authentication.

Hereinafter, applicability of these three methods for the various processing grades and storage temperatures (fresh, refrigerated, frozen, cooked and smoked) as well as the focus of analysis (species identification, delimitation or detection), are discussed separately and at the end of this chapter comprehensively.

7.1. Suitability of loop-mediated isothermal amplification for species delimitation

Because of the reported cases of mislabelling the critically endangered European eel [(*Anguilla anguilla* (Linnaeus, 1758)] as Japanese eel (*Anguilla japonica* Temminck & Schlegel, 1846) as well as the wrong stocking of American eel [(*Anguilla rostrata* (Lesueur, 1817)] instead of European eel in European waters, delimitation of the European eel from other eel species is required. Several PCR-RFLP and real-time PCR methods were developed for eel species differentiation (e.g., Rehbein et al., 2002; Gagnaire et al., 2007; Lago et al., 2012; Trautner, 2013; Espiñeira and Vieites, 2016). One advantage of LAMP to the aforementioned methods is that it only needs easy to handle equipment, making it suitable for on-site analysis. Therefore, two LAMP assays for delimitation of the species European eel (*Anguilla anguilla*) from the other eel species of the genus *Anguilla* were developed and validated in this thesis (Chapter 4).

Application of both LAMP assays was shown on two instruments. In addition to a real-time cycler, the portable Genie instrument (OptiGene, Horsham, United Kingdom) which combines real-time fluorescence detection and subsequent melting curve analysis was used for this purpose. The genus-specific LAMP assay was able to detect specifically down to 500 pg DNA of all four tested eel species [*Anguilla anguilla*, *Anguilla japonica*, *Anguilla rostrata* and *Anguilla australis* Richardson, 1841]. Furthermore, none of the tested 112 fish species (belonging to 49 families) not classified to the genus *Anguilla* was detected (i.e. no false positives). The *Anguilla anguilla*-specific LAMP assay detected down to 500 pg DNA of *Anguilla anguilla*, but none of the other tested 115 fish species.

In addition, smoked eels showed no difference in performance of the LAMP assays compared to the frozen tissues (considering threshold times and melting temperature). This is especially important because eels are often sold smoked (Mafra et al., 2008; Coad, 2016), wherefore food control authorities need techniques suitable for species authentication on this processing grade. Furthermore, combined with a simplified extraction method DNA of *Anguilla anguilla* yielded from a single egg was detectable. The extraction method can be easily performed by homogenizing the sample using a micropistil with 5% Chelex 100 resin and subsequent incubation at 95 °C for 20 min. Moreover, positive LAMP reactions can be detected with reporter dyes, as shown in the study with SYBR Gold. Consequently, the analysis can be conducted with a simple heating block or water bath making these assays suitable for on-site analyses (e.g., for ecological studies or food control investigations in restaurants or retails). In addition, the *Anguilla* genus-specific LAMP assay can be used for verification of a successful DNA extraction as well as a check for occurrence of reaction inhibitors. Currently, samples that are negative in the *Anguilla anguilla* specific LAMP assay require other methods for secure species authentication. This problem can be overcome through development of specific LAMP assays for the other commercially important eel species (*Anguilla rostrata, Anguilla japonica* and *Anguilla australis*).

For simultaneous detection of multiple targets using LAMP, various protocols have been provided in the literature (for a review see Wong et al., 2018). For instance, Aonuma et al. (2010) detected two parasites in mosquitos with primers labelled with two different fluorescence dyes. He and Xu (2011) used subsequent restriction enzyme digestion. However, digestion could be incomplete because of the various structures of LAMP products (Liang et al., 2012). Besides, all these protocols require additional equipment, for instance real-time detection of different fluorophores or gel electrophoresis devices.

Due to the shown applicability of LAMP for eel eggs, this technique offers a fast approach for detection of fish species of one of the most expensive animal product in the world trade: caviar from sturgeons and paddlefish (Order Acipenseriformes). Additionally, this group is one of the most critically endangered, wherefore its international trade is controlled. Mislabelling of caviar concerning the fish species has been reported frequently and commercially relevant (Chapter 1). Furthermore, Ludwig et al. (2015) revealed in a market survey four cases in which the sold product does not contain sturgeon DNA. Moreover, only in one of these products animal DNA, namely of lumpfish (*Cyclopterus lumpus* Linnaeus, 1758; order Scorpaeniformes), was present.

Additionally, the finding that LAMP is also suitable for smoked products offers development of further LAMP assays enabling food control investigators to directly analyse potentially mislabelled food, for instance in restaurants. Therefore, LAMP assays are an appropriate tool for food control authorities to detect mislabelling as well as for custom authorities for the protection of endangered species from illegal trade.

