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Analytical Methods

Survey of the authenticity of prawn and shrimp species in commercial food products by PCR-RFLP analysis of a 16S rRNA/tRNA^{Val} mitochondrial region ☆

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Abstract

A novel PCR-RFLP method was evaluated as a tool to assess the incidence of incorrect labelling of prawns and shrimps in commercial food products. The whole method can be performed in less than 8 h in only one day of work. PCR amplification with primers 16Scru4/16Scru3, targeted to the amplification of a *ca.* 530 bp region of 16S rRNA and tRNA^{Val} mitochondrial genes, was coupled to restriction analysis with *Alu*I, *Taq*I or *Hin*fI. Forty-one commercial food products were considered. The molecular method considered allowed the identification of up to 17 different prawn and shrimp species in all the processed products considered. Seven (28%) of the 25 food products declaring one or more species in their labels were incorrectly labelled. Authentication was successfully assessed in commercial peeled products subjected to industrial processing, in which none of the products displayed labelling at species level. Overall, incorrect labelling was detected in 10 (24.4%) of the 41 commercial products tested, while another 16 samples (39%) exhibited incomplete labelling. The molecular method evaluated in this study proved to be a rapid and easy-to-perform two-step analytical approach to achieve species identification of commercial whole specimens of frozen prawns and shrimps and in peeled processed products where such raw materials are included as added-value ingredients.

Keywords: Food authenticity; Species identification; PCR-RFLP; mtDNA; Decapoda crustaceans; Penaeid shrimps; Prawns

1. Introduction

Food authenticity is an issue of major concern for food authorities, since incorrect food labelling represents commercial fraud to the consumers, in particular when this implies the replacement of one ingredient by another of lower commercial value (Lockley & Bardsley, 2000). Incorrect labelling may also have negative sanitary implications – such as allergy or toxic syndromes – derived from the inadvertent introduction of any food ingredient that might be harmful to human health (Mermelstein, 1993; Patterson & Jones, 2000; Sotelo, Piñeiro, Gallardo, & Pérez-Martín, 1993).

The fish products sector is characterized by the high commercial value of many of its products, this being specially relevant in the case of prawn and penaeid shrimp species, both when they are sold as whole specimens and when they are included as high-value ingredients in pre-cooked food products. Prawns and penaeid shrimps are Decapoda crustaceans belonging to the superfamily *Penaeoidae,* and

☆ The PCR-RFLP protocol evaluated in the present study for prawn and penaeid shrimp species identification is freely available for research purposes but protected for industrial exploitation by Spanish Patent No.

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they are important resources from both commercial fisheries and for aquaculture in many countries, accounting for more than 30% of the global consumption of this type of crustacean worldwide (Pérez-Farfante & Kensley, 1997; Rosenberry, 2001). Among prawn and shrimp species, morphological characters are particularly difficult to use for species differentiation due to their phenotypic similarities and to the fact that their industrial processing often removes their external carapace (Vondruska, Otwell, & Martin, 1988).

To overcome these problems, molecular methods based on protein and DNA analysis have been developed. Thus, electrophoretic and immunological methods have been proposed for the detection and differentiation of Decapoda crustaceans (An, Marshall, Otwell, & Wei, 1989; Shanti, Martin, Nagpal, Metcalfe, & Subba Rao, 1993). However, such methods are laborious, time-consuming, and their application may be hampered by the lack of stability of the polypeptide targets as a result of industrial processing (Piñeiro, Vázquez, Figueras, Barros-Velázquez, & Gallardo, 2003; Piñeiro et al., 1999). Such limitations have been solved with the introduction of methods based on DNA amplification and DNA hybridization (Gutiérrez-Millan, Peregrino-Uriarte, Sotelo-Mundo, Vargas-Albores, & Yépiz-Plasencia, 2002; Khamnamtong, Klinbunga, & Menasveta, 2005; Klinbunga et al., 2001; Lavery, Chan, Tam, & Chu, 2004; Quan, Zhuang, Deng, Dai, & Zhang, 2004). Among the DNA targets, mitochondrial DNA (mtDNA) has been used in PCR-based studies, the 16S rRNA gene and, to a lesser degree, the cytochrome oxidase I (COI) gene, having been reported as good molecular markers for some crustacean species in phylogenetic studies (Baldwin, Bass, Bowen, & Clark, 1998; Bellis, Ashton, Freney, Blair, & Griffiths, 2003; Brzezinski, 2005; Bucklin, Frost, & Köcher, 1992; Maggioni, Rogers, Maclean, & D'Incao, 2001).

