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Review

Food authentication of commercially-relevant shrimp and prawn species: From classical methods to Foodomics

Although seafood species identification has traditionally relied on morphological analysis, sometimes this is difficult to apply for the differentiation among penaeid shrimps owing to their phenotypic similarities and to the frequent removal of external carapace during processing. The objective of this review is to provide an updated and extensive overview on the molecular methods for shrimp and prawn species authentication, in which several omics approaches based on protein and DNA analysis are described. DNA-based methods include the amplification by PCR of different genes, commonly the mitochondrial 16S ribosomal RNA and cytochrome oxidase I genes. A recently described method based on RFLP coupled to PCR turned out to be particularly interesting for species differentiation and origin identification. Protein analysis methods for the characterization and detection of species-specific peptides are also summarized, emphasizing some novel proteomics-based approaches, such as phyloproteomics, peptide fragmentation, and species-specific peptide detection by HPLC coupled to multiple reaction monitoring (MRM) MS, the latter representing the fastest method described to date for species authentication in food.

Keywords:
Decapoda shrimps / Food authentication / Foodomics / Genomics / Proteomics
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1 Introduction

Food safety and quality is demanding great attention by the food industry and consumers. One of the most relevant points concerning food quality is the authentication of food components. Food products may be adulterated, and highly valuable species may be substituted, partially or entirely, by similar but cheaper ones. Food authentication is a major concern not only for the prevention of commercial fraud, but also for the assessment of safety risks derived from the nondeclared introduction of any food ingredient that might be harmful to human health [1–3], such as potential allergenic or toxic compounds, or others that might alter eating habits of a certain group of consumers, this dealing with lifestyles such as vegetarianism or religious practices. Regarding allergens, crustaceans are one of the foods with more prevalence of food allergies [4]. In addition to the negative effects that adulteration can induce to the food industry and the consumers, and to the safety risks derived, this practice can also reduce the effectiveness of marine conservation and management programs that help to protect overexploited endangered species [5].

Fishery products are among the most internationally traded food commodities. Among them, crustaceans belonging to the order Decapoda are of significant commercial relevance [6]. Decapoda marine shrimps and prawns, especially those belonging to the superfamily Penaeoidea, the penaeid shrimps, represent important resources for both commercial fisheries and aquafarming facilities in many countries, accounting for more than 17% of the global consumption of seafood products worldwide [7]. Shrimps and prawns are increasingly produced by aquaculture especially in some areas of China, Ecuador, Mozambique, Southeast Asia (in particular, in countries such as Thailand), in addition to catches in different areas around the world, mainly Food and Agriculture Organization of the United Nations (FAO) Areas 87 (Southern Eastern Pacific) and 71 (Western Central Pacific).

The identification of species in food products has traditionally relied on morphological analysis. However, morphological features are particularly difficult to use in species differentiation among seafood species in general, and Decapoda shrimps in particular, because of their phenotypic similarities and to the fact that during processing, their external carapace is often removed. Therefore, unintentional fraud may occur due to interspecies phenotypic similarities, which may lead to

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Abbreviations: AK, arginine kinase; **COI**, cytochrome c oxidase subunit I; **cytb**, cytochrome b; **FAO**, Food and Agriculture Organization of the United Nations; **FDA**, Food and Drug Administration; **FINS**, forensically informative nucleotide sequencing; **MRM**, multiple reaction monitoring; **SSRs**, Simple sequence repeats

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inadvertent adulteration and mislabeling of products [8]. In addition, the increasing demand of crustaceans in general, and of high-quality shrimps in particular, may be compromised by deliberate adulteration along the food chain, due to the substitution of high-quality species by lower quality counterparts. Therefore, there is a remarkable need for developing fast and reliable methods to identify seafood species and to quantify their levels in seafood products in order to ensure product quality and thus to protect consumers.

A great variety of molecular methods based on protein and DNA analysis have been developed, and several review articles on this topic have been published [9–11]. Classical electrophoretic and immunological methods have been used for the detection and authentication of fish and seafood species [1, 12]. In fact, electrophoretic methodologies are currently being used to officially differentiate the species presented in seafood products [13], although their application may be hampered by the lack of stability of some proteins during thermal processing [14,15]. Limitations of these classic methods, such as laboriousity and time consumption, were solved with the introduction of methods based on DNA amplification and DNA hybridization [1, 11, 16–18].

mtDNA markers such as the 16S ribosomal RNA (rRNA), 12S rRNA, cytochrome c oxidase subunit I (COI), and cytochrome b (cytb) genes, and the mtDNA control region, have been extensively used in PCR-based studies on food authenticity [11,18], population structures, phylogeography, and phylogenetic relationships at different taxonomic levels [19–23]. The 16S rRNA gene [19,23,24] and the COI gene [20,25], in addition to several nuclear genes [8,26,27], have been described for inferring phylogenetic relationships within shrimp and prawn species.

Proteomics tools have recently been proposed as faster, sensitive, and high-throughput approaches for the assessment of the authenticity and traceability of marine species in seafood products [15, 28]. Thus, mass spectrometry (MS) has been used for the characterization of species-specific peptidic markers [29, 30].

In this review, an extensive and updated overview of the molecular methodologies for the authentication of prawn and shrimp species is provided, emphasizing different strategies that can be applied in the new field of Foodomics [31], such as the state-of-the-art proteomics and the latest DNA-based approaches.