7.2. Suitability of matrix-assisted laser desorption ionization time of-flight mass spectrometry for species identification

Several working groups studied the potential of MALDI-TOF MS for seafood species identification of fresh, frozen and ethanol fixed tissues using various protein preparation protocols (Chapter 2.1.2). However, the influences of fat-content, storage temperature or level of food processing to the identification reliability have not yet been fully investigated. It also needed examination, whether the same protein preparation protocol has to be used for MSP generation as well as for identification to ensure a reliable species identification.

Therefore, in this thesis (Chapter 5) the influence of the protein preparation protocol, fat-content, as well as storage temperature and processing grade (fresh, refrigerated, frozen, cooked and smoked) on the reliability of identification of fish was examined. The high-fat Atlantic mackerel (*Scomber scombrus* Linnaeus, 1758; order: Perciformes) and the low-fat rainbow trout [*Oncorhynchus mykiss* (Walbaum, 1792), order: Salmoniformes) were used for this study as representatives.

The reliability of the five protein preparation protocols was determined based on the suitability to generate high quality main spectra projections (MSP) of both fishes with different storage temperature and processing grade. MSP are reduced reference spectra calculated from sum spectra by considering only a predefined number of reproducible peaks with high intensities and signal-to noise ratios. Generation of MSP of fresh and frozen tissues from both fish species was possible with all five tested protein preparation protocols. However, only one protocol (using 25% formic acid followed by chloroform-methanol defatting) resulted in high quality MSP (yield of sum spectra, resulting number of peaks and spectra reproducibility). Regarding the high-fat Atlantic mackerel, this could be due the defatting step of the used protocol. Hence, this protocol was selected as standard protocol for protein preparation of fish for subsequent MALDI-TOF MS analysis.

However, suitability of this protocol has to be examined case-by-case for each target group of fish. Furthermore, the applicability of this protocol for other animal groups (e.g., crustacean) has to be evaluated. Since this protocol has already been used successfully for the identification of another seafood group, namely molluscs (Stephan et al., 2014), there is the potential that it is also applicable for crustacean species. However, if this method proves to be unsuitable for crustacean, other protein extraction methods that have already been successfully applied to crustacean (e.g., Salla and Murray, 2013) can be used. Nevertheless, it is desirable for the practicability in routine analytics to apply a single protein preparation protocol for all groups.

In addition, it was ascertained whether the same protein preparation protocol as well as the same storage temperature or processing grade of the tissue has to be used for MSP generation as well as for identification to ensure a reliable species identification. Hence, proteins from tissues with varying storage temperatures and processing grades were prepared with all five protocols and analysed using the MSP obtained with the chosen standard protein preparation protocol of frozen fish. The varying storage temperatures and processing grades were fresh, frozen (1 day up to 14 months), refrigerated (7 days), cooked (99 °C for 5 min), and smoked. It was not possible to obtain identifiable protein spectra of smoked and refrigerated fish using the five protein preparation protocols. Fresh, frozen and cooked Atlantic mackerel and rainbow trout were successfully identified by using the chosen standard protein preparation for analysis as well as for MSP generation. This was not possible when using one of the other four tested preparation protocols. Consequently, the same protein preparation protocol should be used for MSP generation as well as for identification.

Besides this, it was shown that MSP generated from frozen tissues are sufficient for analysis of fresh, frozen or cooked fish, which simplifies the development of a protein spectra database for fish species and offers a fast and reliable approach for species identification in the context of the official control of foodstuff for these processing grades and storage conditions. However, when analysing unknown long refrigerated or smoked fish, DNA sequencing is recommended to ensure reliable species identification.

Nevertheless, correct species identification depends on the quality and size of the underlying reference database. Stahl and Schröder (2017) pointed out that the lack of reference spectra may lead to incorrect identification results. For instance, because no protein spectrum of *Gadus chalcogrammus* Pallas, 1814 was deposited in the database, this species was wrongly identified as *Gadus macrocephalus* Tilesius, 1810 due to the high similarity of the protein spectra in both species. Consequently, until a database containing spectra of all food relevant and closely related marine animal species as well as exotic species is available no unambiguous species identification is possible.