Nonetheless, a limited number of reports have focused their interest on the development of PCR methodologies for the identification of prawn and shrimp species in foodstuffs. Although only focused on three Eastern Pacific species, a previous study described the usefulness of a 1.38 kb mitochondrial region that comprised fragments of the 16S rRNA and 12S rRNA genes and the entire $tRNA^{Val}$ region for phylogenetic analysis of penaeid shrimp species (Gutiérrez-Millan et al., 2002). These species were: *Farfantepenaeus californiensis*, *Litopenaeus vannamei* and *Litopenaeus stylirostris*. Another previous study provided a molecular method – targeted to COI, cytochrome oxidase II (COII) and 16S rRNA mitochondrial genes – for the identification of species using restriction analysis of a 312 bp fragment (Khamnamtong et al., 2005). Likewise, that study considered the identification of only five species: *Penaeus monodon*, *Penaeus semisulcatus*, *L. vannamei*, *Fenneropenaeus merguiensis* and *Marsupenaeus japonicus*. More recently, a method for the detection of crustacean DNA based on a PCR-RFLP approach has been proposed (Brzezinski, 2005). The method, aimed at the detection of potentially allergenic proteins, did not allow prawn and shrimp species identification, although it did permit their generic detection and differentiation with respect to crab, lobster and crawfish species (Brzezinski, 2005).

Accordingly, the main goal of the present work was to evaluate the usefulness of a novel PCR-RFLP method based on the generic primers 16ScruC4/16ScruC3, targeted to a 16S rRNA/$tRNA^{Val}$ 530 bp mitochondrial region, for the identification of a broad number of prawn and crustacean species in commercial food products subjected to different technological processes.

2. Materials and methods

2.1. Commercial food products

Forty-one commercial food products containing or consisting of prawns or shrimps were considered. The products were purchased from supermarkets in Northwestern Spain or directly from Spanish companies involved in the commercialization of aquatic food products. Thirty-two samples concerned frozen penaeid shrimps while the remaining nine products were more processed commercial products containing prawns or shrimps as food ingredients. Reference samples consisting of nearly twenty penaeid species were used for purposes of comparison (Table 1).

2.2. DNA extraction and purification

Samples from the prawns and shrimps were scraped from the food products with sterile surgical blades. Representative portions of 0.2 g of each sample were placed in sterile 2 ml tubes and subjected to DNA extraction. A commercial DNA extraction kit (DNeasy tissue minikit, QIAGEN, Valencia, CA, USA) based on the use of purification micro-columns was used. The concentration of the purified DNA extracts was determined by measuring the fluorescence developed after mixing with Hoechst 33258 reagent (Sigma, St. Louis, MO, USA) in a LS 50 fluorimeter (Perkin Elmer, Wellesley, MA, USA).

2.3. Evaluation of a PCR-RFLP method for prawn and shrimp species identification

Primers 16ScruC4 (5́-AATATGGCTGTTTTTAAGCCTAATTCA-3') and 16ScruC3 (5́-CGTTGAGAAGTTCGTTGTGCA-3́), constructed in two well-conserved regions of the 16S rRNA/$tRNA^{Val}$ mitochondrial genes of prawns and penaeid shrimp species, were evaluated. The GenBank accession numbers of the penaeid species considered for the development of such primers are detailed in Table 1. Such primers allowed the amplification of a *ca.* 530 bp fragment of the 16S rRNA/$tRNA^{Val}$ mtDNA genes in the case of prawns and penaeid shrimps (Fig. 1A). The small size of this molecular target facilitates amplification from fresh, frozen or pre-cooked samples,

Table 1
Reference penaeid shrimp species considered for the development of the 16ScruC4/16ScruC3 primers