2 Seafood labeling and traceability regulations

The increasing concern of consumers about the composition of the food they eat has resulted in the public awareness of the need for standardizing food labeling. Due to the great number and variety of species, it can be found that, in some cases, several species share the same commercial name. In this sense, different species can be commercialized under the name "banana shrimp", such as *Fenneropenaeus indicus* (FAO: Indian white prawn), *Fenneropenaeus merguiensis* (FAO: Banana prawn), *Penaeus semisulcatus* (FAO: Green tiger prawn), *Melicertus latisulcatus* (FAO: Western king prawn), *Melicertus longistylus* (FAO: Redspot king prawn), *Melicertus plebejus* (FAO: Eastern king prawn), *Metapenaeus endeavouri* (FAO: Endeavour shrimp), *Metapenaeus ensis* (FAO: Greasyback shrimp), and *Metapenaeus monoceros* (FAO: speckled shrimp) [32]. In US, the seafood substitution has been prohibited according to the Federal Food, Drug, and Cosmetic Act Section 403, Misbranded Food [33], which declares that a food shall be deemed misbranded if it is offered for sale under the name of another food. The need for commonly acceptable market names for seafood sold in interstate commerce and the need for assisting manufacturers in labeling seafood products has led to the publication of *The Fish List* in 1988, which was later renamed as *The Seafood List*, by the US Food and Drug Administration (FDA) (http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/Seafood/ucm113260.htm#thelist; accessed date Sep 2011) in collaboration with the National Marine Fisheries Service. This list provides four types of names for domestic and imported fish and invertebrated species, i.e. market name, scientific common name, scientific name, and vernacular name, with the objective of reducing confusion within producers and consumers.

In the European Union, in response to consumer concerns, regulations have been implemented to ensure complete and correct information, guaranteeing market transparency and avoiding substitution of species. The Council Regulation (EC) No. 104/2000 [34] on the common organization of the markets in fishery and aquaculture products advises that seafood products should be labeled indicating: (i) the commercial designation of the species, (ii) the production method (wild or farmed) and (iii) the geographical origin. This regulation has enforced all the member states to publish a list of the commercial designations accepted in their territory indicating the scientific name and the common name in the language(s) of each state. Commercial designation, scientific name, production method, and catch area must be available at each stage of the food chain to guarantee its traceability. These requirements have been implemented in each of the European States, such as Spain, where several regulations have been promulgated to assure the correct labeling and identification of seafood products [35–37]. In addition, the Codex Alimentarius FAO/World Health Organization and the European Commission have recently proposed a list of allergens that should be labeled in prepacked foods, from which crustaceans are emphasized [38]. Moreover, the European Food Safety Authority has established a system of traceability for food, including seafood, and feed business in order to ensure food safety [39].

3 Classical protein-based methods for shrimp and prawn species identification

Methodologies based on protein analysis have been extensively used for authentication of seafood species [5], but few

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Table 1. Summary of protein-based methods applied in the authentication of shrimp and prawn species

Technique		Species[a)]	Target[b)]	Reference
Electrophoresis	SDS-PAGE	Shrimp and crab	Proteins < 30 kDa	[40]
		Far. duorarum, L. setiferus, S. brevirostris	Sarcoplasmic proteins	[41]
	IEF	Shrimp, fish, and lobster	Calcium-binding proteins	[42]
		Shrimp, fish, and cephalopod	Nonsarcoplasmic proteins	[42]
		Far. duorarum, L. setiferus, S. brevirostris	Sarcoplasmic proteins	[43, 44]
		Fourteen shrimp and prawn species	SCPs	[45]
	2DE	Seven shrimp and prawn species	AK	[46]
Immunological	ELISA	Crustaceans	Tropomyosin	[47]
		Crustaceans	Tropomyosin	[48]
		S. brevirostris	Protein M	[49]
	Immunodot	Crustaceans	AK	[50]
		S. brevirostris	Protein M	[49]
	Western blot	*S. brevirostris*	Protein M	[49]
MS	MALDI-TOF PMF	*P. monodon, Fen. indicus*	AK	[51]
		Six shrimp and prawn species	AK	[52]
		Pan. borealis	AK	[53]
	MS/MS	Seven shrimp and prawn species	AK	[46]
		Pan. borealis	AK	[53]
	MRM	Seven shrimp and prawn species	AK peptides	[54]

a) Genera abbreviations: *Far.*, *Farfantepenaeus*; *L.*, *Litopenaeus*; *P.*, *Penaeus*; *Fen.*, *Fenneropenaeus*; *Pan.*, *Pandalus*.
b) Target abbreviations: AK, arginine kinase; SCPs, sarcoplasmic calcium-binding proteins.

studies have been made to elucidate differences among the closely related group of Decapoda shrimps and prawns. The historic application of protein-based techniques in the authenticity assessment of shrimp and prawn species is compiled in Table 1.

3.1 Electrophoretic methods

Electrophoretic techniques such as SDS-PAGE [55], IEF [56], and 2DE [57] have been used for fish species identification. Civera and Parisi [40] reported a faint variability in SDS-PAGE patterns between generic shrimps and crabs, although they were not able to differentiate among species within each of these two groups. An et al. [41] demonstrated the effectiveness of SDS-PAGE to differentiate among three species of shrimp, namely pink (*Farfantepenaeus duorarum*), white (*Litopenaeus setiferus*), and rock shrimp (*Sicyonia brevirostris*). The electrophoretic pattern of the water-extractable protein fraction from the raw specimens resulted to be specific for each of the species. Heating the water-soluble samples prevented from identifying the species due to the precipitation of proteins, and only an extraction with SDS made the differentiation of the two genera possible under that condition.