However, due to the growing application of MALDI-TOF MS the demand for comparing protein spectra of seafood species will increase (Mazzeo et al., 2008; Volta et al., 2012; Laakmann et al., 2013; Stahl and Schröder, 2017). Currently, there is no commercial or non-commercial MALDI-TOF MS database available containing protein spectra of all food relevant and closely related marine animal species. Creation, maintenance and validation of a protein spectra database is time-consuming. The collective work of an institutional network of several laboratories on a common, ideally global database may overcome this obstacle. As true for all reference libraries, the greatest challenge in developing a protein spectra database for species identification in the context of official control of foodstuffs using MALDI-TOF MS is to obtain material from all food relevant and closely related marine animal species as well as exotic species.

The MALDI-User-Platform (MALDI-UP, http://maldi-tof-ms-user-platform.ua-bw.de/) may facilitate the development of a protein spectra database. This platform provides a catalogue hosted by the State Institute of Chemical and Veterinarian Analysis (the food control authority of Baden-Wuerttemberg) containing taxonomic information, MALDI-TOF MS instrument, sample preparation parameters and further metadata (Rau, 2016). The interested users can obtain these information as well as contact information of the spectra creators for exchanging spectra under own merchandising criteria. This catalogue is updated on a regular basis and contained 111 entries (1269 entries in total) of 47 fish species in March 2019. Furthermore, 11 crustacean species (all of the order Decapoda) were listed in the catalogue. However, only in case of five fish species spectra were obtained with the chosen protein preparation protocol (25% formic acid followed by chloroform-methanol defatting).

As shown above, the same protein preparation protocol should be used for MSP generation as well as for identification to ensure reliable species identification. It will take a lot of time and effort until protein spectra of all requested species created with the selected protocol are available. Consequently, MALDI-TOF MS can currently only be used as a screening method or to analyse a limited species group of which reference spectra of all species are available.

7.3. Suitability of DNA sequencing for species identification

The official analytical method in the ASU for crustacean species identification is sequencing of an approximately 312 bp fragment of the 16S rDNA (16S rDNA_312 bp). However, this method is not recommended for all crustacean species. For instance, it cannot be applied to the important shrimp genus *Crangon* due to the lack of amplification (BVL, 2014). Therefore, in comparison with the official method, the applicability of two other marker regions commonly used for crustacean species identification were examined in this thesis on 19 food relevant decapod species (Chapter 6). One marker was another 16S rDNA region of about 570 bp (16S rDNA_570 bp). This region enclosed the 16S rDNA_312 bp fragment. The other marker was the *COI* barcoding region.

It was shown that the *COI* barcoding region is the most suitable gene marker for reliable identification of all 19 examined species belonging to ten different crustacean families (Aristeidae, Cambaridae, Cancridae, Crangonidae, Nephropidae, Palaemonidae, Pandalidae, Parastacidae, Penaeidae and Solenoceridae). The official German control method showed some disadvantages compared to *COI* and the other tested 16S rDNA marker region (16S rDNA_570 bp) as described hereafter. In case of four decapod species, identification using the official 16S rDNA marker region was hampered by insufficient sequences (lack of amplification or heterogeneous sequences). These four species were *Nephrops norvegicus* (Linnaeus, 1758), *Cancer pagurus* Linnaeus, 1758, *Procambarus clarkii* (Girard, 1852) and *Crangon crangon* (Linnaeus, 1758). With the further tested 16S rDNA marker region and the *COI* marker region, sufficient sequences were obtained from all 19 decapod species. However, in case of the species *Pandalus jordani* Rathbun, 1902 no 16S rDNA reference sequence was available in GenBank in contrast to *COI*. Identification of one decapod species (declared as *Heterocarpus reedi* Bahamonde, 1955) failed, because no sequences of either of the two analysed genes of the species *Heterocarpus reedi* were deposited in GenBank or BOLD in July 2018 when the study was conducted. In addition, in March 2019 no sequences were available.

The BOLD database (http://www.boldsystems.org) contained *COI* barcodes of about 200.000 animal species and about 40.000 public *COI* barcodes of decapod species. In addition, over 43.000 *COI* and 22.000 16S rDNA records were available in GenBank (GenBank search, March 2019). Both databases are continuously growing. For instance, in 2018 Mantelatto et al. (2018) deposited about 100 *COI* and 16S rDNA sequences of decapod species that were not yet present in GenBank at that time. Therefore, it seems to be only a matter of time until *COI* sequences of the species *Heterocarpus reedi* Bahamonde, 1955 become available in public databases. Furthermore, about more than twice as many *COI* than 16S rDNA sequences of decapods are published in GenBank, making *COI*, in addition to the other aforementioned advantages, with the current state of knowledge, the marker of choice for reliable decapod species identification.

