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

Ignacio Ortea[1], Ananías Pascoal[2], Benito Cañas[3], José M. Gallardo[1], Jorge Barros-Velázquez[2], Pilar Calo-Mata[2].

[1]Department of Food Technology, Institute for Marine Research, Spanish National Research Council (CSIC), c/ Eduardo Cabello 6, E-36208 Vigo, Spain.

[2]Department of Analytical Chemistry, Nutrition and Food Science, School of Veterinary Sciences/College of Biotechnology, University of Santiago de Compostela, rúa Carballo Calero s/n, E-27002 Lugo, Spain.

[3]Department of Analytical Chemistry, University Complutense of Madrid, E-28040 Madrid, Spain.

Author to whom all correspondence is to be sent: Dr. Ignacio Ortea, Instituto de Investigaciones Marinas (IIM-CSIC), c/Eduardo Cabello 6, E-36208 Vigo, Spain.

E-mail: nachoog@iim.csic.es

Abbreviations: AFLP, Amplified Fragment Length Polymorphism; AK, Arginine Kinase; COI, cytochrome c oxidase subunit I; COII, cytochrome oxidase II; cytb, cytochrome b; ESI-IT MS, electrospray-ion trap mass spectrometry; FAO, Food and Agriculture Organization of the United Nations; FDA, Food and Drug Administration; FINS, Forensically Informative Nucleotide Sequencing; ITS1, internal transcribed spacer 1; MALDI-TOF MS, matrix assisted laser desorption/ionization-time of flight mass spectrometry; MRM, Multiple Reaction Monitoring; MS, mass spectrometry; mtDNA, mitochondrial DNA; m/z, mass-to-charge ratio; PMF, Peptide Mass

1   Fingerprinting; rRNA, ribosomal RNA; SCP, sarcoplasmic calcium-binding protein; SMIM, Selected

2   MS/MS Ion Monitoring; SSRs, Simple Sequence Repeats;

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1 **Abstract**

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3 Although seafood species identification has traditionally relied on morphological analysis, sometimes

4 this is difficult to apply for the differentiation among penaeid shrimps owing to their phenotypic

5 similarities and to the frequent removal of external carapace during processing. The objective of this

6 review is to provide an updated and extensive overview on the molecular methods for shrimp and

7 prawn species authentication, in which several omics approaches based on protein and DNA analysis

8 are described. DNA-based methods include the amplification by PCR of different genes, commonly

9 the mitochondrial 16S ribosomal RNA and cytochrome oxidase I genes. A recently described method

10 based on restriction fragment length polymorphism coupled to PCR turned out to be particularly

11 interesting for species differentiation and origin identification. Protein analysis methods for the

12 characterization and detection of species-specific peptides are also summarized, emphasizing some

13 novel proteomics-based approaches, such as phyloproteomics, peptide fragmentation and species-

14 specific peptide detection by HPLC coupled to Multiple Reaction Monitoring (MRM) mass

15 spectrometry, the latter representing the fastest method described to date for species authentication in

16 food.

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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 high valuable species may be substituted, partial 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 non-declared 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

1   among seafood species in general, and Decapoda shrimps in particular, because of their phenotypic

2   similarities and to the fact that during processing their external carapace is often removed. Therefore,

3   unintentional fraud may occur due to interspecies phenotypic similarities, which may lead to

4   inadvertent adulteration and mislabelling of products [8]. In addition, the increasing demand of

5   crustaceans in general, and of high-quality shrimps in particular, may be compromised by deliberate

6   adulteration along the food chain, due to the substitution of high quality species by lower quality

7   counterparts. Therefore, there is a remarkable need for developing fast and reliable methods to identify

8   seafood species and to quantify their levels in seafood products in order to ensure product quality and

9   thus to protect consumers.

10   A great variety of molecular methods based on protein and DNA analysis have been developed,

11   and several review articles on this topic have been published [9-11]. Classical electrophoretic and

12   immunological methods have been used for the detection and authentication of fish and seafood

13   species [1, 12]. In fact, electrophoretic methodologies are currently being used to officially

14   differentiate the species presented in seafood products [13], although their application may be

15   hampered by the lack of stability of some proteins during thermal processing [14, 15]. Limitations of

16   these classic methods, such as laboriousity and time-consumption, were solved with the introduction

17   of methods based on DNA amplification and DNA hybridization [1, 11, 16-18].

18   mtDNA markers such as the 16S ribosomal RNA (rRNA), 12S rRNA, cytochrome c oxidase

19   subunit I (COI), and cytochrome b (cytb) genes, and the mtDNA control region, have been extensively

20   used in PCR based studies on food authenticity [11, 18], population structures, phylogeography and

21   phylogenetic relationships at different taxonomic levels [19-23,]. The 16S rRNA gene [19, 23, 24]

22   and the COI gene [20, 25], in addition to several nuclear genes [8, 26, 27], have been described for

23   inferring phylogenetic relationships within shrimp and prawn species.

24   Proteomics tools have recently been proposed as faster, sensitive and high-throughput

25   approaches for the assessment of the authenticity and traceability of marine species in seafood

1   products [15, 28]. Thus, mass spectrometry (MS) has been used for the characterization of species-

2   specific peptidic markers [29, 30].