IEF is the most commonly used protein-based technique for species identification [58]. IEF is based on the separation of proteins on polyacrylamide gel by the use of a pH gradient and the subsequent staining of the proteins. IEF was adopted by the Association of Official Analytical Chemists as the only official validated method with species identification purposes [13], and the US FDA offers a library of IEF patterns from different fishes on the Internet (http://www.fda.gov/Food/FoodSafety/Product-specificInformation/Seafood/RegulatoryFishEncyclopediaRFE/default.htm; accessed date: Sep 2011). However, several studies reported that IEF is not suitable for heated or marinated products due to the degradation of some of the muscle proteins under such conditions [12].

Regarding the application of IEF to shrimp authentication, a study with sarcoplasmic proteins succeeded to differentiate generic shrimp from fish and lobster [42]. The specific proteins, which belonged to the class of calcium-binding polypeptides, were in the 4–5 p*I* range, and resulted to be heat stable. For this reason, such proteins provided species-specific patterns for cooked products as well as in raw samples. In the same work, a similar method based on IEF in urea gels was applied to the analysis of products in which the sarcoplasmic proteins had been previously removed by washing steps during processing, this strategy allowed the differentiation of shrimp from fish and cephalopod meat. In another work [43, 44], IEF was used for the differentiation of pink, white, and rock shrimps. Several electrophoretic conditions were tested, and under the optimized conditions using gels containing 9.2 M urea and 6.2% ampholytes, the three shrimp species were successfully identified by the species-specific patterns of the major water-extracted protein bands. The use of pH 4.0–6.5 ampholytes in IEF gels was found to enhance protein separation as compared to gels containing pH 3–10 ampholytes, making easier the differentiation of species. In heated samples, some specific bands were observed, although the loss of some proteins and the clustering of the remaining bands made the differentiation of the shrimp species fail.

In a recent work, native IEF of sarcoplasmic proteins was used for the identification of 14 shrimp species of commercial interest for the seafood industry [45]. Such study

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included the Northern shrimp *Pandalus borealis* and the Argentine red shrimp *Pleoticus muelleri* in addition to other more studied penaeid shrimps, representing the most complete electrophoretic study described to date for the identification of shrimp and prawn species. Sarcoplasmic protein extracts were analyzed in a narrow acidic pH range (pH 4.0–6.5) and the p*I* of the resulting bands was measured using specific image analysis software. Band patterns observed for each of the 14 species resulted to be specific, intraspecific polymorphism being low, this allowing the unambiguous differentiation of all the tested species. Thus, this fast, cheap, and easy to apply technique could be useful as a robust tool for preventing mislabeling in this group of species. In addition, species-specific protein bands were identified by MS as sarcoplasmic calcium-binding proteins (SCPs) [45], opening the way to further studies regarding their potential use as specific biomarkers.

3.2 Immunological methods

Certain immunoassays have been shown to work in heated protein extracts [59]. ELISA is a technique frequently used as a diagnostic tool, as well as in quality control at industry. Such assays are commercially available as kits to enable the detection of a wide range of proteins, succeeding in some cases even with thermally processed foods. The disadvantages of immunoassays are the nonavailability of commercial antibodies for many species and the crossreaction of the antibodies with closely related species [60]. On the other hand, immunological techniques present some advantages, such as simplicity, specificity, and sensitivity. In addition, some of them (e.g. ELISA) are quantitative assays. Antibodies against shrimp tropomyosin have been used for the detection of crustacean meat in food [47,48]. A monoclonal antibody-based sandwich ELISA was applied to the quantification of *Far. aztecus* brown shrimp tropomyosin (Pen a 1) in shellfish extracts [47]. Although this immunoassay relied on a monoclonal antibody, it was found to be suitable for the detection of generic crustaceans, rather than for the specific detection of the brown shrimp. A more recent study [48] described an ELISA protocol using polyclonal antibodies against Western king prawn *Mel. latisulcatus* tropomyosin that could be used for the detection of crustaceans in raw and cooked products, being able to detect as low as 1 ng/mL.

Arginine kinase (AK) from lobster (*Homarus vulgaris*) muscle has also been used for the development of polyclonal antibodies that succeeded to detect the presence of crustacean muscle in heated mixtures of surimi by immunodot [50]. All these assays are generic for crustacean species, but they are not useful for shrimp species differentiation. With species identification purposes, An et al. [49] developed a monoclonal antibody against a specific protein from the rock shrimp (*S. brevirostris*). This antibody proved to be specific for *S. brevirostris* by ELISA, Western blot, and immunodot blot tests, reaching a detection limit of 4.3 ng of rock shrimp in sample mixtures, not being affected by heat treatment.