The results obtained from this part of the thesis showed the potential of the *COI* barcoding region for the complementation or even the replacement of the official method in the official control of foodstuff for crustacean species identification to detect and combat food fraud. However, accuracy of species identification depends on the available reference sequences and the genetic differences among the investigated species (Almerón-Souza et al., 2018). Therefore, in some cases additional markers are required for unambiguous species identification. For instance, Moftah et al. (2011) suggested *cytb* sequencing, additionally to *COI* sequencing, when analysing the shark genus *Carcharhinus* due to the higher evolution rate of *cytb* in this genus. Viñas and Tudela (2009) suggested using the first internal transcribed spacer (*ITS*1) in addition to the mitochondrial control region (CR) as a nuclear gene marker when analysing certain tuna species, for instance in distinguishing between *Thunnus alalunga* (Bonnaterre, 1788) and *Thunnus thynnus* (Linnaeus, 1758) with introgressed mitochondrial DNA of *Thunnus alalunga*. Moreover, Abdullah and Rehbein (2017) used the nuclear rhodopsin gene fragment complementary to *COI* for identification of fishery products to detect possible hybrids. Besides, additional markers are advantageous, if reference sequences are lacking or ambiguous, such as in case of the shark species *Squalus cubensis* Howell Rivero, 1936 (Almerón-Souza et al., 2018). Additionally, they can lead to better amplification result as in case of 16S rDNA sequencing of portunid crabs in contrast to *COI* (Brandão et al., 2016) or confirm the identification results as done by Cawthorn and Hoffman (2017) with 16S rDNA supplementary to *COI* for decapod species identification. However, traditional Sanger sequencing reaches its limits when mixed products (containing more than one animal species, for example surimi or canned tuna) are analysed. In this case, time-consuming and unsuitable for routine analysis separation of amplicons (e.g., PCR cloning) is needed prior to sequencing (Bottero and Dalmasso, 2011; Kumar et al., 2015).

In recent times, Next Generation Sequencing (NGS) has been becoming a useful approach for seafood species identification in products with mixed species content by parallel sequencing of different target sequences in one reaction. Especially metabarcoding (combination of DNA barcoding with NGS) is becoming a suitable application for simultaneous detection of several species in food (Staats et al., 2016). This was shown by multiple examples also within the area of seafood. Galal-Khallaf et al. (2016) used *COI* metabarcoding for fish identification of aquaculture feeds, Kappel et al. (2017) showed the potential of *cytb* metabarcoding for discrimination of tuna species, Giusti et al. (2017) identified fish and cephalopod species of surimi-based products using 16S rDNA metabarcoding, and Maggia et al. (2017) applied *COI* metabarcoding to discriminate fish larval of the Amazonian catfish.

7.4. Suitability of the examined methods depending on the issue

There are many molecular biological methods for seafood authentication and it is a case-by-case decision which method is the most suitable for which focus of analysis, processing grade and storage temperature. The suitability of the three techniques examined in this thesis (MALDI-TOF MS, DNA sequencing and LAMP), depending on the aforementioned issues, is described hereinafter for crustacean and fish species authentication (for an overview see Table 4).

Considering delimitation of two species or detection of few known species, the LAMP technique has the potential to serve as a fast and easy to handle alternative to MALDI-TOF MS and DNA sequencing which are time-consuming and/or need bulky equipment. For instance, in the case of the common substitution of Atlantic halibut [(*Hippoglossus hippoglossus* (Linnaeus, 1758)] with Greenland halibut [*Reinhardtius hippoglossoides* (Walbaum, 1792)] development of LAMP assays for each of the two species would be appropriate for a fast on-site detection (e.g., investigation of food samples in restaurants or retailers). Furthermore, a LAMP assay for detection of the family Tetraodontidae (puffer fish) or the genus *Lophius* (monkfish) could serve as a fast screening method (even on board of a fishing vessel) to distinguish the potentially toxic puffer fish from the non-toxic monkfish. Additionally, delimitation of the higher priced beluga [*Huso huso* (Linnaeus, 1758)] from the lower priced sturgeon species (*Acipenser* spp.) in caviar is a further possible approach due to the shown application of LAMP on fish eggs.