Scientific name[a]	Commercial name	Origin	Accession numbers
L. vannamei	Pacific white shrimp	Eastern Pacific	EF589702/EF589703 AJ132780/AY046914
L. stylirostris	Blue shrimp	Eastern Pacific	AY046913/AJ297970
L. setiferus	White shrimp	Western Atlantic	AJ297971
Farf. notialis	Southern pink shrimp	Western Atlantic	EF589698/ X84350
Farf. notialis	Southern pink shrimp	Eastern Atlantic	EF589694/EF589695 EF589696/EF589697
Farf. brasiliensis	Red spotted shrimp	Western Atlantic	EF589701
Farf. brevirostris	Crystal shrimp	Eastern Pacific	EF589700
Farf. aztecus	Brown shrimp	Western Atlantic	EF589699
Farf. californiensis	Brown shrimp	Eastern Pacific	AY046912
Fen. indicus	Indian white prawn	Indo-West Pacific	EF589688/EF589689 EF589690
Fen. indicus	Indian white prawn	Western Indian	EF589686/EF589687
Fen. merguiensis	Banana prawn	West Central Pacific	EF589691/EF589693
Fen. merguiensis	Banana prawn	Indo West Pacific	EF589692
P. monodon	Giant tiger prawn	Indo West Pacific	EF589682/EF589683 EF589684/EF589685 NC002184
P. semisulcatus	Green tiger prawn	Indo West Pacific	EF589706/EF589707
P. semisulcatus	Green tiger prawn	Western Indian	EF589704/EF589705
Metapenaeus sp.	Penaeid prawn	Western Indian	EF589713/EF589714
Par. longirostris	Deepwater rose shrimp	Eastern Atlantic	EF589715
Mars. japonicus	Kuruma prawn	South West Pacific	EF589712/NC007010
Mel. latisulcatus	Western king prawn	Indo West Pacific	EF589708/EF589709 EF589710/EF589711
Sol. agassizii	Kolibri shrimp	Eastern Pacific	EF589719
Pl. muelleri	Argentine red shrimp	South West Atlantic	EF589716/EF589717 EF589718
Ars. foliacea	Giant red shrimp	Eastern Atlantic	EF589720

[a] Genera abbreviations: *L.*: *Litopenaeus*; *Farf.*: *Farfantepenaeus*; *Fen.*: *Fenneropenaeus*; *P.*: *Penaeus*; *Par.*: *Parapenaeus*; *Mars.*: *Marsupenaeus*; *Mel.*: *Melicertus*; *Sol.*: *Solenocera*; *Pl.*: *Pleoticus*; *Ars.*: *Aristeomorpha*.

where DNA fragmentation may be relevant and fragment size critical.

Amplification assays used 100 ng of template DNA, 25 μl of a master mix (BioMix, Bioline Ltd., London, UK) – this including reaction buffer, dNTPs, $MgCl_2$ and *Taq* DNA polymerase, – PCR water (Genaxis, Montigny le Bretonneaux, France) and 25 pmol of each primer, to achieve a final volume of 50 μl. Amplification conditions were as follows: a previous denaturing step at 94 °C for 1 min 30 s was coupled to 35 cycles of denaturation (94 °C for 20 s), annealing (temperature gradient: 51–55 °C for 20 s), and extension (72 °C for 30 s), and with a final extension at 72 °C for 15 min. All PCR assays were performed on a MyCycler thermocycler (BioRad, Hercules, CA, USA).

The restriction profiles of the PCR products were investigated using the endonucleases *Alu*I, *Taq*I and *Hin*fI, all of them from Sigma. Restriction assays were carried out for 2 h at 37 °C or 65 °C in a final volume of 20 μl. Species identification was achieved by comparing the number and sizes of the restriction fragments obtained with the commercial products with respect to the restriction patterns of the reference specimens compiled in Table 2.

2.4. Electrophoresis and image analysis

PCR products were processed in 2.5% horizontal agarose (MS-8, Pronadisa, Madrid, Spain) electrophoresis. PCR-RFLP analyses were carried out with agarose electrophoresis in 2.5% gels and with SDS–PAGE in 15% ExcelGel homogeneous gels (GE Healthcare) at 15 °C in a Multiphor II electrophoresis unit (Amersham Biosciences, Uppsala, Sweden). The latter gels were stained using a standard silver staining protocol (Amersham Biosciences). When required, PCR products were purified from the agarose gels by means of the MinElute Gel Extraction kit (QIAGEN). Image analysis was carried out by means of the 1-D Manager software (TDI, Madrid, Spain). DNA sequencing was performed as described below.