3   In this review, an extensive and updated overview of the molecular methodologies for the

4   authentication of prawn and shrimp species is provided, emphasizing the different strategies that can

5   be applied in the new field of Foodomics [31], such as the state-of-the-art proteomics and the latest

6   DNA-based approaches.

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8   **2. Seafood labelling and traceability regulations**

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10 The increasing concern of consumers about the composition of the food they eat has resulted in the

11 public awareness of the need for standardizing food labelling. Due to the great number and variety of

12 species, it can be found that, in same cases, several species share the same commercial name. In this

13 sense, different species can be commercialized under the name "banana shrimp", such as

14 *Fenneropenaeus indicus* (FAO: Indian white prawn), *Fenneropenaeus merguiensis* (FAO: Banana

15 prawn), *Penaeus semisulcatus* (FAO: Green tiger prawn), *Melicertus latisulcatus* (FAO: Western king

16 prawn), *Melicertus longistylus* (FAO: Redspot king prawn), *Melicertus plebejus* (FAO: Eastern king

17 prawn), *Metapenaeus endeavouri* (FAO: Endeavour shrimp), *Metapenaeus ensis* (FAO: Greasyback

18 shrimp) and *Metapenaeus monoceros* (FAO: speckled shrimp) [32]. In the U.S., the seafood

19 substitution has been prohibited according to the Federal Food, Drug and Cosmetic Act Section 403,

20 Misbranded Food [33], which declares that a food shall be deemed to be misbranded if it is offered for

21 sale under the name of another food. The need for commonly acceptable market names for seafood

22 sold in interstate commerce and the need for assisting manufacturers in labelling seafood products has

23 led in 1988 to the publication of *The Fish List*, later renamed *The Seafood List*, by the US Food and

24 Drug Administration (FDA) [34] in collaboration with the National Marine Fisheries Service. This list

25 provides four types of names for domestic and imported fish and invertebrated species, i.e., market

1   name, scientific common name, scientific name, and vernacular name, with the objective of reducing

2   confusion within producers and consumers.

3   In the European Union, in response to consumer concerns, regulations have been implemented

4   to ensure complete and correct information, guaranteeing market transparency and avoiding

5   substitution of species. The Council Regulation (EC) No 104/2000 [35] on the common organization

6   of the markets in fishery and aquaculture products advises that seafood products should be labeled

7   indicating: (i) the commercial designation of the species, (ii) the production method (wild or farmed)

8   and (iii) the geographical origin. This regulation has enforced all the member states to publish a list of

9   the commercial designations accepted in their territory indicating the scientific name and the common

10   name in the language(s) of each state. Commercial designation, scientific name, production method

11   and catch area must be available at each stage of the food chain to guarantee its traceability. These

12   requirements have been implemented in each of the European States, such as Spain, where several

13   regulations have been promulgated to assure the correct labelling and identification of seafood

14   products [36-38]. In addition, the Codex Alimentarius FAO/World Health Organization and the

15   European Commission have recently proposed a list of allergens that should be labeled in pre-packed

16   foods, from which crustaceans are emphasized [39]. Moreover, the European Food Safety Authority

17   has established a system of traceability for food, including seafood, and feed business in order to

18   ensure food safety [40].

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

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22   Methodologies based on protein analysis have been extensively used for authentication of seafood

23   species [5], but few studies have been made to elucidate differences among the closely related group

24   of Decapoda shrimps and prawns. The historic application of protein-based techniques in the

25   authenticity assessment of shrimp and prawn species is compiled in Table 1.

1

2 **Electrophoretic methods**

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4 Electrophoretic techniques such as SDS-PAGE [56], IEF [57] and 2-DE [58] have been used for fish

5 species identification. Civera and Parisi [41] reported a faint variability in SDS-PAGE patterns

6 between generic shrimps and crabs, although they were not able to differentiate among species within

7 each of these two groups. An et al. [42] demonstrated the effectiveness of SDS-PAGE to differentiate

8 among three species of shrimp, namely pink (*Farfantepenaeus. duorarum*), white (*Litopenaeus.*

9 *setiferus*) and rock shrimp (*Sicyonia brevirostris*). The electrophoretic pattern of the water-extractable

10 protein fraction from the raw specimens resulted to be specific for each of the species. Heating the

11 water-soluble samples prevented from identifying the species due to the precipitation of proteins, and

12 only an extraction with SDS made the differentiation of the two genera possible under that condition.

13 IEF is the most commonly used protein-based technique for species identification [59]. IEF is

14 based on the separation of proteins on polyacrylamide gel by the use of a pH gradient and the

15 subsequent staining of the proteins. IEF was adopted by the Association of Official Analytical

16 Chemists as the only official validated method with species identification purposes [13], and the US

17 FDA offers a library of IEF patterns from different fishes on the internet [60]. However, several

18 studies reported that IEF is not suitable for heated or marinated products due to the degradation of

19 some of the muscle proteins under such conditions [12].