A further common case of food fraud is adulteration of sole [*Solea solea* (Linnaeus, 1758)]. However, in this case several species are used for substitution. In 2013 during an undercover restaurant investigation in Germany 50% of the analysed samples were not sole as declared. Several species were used for substitution, in particular Senegalese tonguesole [(*Cynoglossus senegalensis* (Kaup, 1858)], Portuguese sole (*Synaptura lusitanica* de Brito Capello, 1868), even pangasius [*Pangasianodon hypophthalmus* (Sauvage, 1878)] as well as two unidentified species (Kappel and Schröder, 2016). In a further undercover investigation in Brussels' restaurants and canteens conducted by Christiansen et al. (2018) sole was replaced with yellowfin sole [*Limanda aspera* (Pallas, 1814)] and Northern rock sole [*Lepidopsetta polyxystra* Orr & Matarese, 2000] in addition to the abovementioned species. In such cases, LAMP assays reach their limits, because LAMP is restricted to the specified target species. Nevertheless, application of LAMP for detection or exclusion of sole is a potential approach. However, it does not offer the possibility to specify the variety of other species used for substitution.

In such cases, MALDI-TOF MS can serve as a fast and easy to handle alternative approach. Currently, the drawback of MALDI-TOF MS is the incomplete protein spectra database. For instance, the developed MALDI-TOF MS database of Stahl and Schröder (2017) contains protein spectra of sole and pangasius but spectra of Senegalese tonguesole and Portuguese sole are missing. Additionally, Stahl and Schröder (2017) showed that the lack of reference spectra can lead to incorrect identification results. Consequently, the application of MALDI-TOF MS is still limited.

However, due to the increasing application of MALDI-TOF MS for seafood species identification, it is a matter of time until a protein spectra database containing spectra of all food relevant and closely related marine animal species as well as exotic species will be available. To build such a database in a non-commercial way it will take several years or decades, considering the DNA database BOLD which still lacks DNA sequences from some species, for instance the crustacean species *Heterocarpus reedi* Bahamonde, 1955.

Furthermore, this thesis showed that secure species identification using MALDI-TOF MS was not possible for smoked fish. DNA-based methods are more suitable for eels as they are often sold smoked (Mafra et al., 2008; Coad, 2016). When the expected number of species is low and known, detection methods such as LAMP assays may be used for fast analysis of processed seafood samples. However, when there is a broad range of expected species or the target species is unknown, DNA sequencing is recommended for reliable authentication of highly processed seafood products. As shown in this thesis the *COI* barcoding region should be applied for crustacean species identification.

Table 4 Comparison of suitability of the molecular biological methods for marine species authentication examined in this study depending on the focus of analysis and available equipment as well as on the handling.

	LAMP[1]	MALDI-TOF MS[2]	DNA sequencing
On-site analysis	x	-	-
No Database required	x	-	-
Database available	n.a.[3]	-	x
Broad species spectrum	-	x	x
Suitable for processed food	x	-	x
Fast	x	x	-
Easy to handle	x	x	-

Methods marked with an 'x' indicate that they exhibit the corresponding feature

[1]LAMP: Loop-mediated isothermal amplification

[2]MALDI-TOF MS: Matrix-assisted laser desorption ionization time of-flight mass spectrometry

[3]n.a.: not applicable

Summing up, using the three methods examined in this thesis, depending on the processing grade and the storage temperature (fresh, refrigerated, frozen, cooked and smoked) as well as the focus of analysis (species identification, delimitation or detection), reliable species identification of seafood is possible in most of the cases.

8. Conclusion

Overall, this work has improved seafood species authentication, in particular fish and crustacean, in the context of the official control of foodstuff to detect and combat food fraud.

The results of this thesis show the limits and possibilities of MALDI-TOF MS depending on the influence of storage temperature and level of food processing as well as the used protein preparation protocols. Additionally, the most appropriate protein preparation protocol for subsequent MALDI-TOF MS analysis was determined. This may be used for standardized generation of protein reference spectra for development of a common database as well as for species identification. However, suitability of this protocol has to be examined case-by-case for each target group of fish. In addition, it is recommended to examine the applicability of this protocol to other groups of animals in order to enable easy and fast routine analysis.

Concerning crustacean, the reliability of species identification was enhanced due to the shown suitability of *COI* sequencing. Besides, suitability of LAMP for smoked fish as well as fish eggs with eel as representative was demonstrated.

Due to the establishment of the three molecular biological methods, including preparation protocols, the food control authorities can combat food fraud more effectively. All three methods have the potential to be implemented in the ASU leading to a nationwide standardized quality of seafood examination in Germany. Furthermore, this thesis has shown that there is no molecular biological approach suitable for every analytical focus. However, this work can be useful as a guidance for food control authorities to choose the appropriate method depending on the analysed food, not only for seafood.

Besides the suitability of the well-established methods DNA sequencing and MALDI-TOF MS, the applicability of LAMP for on-site analysis was shown in this thesis. On-site analyses are beneficial for species determination both for ecological studies as well as on board of research or fishing vessels. Besides, LAMP offers food control investigators direct analysis of potentially mislabelled food, for instance in restaurants or retails. In addition, due to the reported applications for other animals as well as plants in the literature, there are (almost) no limits to the possible applications.