2.5. DNA sequencing and genetic analysis

Prior to sequencing, the PCR products were purified by means of the ExoSAP-IT kit (GE Healthcare, Uppsala, Sweden). Direct sequencing was performed with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The same primers used for PCR were used for the sequencing of both strands of the PCR products, respectively. Sequencing reactions were analyzed in an automatic sequencing system (ABI 3730XL DNA Analyser, Applied Biosystems) provided with the POP-7 system. SNP events in DNA sequences were reviewed with the Chromas software. Alignment of sequences was accomplished using the CLUSTALW software (Thompson, Higgins, & Gibson, 1994). The homologies of the nucleotide sequences were searched with the BLAST tool (NCBI). Phylogenetic and molecular evolutionary analyses were conducted with the MEGA software (Kumar, Tamura, Jakobsen, & Nei, 2001) using the neighbour-joining

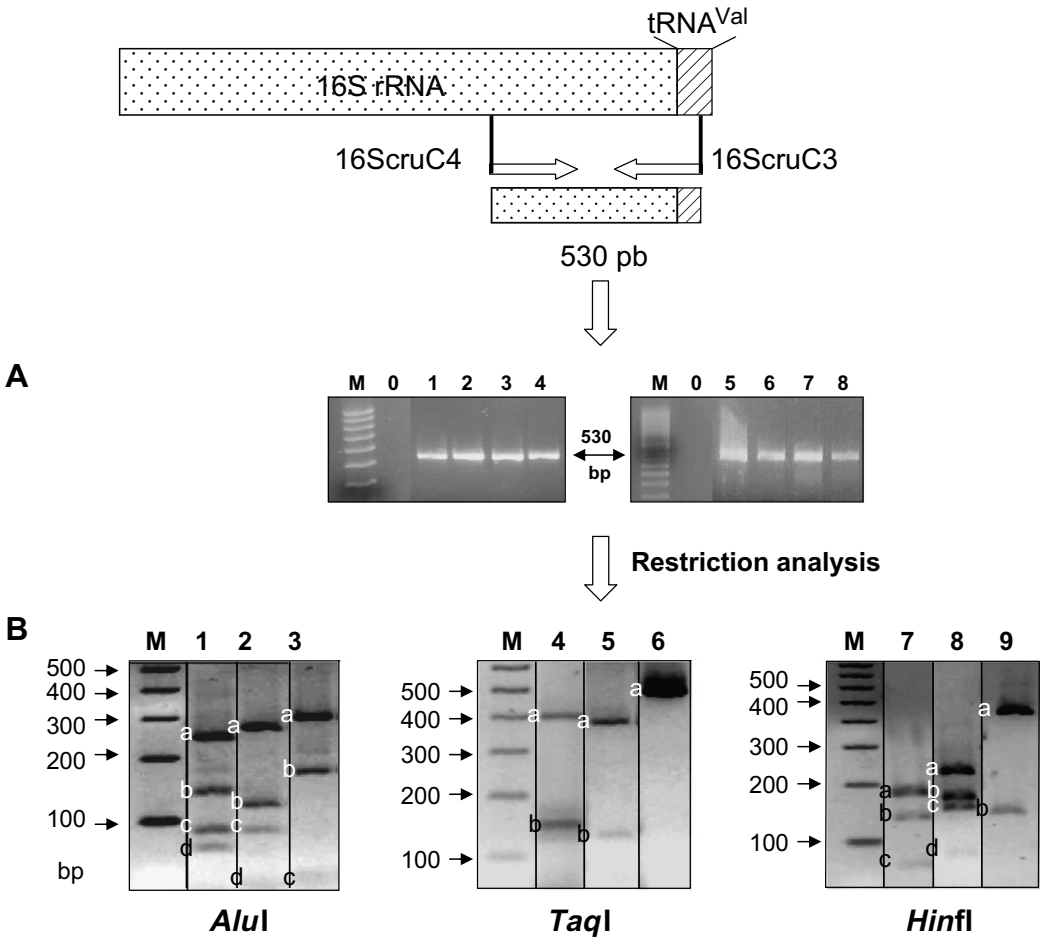

Fig. 1. (A) PCR-amplification of the *ca.* 530 bp mitochondrial fragment considered in the RFLP study in commercial frozen products (lanes 1–4) and in processed peeled products (lanes 5–8); i.e. lanes M: molecular weight marker; lanes 0: negative control; lane 1: sample 2; lane 2: sample 6; lane 3: sample 1; lane 4: sample 10; lane 5: sample 14P; lane 6: sample 15P; lane 7: sample 16P; lane 8: sample 17P. Sample codes are as referred in Tables 3 and 4; (B) PCR-RFLP analysis of the 530 bp fragment with endonuclease *Alu*I, *Taq*I and *Hin*fI; i.e. lanes M: molecular weight marker; lane 1: sample 7 (*L. vannamei*) cleaved with *Alu*I: restriction fragments of 230(a), 130(b), 77(c) and 62(d) bp; lane 2: sample 24 (*Farf. notialis*) cleaved with *Alu*I: restriction fragments of 256(a), 110(b), 77(c) and 34/31(d) bp; lane 3: sample 30 (*Mel. latisulcatus*) cleaved with *Alu*I: restriction fragments of 291(a), 165(b) and 38/35(c) bp; lane 4: sample 26 (*Farf. brevirostris*) cleaved with *Taq*I: restriction fragments of 401(a) and 130(b) bp; lane 5: sample 12 (*Fen. indicus*) cleaved with *Taq*I: restriction fragments of 397(a) and 127(b) bp; lane 6: sample 2 (*Mel. latisulcatus*) cleaved with *Taq*I: undigested 526(a) bp amplicon; lane 7: sample 2 (*Mel. latisulcatus*) cleaved with *Hin*fI: restriction fragments of 172/169(a), 126(b) and 62(c) bp; lane 8: sample 19 (*Fen. merguiensis*) cleaved with *Hin*fI: restriction fragments of 202(a), 144(b), 125(c) and 77(d) bp; lane 9: sample 37 (*Farf. brasiliensis*) cleaved with *Hin*fI: restriction fragments of 403(a) and 129(b) bp. Restriction fragments lower than 31 bp are not visualized.

method (Saitou & Nei, 1987) with 1000 bootstrap replicates to construct distance-based trees.

3. Results and discussion

3.1. Recovery and amplification of prawn and shrimp mtDNA from commercial food products

A total of 41 different commercial food products containing or consisting of prawns or shrimps were subjected to DNA extraction and purification. DNA amplification was successful in all samples analyzed. Accordingly, the size of the molecular target – *ca.* 530 bp – proved to be accurate to achieve PCR amplification even in processed foods that included prawns and penaeid shrimps as ingredients and that had been subjected to a variety of technological treatments such as peeling, frying or freezing (Fig. 1A).

Although DNA exhibits fairly high thermal stability, it is well known that intense heat coupled with overpressure conditions may cause severe DNA degradation (Bellagamba, Moretti, Comincini, & Valfrè, 2001; Borgo, Souty-Grosset, Bouchon, & Gomot, 1996; Fairbrother, Hopwood, Lockley, & Bardsley, 1998; Partis et al., 2000; Poser, Detsch, Müller, Fischer, & Schwägele, 2003; Wolf & Luthy, 2001). A direct relationship between heat treatment and the intensity of DNA fragmentation has also been reported, such degradation affecting the quality of DNA (Freeza et al., 2003; Lockley & Bardsley, 2000). Even more than the processing conditions, the presence of additives that may inhibit DNA polymerase has also been reported as an important cause for the lack of amplification.