20 Regarding the application of IEF to shrimp authentication, a study with sarcoplasmic proteins

21 succeeded to differentiate generic shrimp from fish and lobster [43]. The specific proteins, which

22 belonged to the class of calcium-binding polypeptides, were in the 4-5 p*I* range, and resulted to be

23 heat-stable. For this reason, such proteins provided species-specific patterns for cooked products as

24 well as in raw samples. In the same work, a similar method based on IEF in urea gels was applied to

25 the analysis of products in which the sarcoplasmic proteins had been previously removed by washing

1   steps during processing, this strategy allowing the differentiation of shrimp from fish and cephalopod

2   meat. In another work [44, 45], IEF was used for the differentiation of pink, white and rock shrimps.

3   Several electrophoretic conditions were tested, and under the optimized conditions using gels

4   containing 9.2 M urea and 6.2% ampholytes, the three shrimp species were succesfully identified by

5   the species-specific patterns of the major water-extracted protein bands. The use of pH 4.0-6.5

6   ampholytes in IEF gels was found to enhance protein separation as compared to gels containing pH 3-

7   10 ampholytes, making easier the differentiation of species. In heated samples, some specific bands

8   were observed, although the loss of some proteins and the clustering of the remaining bands made the

9   differentiation of the shrimp species fail.

10   In a recent work, native IEF of sarcoplasmic proteins was used for the identification of 14

11   shrimp species of commercial interest for the seafood industry [46]. Such study included the Northern

12   shrimp *Pandalus borealis* and the Argentine red shrimp *Pleoticus muelleri* in addition to other more

13   studied penaeid shrimps, representing the most complete electrophoretic study described to date for the

14   identification of shrimp and prawn species. Sarcoplasmic protein extracts were analyzed in a narrow

15   acidic pH range (pH 4.0-6.5) and the p*I* of the resulting bands was measured using specific image

16   analysis software. Band patterns observed for each of the 14 species resulted to be specific,

17   intraspecific polymorphism being low, this allowing the unambiguous differentiation of all the tested

18   species. Thus, this fast, cheap and easy to apply technique could be useful as a robust tool for

19   preventing mislabelling in this group of species. In addition, species-specific protein bands were

20   identified by MS as sarcoplasmic calcium-binding proteins (SCPs) [46], this opening the way to

21   further studies regarding their potential use as specific biomarkers.

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23   **Immunological methods**

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1   Certain immunoassays have been shown to work in heated protein extracts [61]. ELISA is a technique

2   frequently used as a diagnostic tool, as well as in quality control at industry. Such assays are

3   commercially available as kits to enable the detection of a wide range of proteins, succeeding in some

4   cases even with thermally-processed foods. The disadvantages of immunoassays are the non-

5   availability of commercial antibodies for many species and the cross-reaction of the antibodies with

6   closely related species [62]. On the other hand, immunological techniques present some advantages,

7   such as simplicity, specificity and sensitivity. In addition, some of them (e.g. ELISA) are quantitative

8   assays. Antibodies against shrimp tropomyosin have been used for the detection of crustacean meat in

9   food [48, 49]. A monoclonal antibody-based sandwich ELISA was applied to the quantification of

10   *Farfantepenaeus aztecus* brown shrimp tropomyosin (Pen a 1) in shellfish extracts [48]. Although this

11   immunoassay relied on a monoclonal antibody, it was found to be suitable for the detection of generic

12   crustaceans, rather than for the specific detection of the brown shrimp. A more recent study [49]

13   described an ELISA protocol using polyclonal antibodies against Western king prawn *Mel.*

14   *latisulcatus* tropomyosin that could be used for the detection of crustaceans in raw and cooked

15   products, being able to detect as low as 1 ng/mL.

16   Arginine kinase (AK) from lobster (*Homarus vulgaris*) muscle has also been used for the

17   development of polyclonal antibodies that succeeded to detect the presence of crustacean muscle in

18   heated mixtures of surimi by immunodot [51]. All these assays are generic for crustacean species, but

19   they are not useful for shrimp species differentiation. With species identification purposes, An et al.

20   [50] developed a monoclonal antibody against a specific protein from the rock shrimp (*S. brevirostris*).

21   This antibody proved to be specific for *S. brevirostris* by ELISA, Western blot and immunodot blot

22   tests, reaching a detection limit of 4.3 ng of rock shrimp in sample mixtures, not being affected by heat

23   treatment.

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25   **4. DNA-based methods for shrimp and prawn species identification**

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2   As referred above, limitations of classical electrophoretic and immunological methods have been

3   solved with the introduction of methods based on DNA analysis. A large number of DNA-based

4   studies have been reported for the authentication of species in foods in the last decade. Although at

5   first these reports used hybridization probes, currently most of them are based on PCR amplification

6   followed by the analysis of amplicons in the search for species-specific markers. For this purpose, a

7   variety of methods have been used, such as allozyme analysis, DNA sequencing, species-specific

8   PCR, multiplex PCR, real-time PCR, RFLP, SSCP, RAPD, Amplified Fragment Length

9   Polymorphism (AFLP), microsatellite analysis, Simple Sequence Repeats (SSRs), and SNPs [11]

10 analyses. All these methods have also been used to detect fish and seafood species in commercial

11 products, even when such products have been subjected to a processing step such as freezing,

12 precooking, etc. [11, 17, 18]. Table 2 provides an overview of the most relevant DNA-based methods

13 for the differentiation of shrimp and prawn species to date.