Although seafood authentication using MALDI-TOF MS and DNA sequencing was improved in this thesis, it has to be noted that there is a lack of DNA sequences and protein spectra. Reliable species identification using database-based methods is only as accurate as the coverage of the underlying database. The collective work of the food control authorities of Germany and other countries in generation and exchange of more spectra can enhance the reliability of species authentication, not only for seafood.

9. Summary

Due to the multitude of uncovered cases of food fraud concerning seafood in recent years, reliable methods for species authentication in the context of the official control of foodstuff are required. Most of the sold seafood lacks morphological characteristics due to processing. Consequently, morphological species identification often reaches its limits.

Therefore, in this thesis one protein-based (Matrix-assisted laser desorption ionization time of-flight mass spectrometry: MALDI-TOF MS) and two DNA-based (DNA sequencing and loop-mediated isothermal amplification: LAMP) methods have been established and compared for suitability of application in the context of the official control of foodstuff for fish and crustacean species authentication. In particular, it was examined which of these three methods is suitable for which focus of analysis (species identification, delimitation or detection), processing grade and storage temperature (fresh, refrigerated, frozen, cooked and smoked).

The LAMP technique has the potential to serve as a fast and easy approach for delimitation of two species or detection of few, known species. The advantages of this technique in contrast to MALDI-TOF MS and DNA sequencing are, that it is neither time-consuming nor requires bulky equipment and therefore is also suitable for on-site analysis. The suitability of the LAMP technique was demonstrated in this thesis by developing LAMP assays for delimitation of the endangered European eel [*Anguilla anguilla* (Linnaeus, 1758)] from other eels of the genus *Anguilla*, for frozen and smoked fish as well as in fish eggs.

For the analysis of unknown species, or groups consisting of many species, the LAMP technique reaches its limits. Considering this problem, MALDI-TOF MS can serve as an also fast and easy to handle alternative approach for species authentication. The influence of storage temperature and processing grade (fresh, refrigerated, frozen, cooked and smoked) on the reliability of identification of the high-fat Atlantic mackerel (*Scomber scombrus* Linnaeus, 1758) and the low-fat rainbow trout [*Oncorhynchus mykiss* (Walbaum, 1792)] was examined in this thesis. It was shown, that only fresh, frozen and cooked fish can be identified reliably using MALDI-TOF MS. Furthermore, due to comparison of five protein preparation protocols the most suitable was chosen. Additionally, it was shown, that the same protein preparation protocol should be used for generation of reference spectra as well as for generation of spectra used for identification. However, the disadvantages of this technique are the lack of a protein spectra database containing all food relevant seafood species and the unsuitability for identification of smoked fish. Consequently, the applicability of this approach for the area of food fraud concerning seafood species authentication is currently limited.

DNA sequencing remains the gold standard for seafood species identification in the context of the official control of foodstuff. The method in the Official Collection of Methods of Analysis and Sampling is based on sequencing of a 16S ribosomal DNA (16S rDNA) fragment. However, this method shows some deficiencies concerning amplification of several species due to hampering by insufficient sequences (missing amplification, heterogeneous sequences). Therefore, this marker was compared with a larger 16S rDNA marker region and the cytochrome *c* oxidase subunit 1 (*COI*) barcoding region. Due to the shown suitability of *COI* sequencing for crustacean and the potential of this marker to complement or even replace the official method, the reliability of species identification for this group was enhanced. However, as with MALDI-TOF MS, reliable species identification depends on the coverage within the database. Generation and exchange of protein spectra and DNA-sequences is necessary to overcome this problem.

In summary, the three methods established and compared in this thesis enable in most cases a reliable authentication of seafood, in particular fish and crustacean. This gives the control authorities reliable state of the art methods to detect and combat food fraud in a nationwide standardised quality. Although no molecular biological approach was suitable for every analytical focus, this work can be a useful guide for food control authorities in choosing the method most appropriate for analysis of a given food sample, whether of seafood origin or not.

10. Zusammenfassung

Aufgrund der Vielzahl in den letzten Jahren aufgedeckten Fällen von Lebensmittelbetrug bei Fischereierzeugnissen sind zuverlässige Methoden zur sicheren Artenerkennung im Rahmen der amtlichen Lebensmittelkontrolle erforderlich. Bei den meisten verkauften Fischereierzeugnissen fehlen aufgrund der Verarbeitung morphologische Merkmale. Daher stößt die morphologische Artidentifizierung oft an ihre Grenzen.