4 DNA-based methods for shrimp and prawn species identification

As referred above, limitations of classical electrophoretic and immunological methods have been solved with the introduction of methods based on DNA analysis. A large number of DNA-based studies have been reported for the authentication of species in foods in the last decade. Although at first these reports used hybridization probes, currently most of them are based on PCR amplification followed by the analysis of amplicons in the search for species-specific markers. For this purpose, a variety of methods have been used, such as allozyme analysis, DNA sequencing, species-specific PCR, multiplex PCR, real-time PCR, RFLP, SSCP, RAPD, amplified fragment length polymorphism (AFLP), microsatellite analysis, simple sequence repeats (SSRs), and SNPs [11] analyses. All these methods have also been used to detect fish and seafood species in commercial products, even when such products have been subjected to a processing step such as freezing, precooking, etc. [11, 17, 18]. Table 2 provides an overview of the most relevant DNA-based methods for the differentiation of shrimp and prawn species to date.

Among the DNA targets, mtDNA genes such as the 16S rRNA gene, COI, and cytb genes have been extensively used as interspecific markers in Decapoda species, although most of these studies were focused on population structures and phylogeography more than on species identification with food authentication purposes [19–23]. Regarding the identification of prawn and shrimp species in foodstuffs, there are several works using DNA targets. To our knowledge, the first DNA-based attempt to differentiate two penaeid shrimp species consisted on the sequencing of the mitochondrial 16S rRNA gene from *Far. notialis* and *Litopenaeus schmitti* [24]. When comparing their sequences, an 11% nucleotide divergence between these two species was found. Using RAPD coupled to PCR, 40 primers were screened and two of them were found to be useful for the specific identification of two penaeid prawns: *Marsupenaeus japonicus* and *Met. ensis* [75]. In a similar study, specific fragments of *Fenneropenaeus chinensis* and *Palaemon gravieri* were detected using several arbitrary primers [76].

Phylogenetic analysis of amplified and sequenced DNA fragments has been widely used for food authentication. The first applications of this method to Decapoda species identification were based on the mitochondrial control region to differentiate *Far. aztecus and L. setiferus* [63], on the COI gene for distinguishing among *Fen. merguiensis, Fenneropenaeus silasi, and Fen. indicus* [64], and on a fragment of the genes 16S rRNA, $tRNA^{Val}$, and 12S rRNA in order to differentiate *Litopenaeus vannamei* and *Litopenaeus stylirostris* [65]. Microsatellites (SSRs) markers were considered in an *L. vannamei* population study, combining the data mining of ESTs freely available sequences with in silico analysis [77]. Polymorphism analysis has also been successfully applied for the differentiation of *L. vannamei, L. stylirostris,* and *Trachypenaeus birdy* [77].

A PCR-RFLP approach for the generic detection of crustacean species in food, including several species of shrimp

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Table 2. Summary of DNA-based methods applied in the authentication of shrimp species

Technique[a)]	Target genes[b)]	Species[c)]	Reference
Species-specific PCR primers and multiplex PCR	16S rRNA	*Far. aztecus, Far. duorarum, Far. brasiliensis, L. setiferus*	[61]
		P. monodon, L. vannamei, and *Fen. indicus*	[62]
Sequencing	16S rRNA	*Far. notialis, L.. schmitti*	[24]
Sequencing + phylogenetic analysis (FINS)	mtDNA control region	*Far. aztecus, L. setiferus*	[63]
	COI	*Fen. merguiensis, Fen. silasi, Fen. indicus*	[64]
	16S rRNA//tRNA^{Val} /12S rRNA	*Far. californiensis, L. vannamei,* and *L. stylirostris*	[65]
	16S rRNA/tRNA^{Val}	19 penaeid species	[66]
		17 penaeid species from commercial origin	[67]
	16S rRNA/tRNA^{Val}, 16S rRNA, COI	19 penaeid species	[68]
	cytb	*P. monodon, L. vannamei, Far. notialis, Par. longirostris, Pl. muelleri, Fen. merguiensis*	[69]
	ITS-1	*Mar. japonicus, Mel. latisulcatus, L. vannamei, Fen. merguiensis, Fen. indicus*	[70]
RFLP	ITS-1	*L. vannamei, Metapenaeopsis dalei, Solenocera crassicornis Procambarus clarkii*	[70]
	16S rRNA/tRNA^{Val}	19 penaeid species	[66]
		21 penaeid species	[71]
		17 penaeid species from commercial origin	[67]
		Pan. borealis	[53]
	Cytb	*P. monodon, L. vannamei, Far. notialis, Par. longirostris, Pl. muelleri, Fen. merguiensis*	[69]
		P. semisulcatus, P. kerathurus, Par. longirostris, Met. monoceros	[72]
	16S rRNA	Shrimp, crab, lobster, and crawfish	[73]
	COI-COII, 16S rDNA	*P. monodon, P. semisulcatus, L. vannamei, Fen. Merguiensis, and Mar. japonicus*	[74]
SSCP	16S rDNA	*P. monodon, P. semisulcatus, Fen. merguiensis, L. vannamei, and Mar. japonicus*	[74]
RAPD	–	*Mar. japonicus, Met. ensis*	[75]
	–	*Fen. chinensis, Pal. gravieri*	[76]
Microsatellites or SSRs	ESTs	*L. vannamei, L. stylirostris, Tra. birdy*	[77]

a) Technique abbreviations: FINS, forensically informative nucleotide sequencing; SSRs, simple sequence repeats.
b) Target abbreviations: rRNA, ribosomal RNA; COI, cytochrome c oxidase subunit I; tRNA, transfer RNA; cytb, cytochrome b; ITS1, internal transcribed spacer 1; COII, cytochrome oxidase II.
c) Genera abbreviations: *Far.*, *Farfantepenaeus*; *L.*, *Litopenaeus*; *P.*, *Penaeus*; *Fen.*, *Fenneropenaeus*; *Par.*, *Parapenaeus*; *Pl.*, *Pleoticus*; *Mar.*, *Marsupenaeus*; *Mel.*, *Melicertus*; *Pan.*, *Pandalus*; *Met.*, *Metapenaeus*; *Pal.*, *Palaemon*; *Tra.*, *Trachypenaeus*.