14   Among the DNA targets, mtDNA genes such as the 16S rRNA gene, COI and cytb genes have

15 been extensively used as interspecific markers in Decapoda species, although most of these studies

16 were focused on population structures and phylogeography more than on species identification with

17 food authentication purposes [19-23]. Regarding the identification of prawn and shrimp species in

18 foodstuffs, there are several works using DNA targets. To our knowledge, the first DNA-based attempt

19 to differentiate two penaeid shrimp species consisted on the sequencing of the mitochondrial 16S

20 rRNA gene from *Farfantepenaeus notialis* and *Litopenaeus schmitti* [24]. When comparing their

21 sequences, a 11% nucleotide divergence between these two species was found. Using RAPD coupled

22 to PCR, 40 primers were screened and two of them were found to be useful for the specific

23 identification of two penaeid prawns: *Marsupenaeus japonicus* and *Met. ensis* [77]. In a similar study,

24 specific fragments of *Fenneropenaeus chinensis* and *Palaemon gravieri* were detected using several

25 arbitrary primers [78].

1   Phylogenetic analysis of amplified and sequenced DNA fragments has been widely used for

2   food authentication. The first applications of this method to Decapoda species identification were

3   based on the mitochondrial control region to differentiate *Far. aztecus* and *L. setiferus* [65], on the

4   COI gene for distinguishing among *Fen. merguiensis* , *Fenneropenaeus silasi* and *Fen. indicus* [66],

5   and on a fragment of   the genes 16S rRNA**,** tRNA^{Val} and 12S rRNA in order to differentiate

6   *Litopenaeus vannamei* and *Litopenaeus. stylirostris* [67]. Microsatellites (simple sequence repeats,

7   SSRs) markers were considered in a *L. vannamei* population study, combining the data mining of

8   ESTs freely available sequences with in-silico analysis [79]. Polymorphism analysis has also been

9   succesfully applied for the differentiation of *L. vannamei*, *L. stylirostris*, and *Trachypenaeus birdy*

10   [79].

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

12   several species of shrimp and other decapods, has been reported [75]. Although this method allowed

13   the detection of allergenic crustacean species and the generic differentiation of shrimps from crab,

14   lobster and crawfish species, it did not achieve species identification. Two alternative methods based

15   on restriction patterns or SSCP, and targeting the mitochondrial genes COI, cytochrome osidase II

16   (COII) and 16S rRNA succeeded in differentiating five penaeid species: *Penaeus monodon*, *P.*

17   *semisulcatus*, *L. vannamei*, *Fen. merguiensis* and *Mar. japonicus* [76].   Another PCR-RFLP study

18   targeted to the cytb gene achieved the differentiation of four penaeid species, namely *P. semisulcatus,*

19   *Penaeus kerathurus, Parapenaeus longirostris* and *Met. monoceros* [74].

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21   **Latest genetic methods**

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23   The most complete method described to date for the identification of penaeid shrimp species of

24   commercial interest was carried out by Pascoal et al. [68] using a PCR-RFLP approach. This specific

25   analytical procedure has been protected from commercial purposes by international patent [73], and

1   consisted in the amplification of a 515-535 bp region of the 16S rRNA/ tRNA^{Val} mitochondrial genes,

2   and subsequent cleavage of amplicons with endonucleases *Alu*I, *Taq*I and *Hinf*I. Species-specific

3   restriction patterns were obtained in all cases, allowing the differentiation of the 21 species studied.

4   Authentication was also successfully assessed in commercial peeled products subjected to industrial

5   processing. In addition to their interspecific differentiation, different restriction types corresponding to

6   different populations and origins were found in some of these species at the intraspecific level. After

7   sequencing such regions, nucleotide sequences were aligned and phylogenetic analyses were

8   conducted using MEGA software [80], obtaining rooting phylogenetic trees. Such trees, besides

9   allowing to interpret phylogenetic relationships among the shrimp species studied, can help in the

10   species identification of unknown samples, through the comparison with well-established reference

11   samples. This methodology, known as Forensically Informative Nucleotide Sequencing (FINS), has

12   been extensively used for the identification of seafood species [15], and consists on the estimation of

13   genetic distances between some reference sequences (experimentally obtained or extracted from a

14   DNA database) and an unknown sequence, and subsequent building of a phylogenetic tree, in which

15   sequences of the same species are grouped together [81]. An example of such a phylogenetic tree built

16   from 16S rRNA/tRNA^{Val} partial sequences from 61 samples belonging to 21 different shrimp and

17   prawn species is shown in Figure 1. This methodology was also successfully used to assess the

18   incidence of incorrect labelling of prawns and shrimps in commercial food products [69]. The results

19   of this authenticity survey showed a 24% (10 out of 41 commercial products tested) of incorrect

20   labelling, while another 39% of the commercial samples showed incomplete labelling.

21   In a similar study but based on the amplification of a *ca.* 181 bp region of the cytb gene, using

22   FINS and RFLP with endonucleases *Cvi*JI, *Dde*I and *Nla*IV, Pascoal et al. [71] demonstrated that this

23   mitochondrial marker can also be useful for identification and phylogenetic studies in Decapoda

24   species. This methodology was successfully applied to the identification of six commercially relevant

25   penaeid shrimp species in complex processed foods, the whole analysis being completed in eight

1   hours. Since DNA fragmentation may be important during food processing, the small size of the target

2   considered in such study made it especially useful for analysis of frozen and precooked samples.