Aus diesem Grund wurden in dieser Dissertation eine proteinbasierte (*Matrix-assisted laser desorption ionization time of-flight mass spectrometry*: MALDI-TOF MS) und zwei DNA-basierte (DNA-Sequenzierung und *loop-mediated isothermal amplification*: LAMP) Methoden auf ihre Eignung für die Anwendung im Rahmen der amtlichen Kontrolle von Lebensmitteln zur Authentifizierung von Fisch und Krustentieren etabliert und verglichen. Im Speziellen wurde untersucht, welches dieser drei Verfahren für welchen Analysenfokus (Artenidentifikation, Abgrenzung oder Nachweis), Verarbeitungsgrad und Lagertemperatur (frisch, gekühlt, gefroren, gekocht und geräuchert) geeignet ist.

Die LAMP-Technik hat das Potential, als schnelle und einfach zu handhabende Anwendung bei der Abgrenzung von zwei Arten oder dem Nachweis von wenigen, bekannten Arten zu dienen. Der Vorteil dieser Technik im Gegensatz zu MALDI-TOF MS und der DNA-Sequenzierung ist, dass sie weder zeitaufwendig ist noch sperrige Geräte erfordert und sich daher auch für die Analyse vor Ort eignet. Die Eignung der LAMP-Technik wurde in dieser Dissertation durch die Abgrenzung des gefährdeten Europäischen Aals *Anguilla anguilla* (Linnaeus, 1758) von anderen Aalen der Gattung *Anguilla* bei gefrorenem und geräuchertem Fisch sowie bei Eiern gezeigt.

Für die Analyse unbekannter Arten oder Gruppen mit vielen Arten stößt die LAMP-Technik an ihre Grenzen. Daher kann MALDI-TOF MS als ein, auch schnell und einfach zu handhabender, alternativer Ansatz zur Artauthentifizierung dienen. Den Einfluss von Lagertemperatur und Verarbeitungsgrad (frisch, gekühlt, gefroren, gekocht und geräuchert) auf die Zuverlässigkeit der Identifizierung der fettreichen Makrele (*Scomber scombrus* Linnaeus, 1758) und der fettarmen Regenbogenforelle [*Oncorhynchus mykiss* (Walbaum, 1792)] wurde in dieser Dissertation untersucht. Es wurde gezeigt, dass nur frische, gefrorene und gekochte Fische sicheren mittels MALDI-TOF MS identifiziert werden können. Darüber hinaus wurde durch den Vergleich von fünf Proteinaufarbeitungsprotokollen das am besten geeignete ausgewählt. Des Weiteren wurde gezeigt, dass das gleiche Proteinaufarbeitungsprotokoll sowohl für die Generierung von Referenzspektren als auch für die Generierung der Spektren zur Identifizierung verwendet werden sollte.

Jedoch sind die Nachteile dieser Methode das Fehlen einer Proteinspektren-Datenbank die alle lebensmittelrelevanten Fischereierzeugnisse beinhaltet und der fehlenden Eignung für die Identifizierung von geräucherten Fischen. Daher ist die Anwendung für die Authentifizierung von Meeresfrüchten zur Bekämpfung von Lebensmittelbetrugs derzeit begrenzt.

Die DNA-Sequenzierung bleibt der Goldstandard für die Identifizierung von Fischereierzeugnissen im Rahmen der amtlichen Kontrolle von Lebensmitteln. Die offizielle Kontrollmethode in der Amtlichen Sammlung von Untersuchungsverfahren für die Identifizierung von Krebsarten basiert auf der Sequenzierung eines 16S ribosomalen DNA (16S rDNA) Fragmentes. Jedoch weist die offizielle Kontrollmethode einige Defizite in Bezug auf die Amplifizierung einiger Arten durch fehlende Amplifikation bzw. heterogene Sequenzen auf. Daher wurde dieser Genmarker mit einer größeren 16S rDNA-Markerregion und der Cytochrom *c* Oxidase Untereinheit 1 (*COI*) Barcode-Region verglichen. Aufgrund der, in dieser Dissertation nachgewiesenen Eignung der *COI*-Sequenzierung für die Identifizierung von Krebsarten, wurde die Zuverlässigkeit der Artenidentifikation für diese Gruppe verbessert. Jedoch hängt die zuverlässige Artenbestimmung wie bei MALDI-TOF MS von der Abdeckung innerhalb der Datenbank ab. Die Generierung und der Austausch von Proteinspektren und DNA-Sequenzen sind notwendig, um dieses Problem zu lösen.