and other decapods, has been reported [73]. Although this method allowed the detection of allergenic crustacean species and the generic differentiation of shrimps from crab, lobster, and crawfish species, it did not achieve species identification. Two alternative methods based on restriction patterns or SSCP, and targeting the mitochondrial genes COI, cytochrome oxidase II (COII) and 16S rRNA succeeded in differentiating five penaeid species: *Penaeus monodon*, *P. semisulcatus*, *L. vannamei*, *Fen. Merguiensis*, and *Mar. japonicus* [74]. Another PCR-RFLP study targeted to the cytb gene achieved the differentiation of four penaeid species, namely *P. semisulcatus, Penaeus kerathurus, Parapenaeus longirostris*, and *Met. monoceros* [72].

4.1 Latest genetic methods

The most complete method described to date for the identification of penaeid shrimp species of commercial interest was carried out by Pascoal et al. [66] using a PCR-RFLP approach. This specific analytical procedure has been protected from commercial purposes by international patent [71], and consisted in the amplification of a 515–535 bp region of the 16S rRNA/ tRNA^{Val} mitochondrial genes, and subsequent cleavage of amplicons with endonucleases *Alu*I, *Taq*I, and *Hinf*I. Species-specific restriction patterns were obtained in all cases, allowing the differentiation of the 21 species studied. Authentication was also successfully assessed in

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commercially peeled products subjected to industrial processing. In addition to their interspecific differentiation, different restriction types corresponding to different populations and origins were found in some of these species at the intraspecific level. After sequencing such regions, nucleotide sequences were aligned and phylogenetic analyses were conducted using MEGA software [78], obtaining rooting phylogenetic trees. Such trees, besides allowing to interpret phylogenetic relationships among the shrimp species studied, can help in the species identification of unknown samples, through the comparison with well-established reference samples. This methodology, known as forensically informative nucleotide sequencing (FINS), has been extensively used for the identification of seafood species [15], and consists on the estimation of genetic distances between some reference sequences (experimentally obtained or extracted from a DNA database) and an unknown sequence, and subsequent building of a phylogenetic tree, in which sequences of the same species are grouped together [79]. An example of such a phylogenetic tree built from 16S rRNA/tRNA^{Val} partial sequences from 61 samples belonging to 21 different shrimp and prawn species is shown in Fig. 1. This methodology was also successfully used to assess the incidence of incorrect labeling of prawns and shrimps in commercial food products [67]. The results of this authenticity survey showed a 24% (10 out of 41 commercial products tested) of incorrect labeling, while another 39% of the commercial samples showed incomplete labeling.

In a similar study but based on the amplification of a *ca.* 181 bp region of the cytb gene, using FINS and RFLP with endonucleases *Cvi*JI, *Dde*I, and *Nla*IV, Pascoal et al. [69] demonstrated that this mitochondrial marker can also be useful for identification and phylogenetic studies in Decapoda species. This methodology was successfully applied to the identification of six commercially relevant penaeid shrimp species in complex processed foods, the whole analysis being completed in 8 h. Since DNA fragmentation may be important during food processing, the small size of the target considered in such study made it especially useful for analysis of frozen and precooked samples.

Recently, three different mtDNA markers from 20 shrimp and prawn species were compared, namely the 16S rRNA/tRNA^{Val}, 16S rRNA, and COI genes. The 16S rRNA/tRNA^{Val} marker was found to contain more variable sites than the other two regions, thus allowing the interspecific differentiation of a large number of shrimp species [68]. In addition, the phylogenetic relationships inferred using such marker proved to be more in agreement with widely accepted taxonomical studies established for this group of Decapoda. Another PCR-RFLP study dealing with shrimp species authentication, reported the rRNA internally transcribed spacer 1 (ITS-1) as an adequate marker to the identification of *L. vannamei, Metapenaeopsis dalei, Solenocera crassicornis,* and *Procambarus clarkii,* [70]. They also used a FINS approach to differentiate five *Penaeus* species from Southeast China, namely *Mar. japonicus, Mel. latisulcatus, L. vannamei, Fen. merguiensis,* and *Fen. indicus* [70]. In a recent work [53], the Northern prawn *Pan. borealis*, one of the most important commercial decapods in the world, was differentiated from a wide range of commercial Decapoda species by a PCR-RFLP method based on the amplification of a *ca.* 966 bp 16S rRNA/tRNA^{Val}/12S rRNA mitochondrial region. The restriction patterns obtained using enzymes *Alu*I, *Taq*I, and *Hinf*I, allowed the differentiation of *Pan. borealis* from other 30 crustacean species, these including prawns, shrimps, crabs, and lobsters, this representing the most complete study of a crustacean species as regards its detection and identification in foodstuffs, either as whole individuals or in processed products.