3   Recently, three different mtDNA markers from 20 shrimp and prawn species were compared,

4   namely the 16S rRNA/tRNA^{Val}, 16S rRNA and COI genes. The 16S rRNA/tRNA^{Val} marker was found

5   to contain more variable sites than the other two regions, thus allowing the interspecific differentiation

6   of a large number of shrimp species [70]. In addition, the phylogenetic relationships inferred using the

7   such marker proved to be more in agreement with widely accepted taxonomical studies established for

8   this group of Decapoda. Another PCR-RFLP study dealing with shrimp species authentication,

9   reported the rRNA internally transcribed spacer 1 (ITS-1) as an adequate marker to the identification

10 of *L. vannamei*, *Metapenaeopsis dalei*, *Solenocera crassicornis* and *Procambarus clarkii*, [72]. They

11 also used a FINS approach to differentiate five *Penaeus* species from Southeast China, namely *Mar.*

12 *japonicus, Mel. latisulcatus, L. vannamei, Fen. merguiensis,* and *Fen. indicus* [72]. In a recent work

13 [54], the Northern prawn *Pan. borealis*, one of the most important commercial decapods in the world,

14 was differentiated from a wide range of commercial Decapoda species by a PCR-RFLP method based

15 on the amplification of a *ca.* 966 bp 16S rRNA/tRNA^{Val}/12S rRNA mitochondrial region. The

16 restriction patterns obtained using enzymes *Alu*I, *Taq*I and *Hinf*I, allowed the differentiation of *Pan.*

17 *borealis* from other 30 crustacean species, these including prawns, shrimps, crabs and lobsters, this

18 representing the most complete study of a crustacean species as regards its detection and identification

19 in foodstuffs, either as whole individuals or in processed products.

20   Latest DNA-based methods currently under development are mainly species-specific PCR of

21 commercially-relevant species, and multiplex PCR methods. Multiplex PCR is a PCR variant aimed to

22 amplifying several DNA fragments of interest by using several pairs of primers in a single reaction.

23 Alvarado-Bremer et al. [63] developed a multiplex PCR for the identification of four commercially-

24 relevant shrimp species from the Gulf of Mexico, namely *Far. aztecus, Far. duorarum,*

25 *Farfantepenaeus brasiliensis*, and *L. setiferus*, and *L. vannamei* from aquaculture origin, using

1   species-specific PCR oligonucleotide primers for each of these species. In a recent study [64], specific

2   primers were designed for the simultaneous identification of *P. monodon*, *L. vannamei* and *Fen.*

3   *indicus*, which represent more than 80% of the aquaculture shrimp production and may be fraudulently

4   replaced by species exhibiting lower value. These species-specific primers, which amplify

5   mitochondrial fragments of 362, 151 and 213 bp in the 16S rRNA gene for *P. monodon*, *L. vannamei*

6   and *Fen. indicus* respectively, allowed the direct identification of these in a food sample, avoiding the

7   potential complications of complex restriction patterns caused by the presence of more than one

8   shrimp species.

9

10   **5. Proteomics**

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12   Proteomics, defined as the large-scale analysis of proteins [82], is aimed at the investigation of the

13   proteome based on MS techniques, such as matrix assisted laser desorption/ionization-time of flight

14   mass spectrometry (MALDI-TOF MS) and electrospray-ion trap mass spectrometry (ESI-IT MS),

15   coupled with the bioinformatics treatment of the data obtained. The combination of different

16   proteomics tools, taking advantage of the high-throughput capacity of MS-based techniques,

17   represents a robust and powerful strategy for protein and peptide identification and characterization.

18   Proteomics techniques have some advantages over other methods: (i) they may be automated,

19   producing fast, reproducible, and sensitive results and allowing high-throughput analysis of foodstuffs;

20   (ii) they can also be applied to species that are poorly characterized at the genomic and proteomic

21   levels in the databases, avoiding the time-consuming steps of DNA amplification and sequencing; and

22   (iii) they open the way to the identification and characterization of species-specific peptides, this

23   representing the first step toward designing fast and cheap detection analysis, such as antibody-based

24   assays or MS detection methods.

1   Proteomics methodologies have been used for the identification of some seafood species such

2   as mussels [29] and hakes [30, 83], but their application for the authentication of decapods has been

3   scarce. Figure 2 shows the different proteomics approaches that are currently under use at our

4   laboratory for the isolation, characterization and detection of species-specific peptides from shrimps

5   with food authentication purposes. Two dimensional electrophoresis (2-DE) has been considered for

6   the separation of proteins in muscle extracts. Potentially species-specific proteins according to 2-DE

7   analysis, are selected and digested with trypsine in order to investigate the amino acidic sequences by

8   means of MS. In this sense, MALDI-TOF MS has been successfully applied for the differentiation of

9   several shrimp species. In a preliminary study, Peptide Mass Fingerprinting (PMF), one of the main

10   applications of MALDI-TOF MS, was successfully applied to the differentiation of Decapoda species,

11   as was ascertained by analyzing two very closely-related species: *P. monodon* and *Fen. indicus* [52].