Insgesamt ermöglichen die drei in dieser Dissertation etablierten und verglichenen Methoden in den meisten Fällen eine zuverlässige Authentifizierung von Fischereierzeugnissen, insbesondere von Fisch und Krebstieren. Damit verfügen die Kontrollbehörden über zuverlässige und hochmoderne Methoden zur Aufdeckung und Bekämpfung von Lebensmittelbetrug. Obwohl kein molekularbiologischer Ansatz für jeden analytischen Fokus geeignet ist, kann diese Arbeit als Orientierungshilfe für die Lebensmittelkontrollbehörden bei der Wahl der geeigneten Methode in Abhängigkeit vom analysierten Lebensmittel - nicht nur für Fischereierzeugnisse - genutzt werden.

11. Abbreviations

16S rDNA	16S ribosomal DNA
16S rRNA	16S ribosomal RNA
16S rRNA/tRNA Val	16S ribosomal RNA/transfer RNA Val
ASU	Official Collection of Methods of Analysis and Sampling
BIP	Reverse inner primer
BOLD	Barcode of Life Data System
bp	Base pairs
CBOL	Consortium for the Barcode of Life
COI	Cytochrome *c* oxidase subunit 1
CR	Control region
cytb	Cytochrome b
FINS	Forensically informative nucleotide sequencing
FIP	Forward inner primer
HRM	High resolution melting
IEF	Isoelectric focusing
ITS1	First internal transcribed spacer
IUCN	International Union for Conservation of Nature
LAMP	Loop-mediated isothermal amplification
MALDI-TOF MS	Matrix-assisted laser desorption ionization time of-flight mass spectrometry
MALDI-UP	MALDI-User-Platform
MSP	Main spectra projection
NCBI	National Center for Biotechnology
NGS	Next Generation Sequencing
NIH	National Institute of Health
PCR	Polymerase chain reaction
PCR-RFLP	Polymerase chain reaction-restriction fragment length polymorphism

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List of Publications and declaration of contribution as a co-author

Under the supervision of Dr. Ingrid Huber and Prof. Dr. Gerhard Haszprunar, Gesche Spielmann was responsible for study design, sample management, laboratory work, data analysis, writing the main part of the manuscript, submission and editing after peer-review of the manuscripts with following exceptions.

All authors read and approved the final manuscript.

Publication I

Spielmann G, Ziegler S, Haszprunar G, Busch U, Huber I and Pavlovic M (2019).
Using loop-mediated isothermal amplification for fast species delimitation in eels (genus *Anguilla*), with special reference to the European eel (*Anguilla anguilla*). Food Control 101: 156-162.

Sonja Ziegler did the main part of the specificity testing and establishing of a fast DNA extraction protocol under the supervision of Gesche Spielmann. Dr. Melanie Pavlovic designed the primers for both detection systems.
Dr. Melanie Pavlovic was involved in supervision of the writing of the manuscript.

Publication II

Spielmann G, Huber I, Maggipinto M, Haszprunar G, Busch U, Pavlovic M (2018).
Comparison of five preparatory protocols for fish species identification using MALDI-TOF MS. Eur Food Res Technol 244: 685-694.

Dr. Melanie Pavlovic was involved in supervision of study design, data analysis and writing of the manuscript.

Publication III

Spielmann G, Diedrich J, Haszprunar G, Busch U and Huber I (2018).
Comparison of three DNA marker regions for identification of food relevant crustaceans of the order Decapoda. Eur Food Res Technol.

Jana Diedrich generated and preliminary analysed approx. 40% of the sequences under the supervision of Gesche Spielmann.

I hereby confirm the above statement.

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10/2011 – 03/2012	Diploma thesis ‚Etablierung der Polymerase-Kettenreaktionen des Random Mutation Capture Assays für das humane Phosphatidylinositol-3-Kinase-Gen‘
10/2007 – 03/2012	Study of Food Chemistry at the Julius Maximilians - University of Würzburg, Germany
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Peer-reviewed publications

Spielmann G, Ziegler S, Haszprunar G, Busch U, Huber I and Pavlovic M (2019).
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Congress contributions

Posters

Widmann T, Spielmann G, Pavlovic M, Busch U and Huber I (2019)
Nachweis der Gattung *Pecten* (Jakobsmuschel) mittels loop-mediated isothermal amplification (LAMP). 70. Arbeitstagung des Regionalverbandes Bayern der Lebensmittelchemischen Gesellschaft, Fachgruppe in der Gesellschaft Deutscher Chemiker.

Hogh K, Spielmann G, Huber I and Pavlovic M (2019)
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