Latest DNA-based methods currently under development are mainly species-specific PCR of commercially relevant species, and multiplex PCR methods. Multiplex PCR is a PCR variant aimed to amplify several DNA fragments of interest by using several pairs of primers in a single reaction. Alvarado-Bremer et al. [61] developed a multiplex PCR for the identification of four commercially relevant shrimp species from the Gulf of Mexico, namely *Far. aztecus, Far. duorarum, Far. brasiliensis,* and *L. setiferus*, and *L. vannamei* from aquaculture origin, using species-specific PCR oligonucleotide primers for each of these species. In a recent study [62], specific primers were designed for the simultaneous identification of *P. monodon, L. vannamei,* and *Fen. indicus,* which represent more than 80% of the aquaculture shrimp production and may be fraudulently replaced by species exhibiting lower value. These species-specific primers, which amplify mitochondrial fragments of 362, 151, and 213 bp in the 16S rRNA gene for *P. monodon, L. vannamei,* and *Fen. Indicus,* respectively, allowed the direct identification of these in a food sample, avoiding the potential complications of complex restriction patterns caused by the presence of more than one shrimp species.

5 Proteomics

Proteomics, defined as the large-scale analysis of proteins [80], is aimed at the investigation of the proteome based on MS techniques, such as MALDI-TOF MS and ESI-IT MS, coupled with the bioinformatics treatment of the data obtained. The combination of different proteomics tools, taking advantage of the high-throughput capacity of MS-based techniques, represents a robust and powerful strategy for protein and peptide identification and characterization. Proteomics techniques have some advantages over other methods: (i) they may be automated, producing fast, reproducible, and sensitive results and allowing high-throughput analysis of foodstuffs; (ii) they can also be applied to species that are poorly characterized at the genomic and proteomic levels in the databases, avoiding the time-consuming steps of DNA amplification and sequencing; and (iii) they open the way to the identification and characterization of species-specific peptides, this representing the first step toward designing fast and cheap detection analysis, such as antibody-based assays or MS detection methods.

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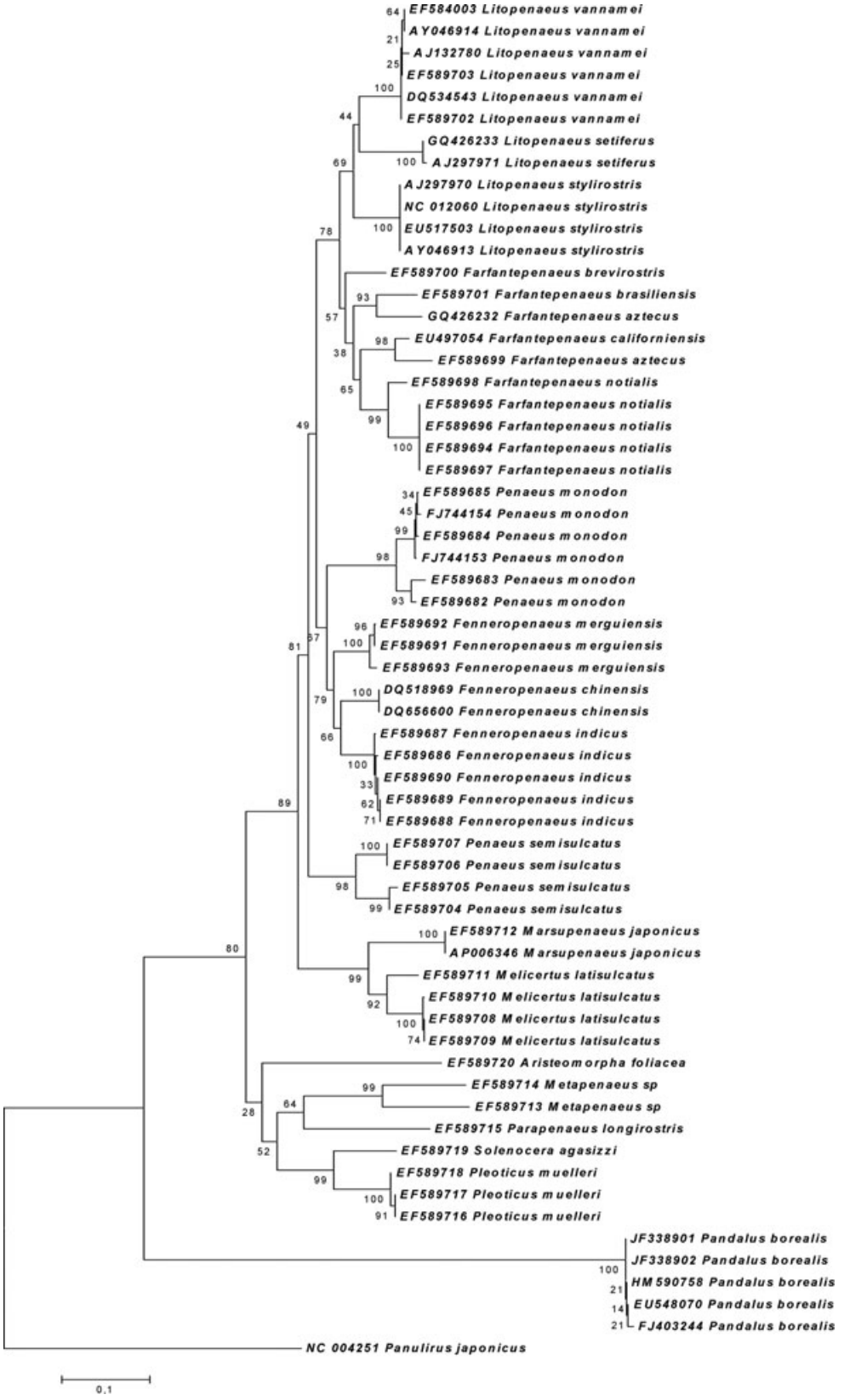