12   Fingerprints of the sarcoplasmic protein Arginine Kinase (AK) exhibited two peaks that resulted to be

13   species-specific of *P. monodon*, and which could be useful as biomarkers for this commercially-

14   relevant species. In a subsequent study [53], six shrimp species of commercial relevance, namely the

15   giant tiger prawn (*P. monodon*), the Pacific white shrimp (*L. vannamei*), the Indian white prawn (*Fen.*

16   *indicus*), the Southern pink shrimp (*Far. notialis*), the Argentine red shrimp (*Pl. muelleri*), and the

17   Northern shrimp (*Pan. borealis*), were successfully differentiated by a proteomic approach combining

18   2-DE, tryptic digestion and PMF of AK. In such work, a mathematical method for the comparison of

19   the PMF spectra was reported, which could be applied to infer taxonomic relationships between

20   specimens. This method consisted on a similarity coefficient between the different PMF peak lists, in

21   order to generate a distance matrix that could be represented as a dendrogram. The use of this

22   mathematical approach instead of visual spectral comparison provides a quantitative measure of how

23   well two species match. Although a high homology was found among the AK PMF spectra from the

24   different shrimp species, species-specific profiles useful for shrimp differentiation were found,

25   providing a selective differentiation method among families Penaeidae, Solenoceridae and Pandalidae,

1   and an unequivocal identification of the shrimp species. Due to the interspecific variability of AK and

2   to its high concentration in shrimp muscle, this enzyme has been proposed as a a biomarker for shrimp

3   species identification [47]. In addition, the phyloproteomic dendrogram generated was consistent with

4   the phylogenies established with mtDNA data [53], thus opening the way to further studies on

5   phyloproteomics. Further identification and characterization of the differential peptides from the AK

6   in seven commercial shrimp species (the previous six shrimp species and *Fen. merguiensis*) was

7   performed by ESI-IT MS/MS, followed by database searching and *de novo* sequence interpretation

8   [47]. Several species-specific peptides were reported, together with the spectra of their Collision

9   Induced Dissociation fragmentation. In addition, in a subsequent work [54], additional *Pan. borealis*

10 specific AK peptides were identified and characterized. Such works allowed the identifiation of

11 diagnostic peptides that could be useful to design fast and cheap analysis kits for the sensitive

12 detection of these commercially-relevant prawn species. The selection of AK is particularly interesting

13 because it is an allergen [84], and therefore an analysis targeting this protein would have a double

14 application, both for species identification and food safety purposes. Thus, this would allow the

15 detection of shrimp allergens in food products and help the manufacturer and the administration to

16 identify, in real time, the existence of a contamination in a production line, and compliance with food

17 labelling regulations, even allowing the allergen quantification [85].

18   In a recent work [55], a shotgun proteomics approach was applied to the detection of some of

19 the previously characterized AK species-specific peptides [47]. Although 2-DE has been the

20 workhorse for the majority of proteome projects in the past [86], when peptidic biomarkers have been

21 already characterized it is much faster to directly search for the specific known peptides in the protein

22 extract, avoiding the laborious and time-consuming 2-DE isolation step. When dealing with complex

23 samples, Multiple Reaction Monitoring (MRM), or its variant when working with an ion-trap, Selected

24 MS/MS Ion Monitoring (SMIM), is the most suitable screening method for the detection and

25 quantification of peptides previously sequenced by MS [87]. In MRM, a precursor ion of a particular

1 mass-to-charge ratio (m/z) is selected in the first stage of MS and several product ions are selected in

2 the second stage [88]. An innovative methodology combining the speed of high-intensity focused

3 ultrasound-assisted tryptic digestion of proteins, the high separation capability of reverse phase HPLC,

4 and the peptide detection ability of MS working in the MRM-SMIM scanning mode, made possible

5 the differentiation of the seven shrimp and prawn species considered in no longer than 90 minutes,

6 from the arrival of the sample to the identification of the species [55]. Alternatively, an off-line

7 analysis of the unseparated tryptic digests also achieved the discrimination of closely-related species

8 studied in an even shorter analysis time [55], although this methodology is less automatized. To the

9 best of our knowledge, these two methodologies are the fastest molecular methods described to date

10 for the authentication of animal species in foods.

11

12 **6. Final considerations and future trends**

13

14 There is a need for developing molecular identification methods that may provide the administration

15 and the seafood industry with the tools necessary to comply with labelling and traceability at species

16 level. Although conventional identification of species has been based on phenotypic traits, genotype-

17 based classification is more accurate, especially in those specimens with less identifiable traits and

18 more easily influenced by environment. Although IEF is the most commonly described method for

19 species identification, genomic analysis is currently considered to be the best approach due to its

20 simplicity and good reproducibility. Major challenges facing research on shrimp authentication still

21 are: (i) the optimization of simple, fast and inexpensive methodologies for routine use; (ii) the analysis

22 of highly processed or complex food matrices, this including the development of methods targeted to

23 smaller fragments, which could be detected both in raw and processed products; (iii) multiple species

24 identification in a food samples; and (iv) quantification of different species in mixed food samples

25 [18]. Omics technologies are already helping to meet these challenges, and several methodologies are

1   currently under development, such as methods based on multiplex PCR, with specific primers targeted

2   to several species and allowing the simultaneous detection of multiple species, real-time PCR, PCR-

3   RFLP, which has been found to detect a specific species in complex matrices and mixtures with a

4   detection limit of 0.1% (wt/wt) [89], microarrays, IEF of native proteins, and MS detection of species-

5   specific peptides previously characterized by means of proteomics tools, which has been found to

6   detect 0.5% contaminant meat [90]. The great advance of proteomics approaches is that the

7   identification and characterization of new specific peptides is a first step toward the design of fast and

8   easy-to-use detection analyses. The characterized species-specific peptides can then be used to prepare

9   antibody-based kits for the fast and sensitive detection of the different species in a sample.