Table 2
RFLP specific patterns (bp) for the penaeid shrimp species considered as references in this study

Scientific name	Restriction type[a]	*Alu*I	*Taq*I	*Hin*fI	Seq. size (bp)
L. vannamei	PNV1	230 + 130 + 77 + 62 + 31	400 + 130	403 + 127	530
L. vannamei	PNV2	230 + 161 + 77 + 62	400 + 130	403 + 127	530
L. stylirostris	PNS	292 + 131 + 77 + 31	229 + 172 + 130	404 + 127	531
L. setiferus	PST	271 + 151 + 77 + 31	230 + 169 + 131	282 + 128 + 120	530
Farf. notialis	SOP1	256 + 110 + 77 + 34 + 31 +	171 + 171 + 129 + 57	402 + 126	528
Farf. notialis	SOP2	294 + 110 + 77 + 51	174 + 171 + 130 + 57	405 + 127	532
Farf. notialis	SOP3	251 + 130 + 77 + 34 + 31	171 + 170 + 105 + 58 + 19	402 + 121	523
Farf. brasiliensis	PNB	294 + 161 + 71 + 6	278 + 132 + 122	403 + 129	532
Farf. brevirostris	CSP	293 + 161 + 77	401 + 130	404 + 127	531
Farf. aztecus	ABS	290 + 161 + 77	276 + 130 + 122	401 + 127	528
Farf. californiensis	YPS	292 + 161 + 77	229 + 171 + 130	403 + 127	530
Fen. indicus	PNI	287 + 163 + 74	397 + 127	400 + 80 + 44	524
Fen. merguiensis	PBA1	255 + 131 + 78 + 33 + 31	227 + 173 + 128	202 + 144	528
Fen. merguiensis	PBA2	255 + 131 + 78 + 33 + 31	400 + 128	202 + 144 + 125 + 77	528
P. monodon	MPN	289 + 131 + 70 + 31 + 6	399 + 128	402 + 125	527
P. semisulcatus	TIP	288 + 161 + 75	227 + 169 + 128	524	524
Metapenaeus sp.	PEN1	459 + 76	535	535	535
Metapenaeus sp.	PEN2	448 + 63 + 11+9	531	531	531
Par. longirostris	DPS	448 + 76	524	306 + 175 + 43	524
Mars. japonicus	KUP	288 + 122 + 75 + 42	451 + 76	357 + 170	527
Mel. latisulcatus	WKP1	291 + 165 + 38 + 35	400 + 129	172 + 169 + 126 + 62	529
Mel. latisulcatus	WKP2	288 + 164 + 68 + 6	526	269 + 169 + 88	526
Sol. agassizii	SOK	272 + 143 + 61 + 31 + 11 + 6	227 + 173 + 124	403 + 121	524
Pl. muelleri	LAA1	304 + 85 + 77 + 61	228 + 172 + 127	403 + 124	527
Pl. muelleri	LAA2	304 + 146 + 77	228 + 172 + 127	403 + 124	527
Ars. foliacea	ARS	441 + 74	347 + 168	397 + 118	515

[a] The three initial letters of the restriction types correspond to the FAO codes. Different numbers after the FAO code indicate more than one restriction pattern.

Remarkably, in our study, the PCR-RFLP protocol evaluated yielded good results when applied to commercial frozen prawns and shrimps, as well as to other more processed products including peeled prawns or shrimps together with other food additives.

3.2. Species identification of commercial frozen prawns and shrimps

Of the 41 different processed food products analyzed, thirty two were frozen prawns and shrimps marketed as whole specimens (Table 3). Of these 32 food products, 23 declared a single species in their labels while another two products declared two (sample 19) or three (sample 12) different species, respectively (Table 3). The remaining nine products declared no species in their labels. A selection of specific *Alu*I, *Taq*I and *Hin*fI restriction patterns is shown in Fig. 1B. Species identification by PCR-RFLP revealed that among the 23 food products that declared a single species five samples (9, 10, 15, 29 and 30), i.e., 21.7%, were not correctly labelled (Table 3). Two of these cases of mislabelling involved partial replacement of the declared species by a non-declared one. Specifically, the partial replacement of *Fen. indicus* by *Metapenaeus* sp. (sample 9), and of *Farf. aztecus* by *Farf. brasiliensis* (sample 10), respectively. Both Western Atlantic species – the red spotted shrimp *Farf. brasiliensis* (PNB) and the brown shrimp *Farf. aztecus* (ABS) – could be differentiated by an additional *Alu*I restriction site in PNB, not present in ABS (Table 2). Moreover, endonuclease *Dra*I also allowed the ready differentiation of *Farf. brasiliensis* from *Farf. aztecus*, since the 532 bp PCR product exhibits a sequence recognized by *Dra*I in the case of *Farf. brasiliensis* thus producing two restriction fragments of 428 bp and 104 bp, while in the case of *Farf. aztecus* there is no cleavage of the amplicon.