Figure 1. Topologies resulting from the phylogenetic analysis of the nucleotide sequences of 515–535 bp 16S rRNA/tRNA^{Val} mitochondrial genes in 21 decapod shrimp and prawn species, by means of the neighbor joining method. Numbers above and below branches indicate bootstrap values from neighbor joining analysis.

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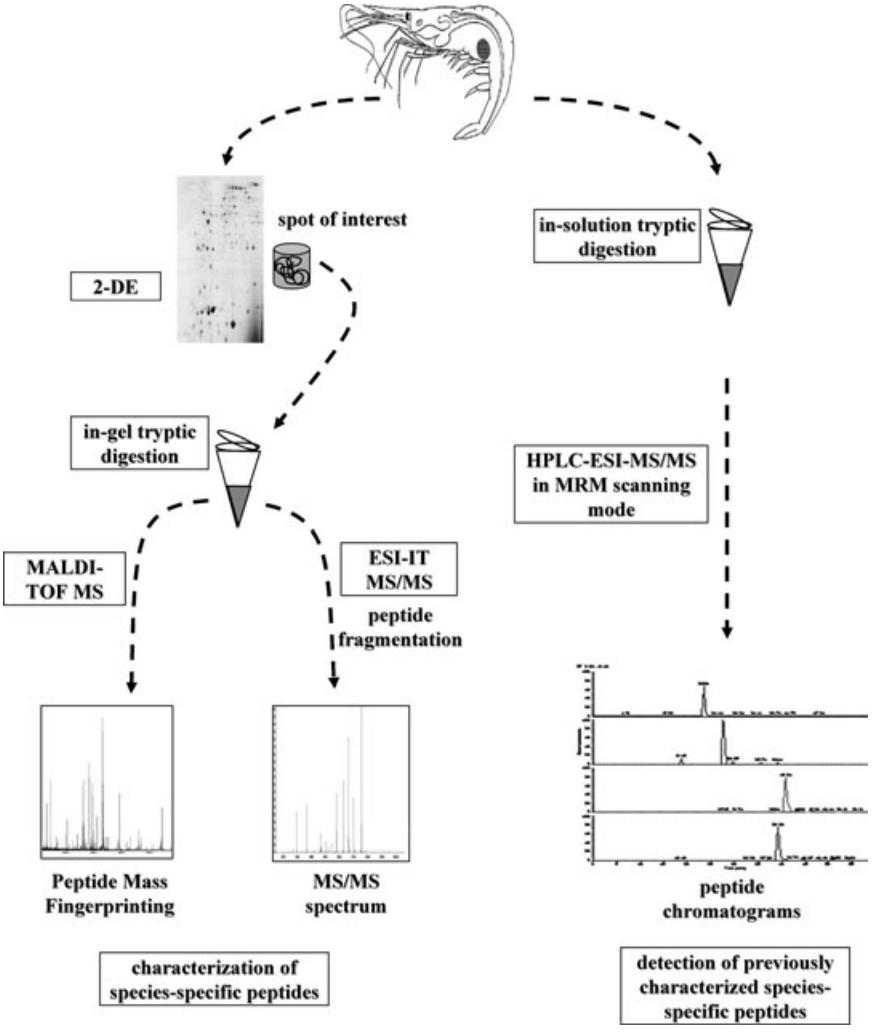

Figure 2. Proteomics approaches considered for the identification, characterization, and detection of species-specific diagnostic peptides from shrimp with food authentication purposes.

Proteomics methodologies have been used for the identification of some seafood species such as mussels [29] and hakes [30, 81], but their application for the authentication of decapods has been scarce. Figure 2 shows the different proteomics approaches that are currently under use at our laboratory for the isolation, characterization, and detection of species-specific peptides from shrimps with food authentication purposes. 2DE has been considered for the separation of proteins in muscle extracts. Potentially species-specific proteins according to 2DE analysis, are selected and digested with trypsine in order to investigate the amino acidic sequences by means of MS. In this sense, MALDI-TOF MS has been successfully applied for the differentiation of several shrimp species. In a preliminary study, PMF, one of the main applications of MALDI-TOF MS, was successfully applied to the differentiation of Decapoda species, as was ascertained by analyzing two very closely related species: *P. monodon* and *Fen. indicus* [51]. Fingerprints of the sarcoplasmic protein arginine kinase (AK) exhibited two peaks that resulted to be species specific of *P. monodon*, and which could be useful as biomarkers for this commercially relevant species. In a subsequent study [52], six shrimp species of commercial relevance, namely the giant tiger prawn (*P. monodon*), the Pacific white shrimp (*L. vannamei*), the Indian white prawn (*Fen. indicus*), the Southern pink shrimp (*Far. notialis*), the Argentine red shrimp (*Pl. muelleri*), and the Northern shrimp (*Pan. borealis*), were successfully differentiated by a proteomic approach combining 2DE, tryptic digestion, and PMF of AK. In such work, a mathematical method for the comparison of the PMF spectra was reported, which could be applied to infer taxonomic relationships between specimens. This method consisted on a similarity coefficient between the different PMF peak lists, in order to generate a distance matrix that could be represented as a dendrogram. The use of this mathematical approach instead of visual spectral comparison provides a quantitative measure of how well two species match. Although a high homology was found among the AK PMF spectra from the different shrimp species, species-specific profiles useful for shrimp differentiation were found, providing a selective differentiation method among families Penaeidae, Solenoceridae, and Pandalidae, and an unequivocal identification of the shrimp species. Due to the interspecific