10   Alternatively, these peptides could be used in MRM MS assays. The potential ability of proteomics

11   approaches for species identification is remarkable, allowing a fast, cheap, high throughput, high

12   accuracy and sensitive screening. In this sense, the characterization and validation of species-specific

13   peptides could allow their use as biomarkers for shrimps species identification, being monitored by

14   MRM MS or by ready-to-use antibody-based kits for the sensitive detection of the species, allowing

15   multiple species identification, detection and even quantification in complex food matrices.

16

17

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25   **Conflict of interest statement**

1   All authors declare there are no financial/commercial conflicts of interest.

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4   **References**

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1 **Figure legends**

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3 **Figure 1.** Topologies resulting from the phylogenetic analysis of the nucleotide sequences of 515–535

4 bp 16S rRNA/tRNA^{Val} mitochondrial genes in 21 decapod shrimp and prawn species, by means of the

5 neighbor-joining method. Numbers above and below branches indicate bootstrap values from

6 neighbor-joining analysis.

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8 **Figure 2.** Proteomics approaches considered for the identification, characterization and detection of

9 species-specific diagnostic peptides from shrimp with food authentication purposes.

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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	[41]
		Far. duorarum, L. setiferus, Sicyonia brevirostris	Sarcoplasmic proteins	[42]
	IEF	Shrimp, fish and lobster	Calcium-binding proteins	[43]
		Shrimp, fish and cephalopod	Non-sarcoplasmic proteins	[43]
		Far. duorarum, L. setiferus, Sicyonia brevirostris	Sarcoplasmic proteins	[44, 45]
		14 shrimp and prawn species	SCPs	[46]
	2-DE	7 shrimp and prawn species	AK	[47]
Immunological	ELISA	Crustaceans	Tropomyosin	[48]
		Crustaceans	Tropomyosin	[49]
		Sicyonia. brevirostris	Protein M	[50]
	Immunodot	Crustaceans	AK	[51]
		Sicyonia brevirostris	Protein M	[50]
	Westernblot	*Sicyonia brevirostris*	Protein M	[50]
MS	MALDI-TOF PMF	*P. monodon, Fen. indicus*	AK	[52]
		6 shrimp and prawn species	AK	[53]
		Pan. borealis	AK	[54]
	MS/MS	7 shrimp and prawn species	AK	[47]
		Pan. borealis	AK	[54]
	MRM	7 shrimp and prawn species	AK peptides	[55]

a) Genera abbreviations: *Far.*, *Farfantepenaeus*; *L.*, *Litopenaeus*; *P.*, *Penaeus*; *Fen.*, *Fenneropenaeus*; *Pan.*, *Pandalus*.

b) Target abbreviations: AK, arginine kinase; MALDI-TOF PMF, matrix assisted laser desorption/ionization-time of flight peptide mass fingerprinting; MRM, multiple reaction monitoring; SCPs, sarcoplasmic calcium-binding proteins.

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*	[63]
		P. monodon, L. vannamei and *Fen. indicus*	[64]
sequencing	16S rRNA	*Far. notialis, L.. schmitti*	[24]
sequencing + phylogenetic analysis (FINS)	mtDNA Control Region	*Far. aztecus, L. setiferus*	[65]
	COI	*Fen. merguiensis, Fen. silasi, Fen. indicus*	[66]
	16S rRNA//tRNA^{Val}/12S rRNA	*Far. californiensis, L. vannamei* and *L. stylirostris*	[67]
	16S rRNA/tRNA^{Val}	19 penaeid species	[68]
		17 penaeid species from commercial origin	[69]
	16S rRNA/tRNA^{Val}, 16S rRNA, COI	19 penaeid species	[70]
	cytb	*P. monodon, L. vannamei, Far. notialis, Par. longirostris, Pl. muelleri, Fen. merguiensis*	[71]
	ITS-1	*Mar. japonicus, Mel. latisulcatus, L. vannamei, Fen. merguiensis, Fen. indicus*	[72]
RFLP	ITS-1	*L. vannamei, Metapenaeopsis dalei, Solenocera crassicornis Procambarus clarkii*	[72]
	16S rRNA/tRNA^{Val}	19 penaeid species	[68]
		21 penaeid species	[73]
		17 penaeid species from commercial origin	[69]
		Pan. borealis	[54]
	cytb	*P. monodon, L. vannamei, Far. notialis, Par. longirostris, Pl. muelleri, Fen. merguiensis*	[71]
		P. semisulcatus, P. kerathurus, Par. longirostris, Met. monoceros	[74]
	16S rRNA	shrimp, crab, lobster and crawfish	[75]
	COI-COII, 16S rDNA	*P. monodon, P. semisulcatus, L. vannamei, Fen. merguiensis* and *Mar. japonicus*	[76]
SSCP	16S rDNA	*P. monodon, P. semisulcatus, Fen. merguiensis, L. vannamei* and *Mar. japonicus*	[76]
RAPD	—	*Mar. japonicus, Met. ensis*	[77]
	—	*Fen. chinensis, Pal. gravieri*	[78]
microsatellites or SSRs	ESTs	*L. vannamei, L. stylirostris, Tra. birdy*	[79]