Other detected cases of incorrect labelling involved the complete substitution of *P. sculptius* by the green tiger prawn *P. semisulcatus* (sample 15); of *Mel. plebejus* by the banana prawn *Fen. merguiensis* (sample 29), and of *Fen. merguiensis* by the Western king prawn *Mel. latisulcatus* (sample 30), respectively.

Remarkably, sample 12 declared three different species – *Fen. indicus, Fen. merguiensis* and *P. monoceros* – on its label, but only the Indian white prawn *Fen. indicus* was detected in the food product. Likewise, sample 19 declared the presence of *Fen. indicus* and *Fen. merguiensis* on the label, but only the latter species was detected in the food product. *Fen. merguiensis*, a banana prawn species of low commercial value, was also easily distinguished from *Fen. indicus* with the restriction enzymes *Alu*I or *Hin*fI (Table 2).

In global terms, seven (28%) of the 25 commercial frozen crustaceans declaring one or more species in their labels were incorrectly labelled. While three products did not contain the declared species, two products declared one species but contained a mixture of two species, and the remaining

Table 3
Authenticity of prawn and shrimp species in commercial frozen products

Sample	Product type	Processing	Declared species	Detected species
1	Frozen shrimps	Freezing	*P. monodon*	*P. monodon*
2	Frozen shrimps	Freezing	*Mel. latisulcatus*	*Mel. latisulcatus*
3	Frozen shrimps	Freezing	Shrimps	*L. vannamei*
5	Frozen shrimps	Freezing	*P. monodon*	*P. monodon*
6	Frozen shrimps	Freezing	*Farf. notialis*	*Farf. notialis*
7	Frozen shrimps	Freezing	*L. vannamei*	*L. vannamei*
8	Frozen shrimps	Freezing	*Pl. muelleri*	*Pl. muelleri*
9	Frozen shrimps	Freezing	*Fen. indicus*	*Metapenaeus* sp. *Fen. indicus*
10	Frozen shrimps	Freezing	*Farf. aztecus*	*Farf. brasiliensis* *Farf. aztecus*
11	Frozen shrimps	Freezing	*Par. longirostris*	*Par. longirostris*
12	Frozen shrimps	Freezing	*Fen. indicus* *Fen. merguiensis* *P. monoceros*	*Fen. indicus*
13	Frozen shrimps	Freezing	*P. semisulcatus*	*P. semisulcatus*
14	Frozen shrimps	Freezing	*L. vannamei*	*L. vannamei*
15	Frozen shrimps	Freezing	*P. sculptius*	*P. semisulcatus*
16	Frozen shrimps	Freezing	*Pl. muelleri*	*Pl. muelleri*
17	Frozen shrimps	Freezing	*Penaeus* sp.	*L. vannamei*
18	Frozen shrimps	Freezing	*Solenocera* sp.	*Solenocera* sp. *Pl. muelleri*
19	Frozen shrimps	Freezing	*Fen. indicus* *Fen. merguiensis*	*Fen. merguiensis*
20	Frozen prawns	Freezing	*Parapenaeopsis* sp.	*Par. longirostris* *Metapenaeus* sp.
21	Frozen shrimps	Freezing	*Metapenaeus* sp.	*Metapenaeus* sp.
22	Frozen shrimps	Freezing	*Penaeus* sp.	*L. vannamei*
23	Frozen shrimps	Freezing	*P. monodon*	*P. monodon*
24	Frozen shrimps	Freezing	*Farf. notialis*	*Farf. notialis*
25	Frozen shrimps	Freezing	*Penaeus* sp.	*Farf. notialis*
26	Frozen shrimps	Freezing	*Farf. brevirostris*	*Farf. brevirostris*
28	Frozen shrimps	Freezing	*Mars. japonicus*	*Mars. japonicus*
29	Frozen shrimps	Freezing	*Mel. plebejus*	*Fen. merguiensis*
30	Frozen shrimps	Freezing	*Fen. merguiensis*	*Mel. latisulcatus*
31	Frozen shrimps	Freezing	*Farf. brevirostris*	*Farf. brevirostris*
32	Frozen shrimps	Freezing	*Sol. agassizii*	*Sol. agassizii*
37	Frozen shrimps	Freezing	*Farf. brasiliensis*	*Farf. brasiliensis*
38	Frozen shrimps	Freezing	*Ars. foliacea*	*Ars. foliacea*

two products declared two and three species, respectively, but only contained a single species.