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variability of AK and to its high concentration in shrimp muscle, this enzyme has been proposed as a biomarker for shrimp species identification [46]. In addition, the phyloproteomic dendrogram generated was consistent with the phylogenies established with mtDNA data [52], thus opening the way to further studies on phyloproteomics. Further identification and characterization of the differential peptides from the AK in seven commercial shrimp species (the previous six shrimp species and *Fen. merguiensis*) was performed by ESI-IT MS/MS, followed by database searching and de novo sequence interpretation [46]. Several species-specific peptides were reported, together with the spectra of their collision induced dissociation fragmentation. In addition, in a subsequent work [53], additional *Pan. Borealis*-specific AK peptides were identified and characterized. Such works allowed the identification of diagnostic peptides that could be useful to design fast and cheap analysis kits for the sensitive detection of these commercially relevant prawn species. The selection of AK is particularly interesting because it is an allergen [82], and therefore an analysis targeting this protein would have a double application, both for species identification and food safety purposes. Thus, this would allow the detection of shrimp allergens in food products and help the manufacturer and the administration to identify, in real time, the existence of a contamination in a production line, and compliance with food labeling regulations, even allowing the allergen quantification [83].

In a recent work [54], a shotgun proteomics approach was applied to the detection of some of the previously characterized AK species-specific peptides [46]. Although 2DE has been the workhorse for the majority of proteome projects in the past [84], when peptidic biomarkers have been already characterized it is much faster to directly search for the specific known peptides in the protein extract, avoiding the laborious and time-consuming 2DE isolation step. When dealing with complex samples, multiple reaction monitoring (MRM), or its variant when working with an ion trap, selected MS/MS ion monitoring (SMIM), is the most suitable screening method for the detection and quantification of peptides previously sequenced by MS [85]. In MRM, a precursor ion of a particular mass-to-charge ratio (m/z) is selected in the first stage of MS and several product ions are selected in the second stage [86]. An innovative methodology combining the speed of high-intensity focused ultrasound-assisted tryptic digestion of proteins, the high separation capability of reverse-phase HPLC, and the peptide detection ability of MS working in the MRM-SMIM scanning mode, made possible the differentiation of seven shrimp and prawn species considered in no longer than 90 min, from the arrival of the sample to the identification of the species [54]. Alternatively, an offline analysis of the unseparated tryptic digests also achieved the discrimination of closely related species studied in an even shorter analysis time [54], although this methodology is less automatized. To the best of our knowledge, these two methodologies are the fastest molecular methods described to date for the authentication of animal species in foods.

6 Final considerations and future trends

There is a need for developing molecular identification methods that may provide the administration and the seafood industry with the tools necessary to comply with labeling and traceability at species level. Although conventional identification of species has been based on phenotypic traits, genotype-based classification is more accurate, especially in those specimens with less identifiable traits and more easily influenced by environment. Although IEF is the most commonly described method for species identification, genomic analysis is currently considered to be the best approach due to its simplicity and good reproducibility. Major challenges facing research on shrimp authentication still are: (i) the optimization of simple, fast, and inexpensive methodologies for routine use; (ii) the analysis of highly processed or complex food matrices, this including the development of methods targeted to smaller fragments, which could be detected both in raw and processed products; (iii) multiple species identification in food samples; and (iv) quantification of different species in mixed food samples [18]. Omics technologies are already helping to meet these challenges, and several methodologies are currently under development, such as methods based on multiplex PCR, with specific primers targeted to several species and allowing the simultaneous detection of multiple species, real-time PCR, PCR-RFLP, which has been found to detect a specific species in complex matrices and mixtures with a detection limit of 0.1% (w/w) [87], microarrays, IEF of native proteins, and MS detection of species-specific peptides previously characterized by means of proteomics tools, which has been found to detect 0.5% contaminant meat [88]. The great advancement of proteomics approaches is that the identification and characterization of new specific peptides is the first step toward the design of fast and easy-to-use detection analyses. The characterized species-specific peptides can then be used to prepare antibody-based kits for the fast and sensitive detection of different species in a sample. Alternatively, these peptides could be used in MRM MS assays. The potential ability of proteomics approaches for species identification is remarkable, allowing a fast, cheap, high throughput, high accuracy, and sensitive screening. In this sense, the characterization and validation of species-specific peptides could allow their use as biomarkers for shrimps species identification, being monitored by MRM MS or by ready-to-use antibody-based kits for the sensitive detection of the species, allowing multiple species identification, detection, and even quantification in complex food matrices.

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