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*.
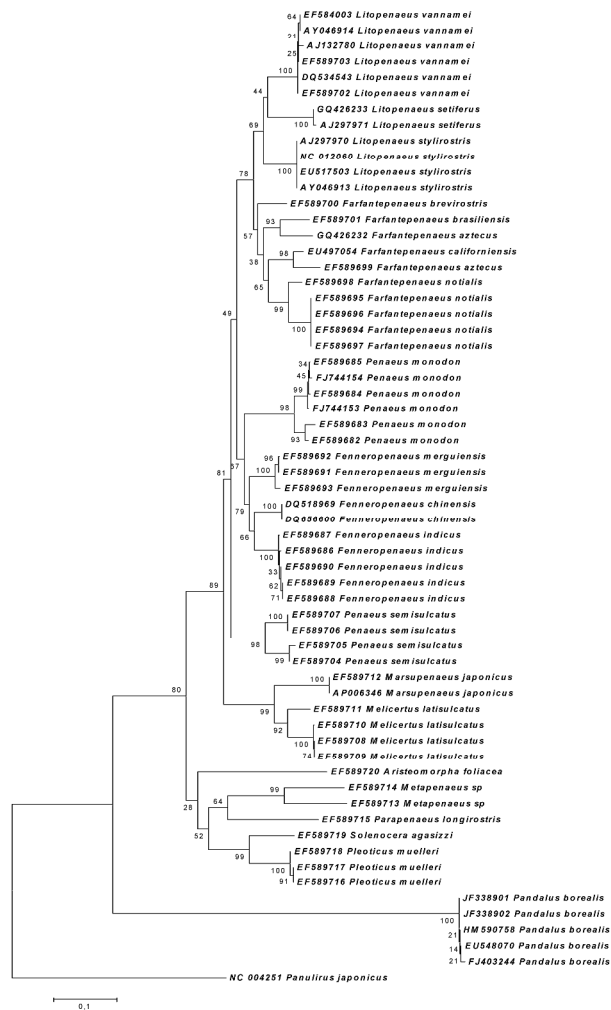
Figure 1. Topologies resulting from the phylogenetic analysis of the nucleotide sequences of 515–535 bp 16S rRNA/tRNAVal mitochondrial genes in 21 decapod shrimp and prawn species, by means of the neighbor-joining method. Numbers above and below branches indicate bootstrap values from neighbor-joining analysis.
1749x2474mm (72 x 72 DPI)

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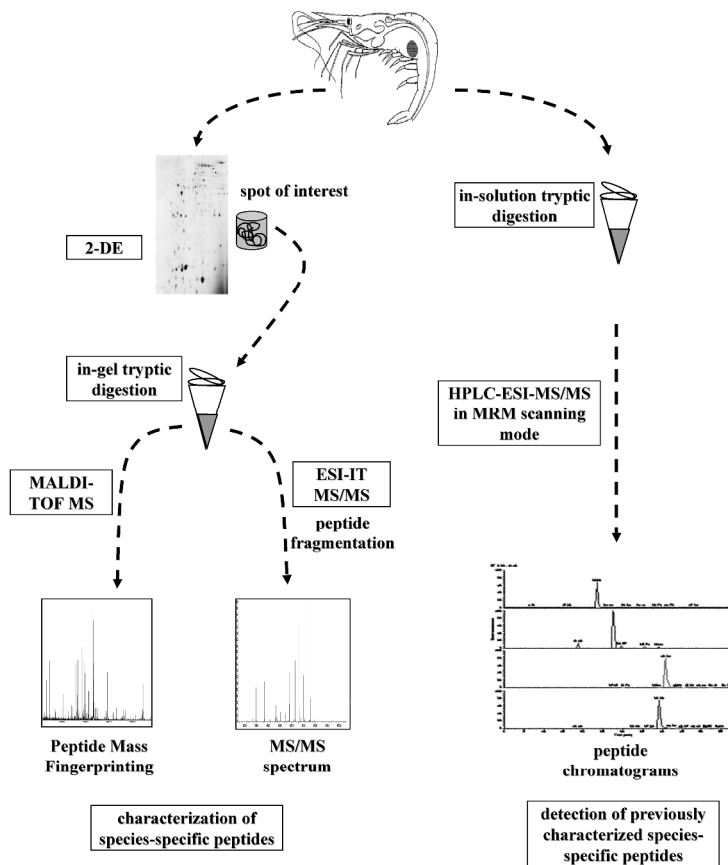51
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Figure 2. Proteomics approaches considered for the identification, characterization and detection of species-specific diagnostic peptides from shrimp with food authentication purposes.
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