Another seven commercial frozen prawn and penaeid shrimp products not displaying labelling at the species level were investigated. Such food products declared the presence of "shrimps" (sample 3), "*Penaeus* sp." (samples 17, 22 and 25), "*Metapenaeus* sp." (sample 21), "*Solenocera* sp." (sample 18) or "*Parapenaeopsis* sp." (sample 20) (Table 3). Sample 3 contained *L. vannamei*, while the remaining samples contained species belonging to the genera indicated on the labels, except for sample 20, which did not contain *Parapenaeopsis* sp. but a mixture of two penaeid shrimps: *Par. longirostris* and *Metapenaeus* sp.

These seven cases, in which the food product exhibited non-specific labelling, highlight the fact that a considerable lack of information is currently associated with the commercialization of this type of Decapoda crustaceans. Thus, our hypothesis is that purveyors devoted to the commercialization of such aquatic products in the food chain may sometimes not provide labelling at species level to avoid the risk of misidentification. The reason would be the lack of confidence in the phenotypic differentiation, based on external anatomical features, which may be especially complicated in the case of closely-related species. At this point, the molecular method evaluated in this work revealed as a valuable tool to circumvent this problem.

The nucleotide sequences determined for the prawn and shrimp specimens present in the commercial frozen products were compared and a phylogenetic tree was constructed using the neighbour-joining method (Fig. 2). The species exhibiting lower values of intraspecific homology were *Par. longirostris* (88.9%) *Mel. latisulcatus* (89.5%), *P. semisulcatus* (90.3%), *Farf. notialis* (92.8%) and *P. monodon* (93.6%). On contrast, the species exhibiting the highest values of intraspecific homology were *Farf. brasiliensis* (100%), *L. vannamei* (99.8%), *Pl. muelleri* (99.6%), *Fen. indicus* (99.4%) and *Fen. merguiensis* (98.9%). Remarkably, and despite the nucleotide variability detected in certain species, none of the single nucleotide polymorphic events detected in the specimens analyzed affected the restriction patterns displayed in Table 2. It should also be remarked that no 16S rRNA is currently available in international databases for the following species: *P. monoceros* and *P. sculptius*, while only a 16S rRNA fragment smaller than 500 bp has been sequenced in *Mel. plebejus*. This situation could have made difficult the authenticity analysis of samples 12, 15 and 29 based on PCR-RFLP analysis. For this reason, DNA sequencing and nucleotide analysis confirmed that sample 12 was *Fen. indicus*, sample 15 was *P. semisulcatus*, and sample 29 was *Fen. merguiensis*, thus confirming mislabelling in all three samples.

3.3. Species identification of peeled prawns and shrimps species in processed food products

Nine processed commercial products containing prawns or shrimps as added-value ingredients were also investigated (Table 4). Six of such nine products did not specify any species or even genera on their labels. This could be due to the complications derived from the fact that such raw materials are handled as peeled products, a situation that prevents their phenotypic identification by external morphological analysis. In six of such processed products, the Decapoda crustacean species included were generically referred to as "prawns" or "shrimps" (Table 4). From the five products that declared "prawns" on their labels, three (samples 8P, 16P and 17P) contained species belonging to the family *Penaeidae* – these were: *Metapenaeus* sp., *Par. longirostris, Fen. indicus* and/or *L. vannamei* – while the remaining two samples (10P and 14P) included species belonging to the family *Solenoceridae* – these were *Sol. agassizii* and *Pl. muelleri* – (Table 4). The only food product analyzed that declared "shrimps" on its label (sample 18P) included two different shrimp species; namely, *L. vannamei* and *Solenocera* sp. (Table 4). The remaining three processed food products (samples 12P, 13P and 15P) also failed to provide identification of shrimps at

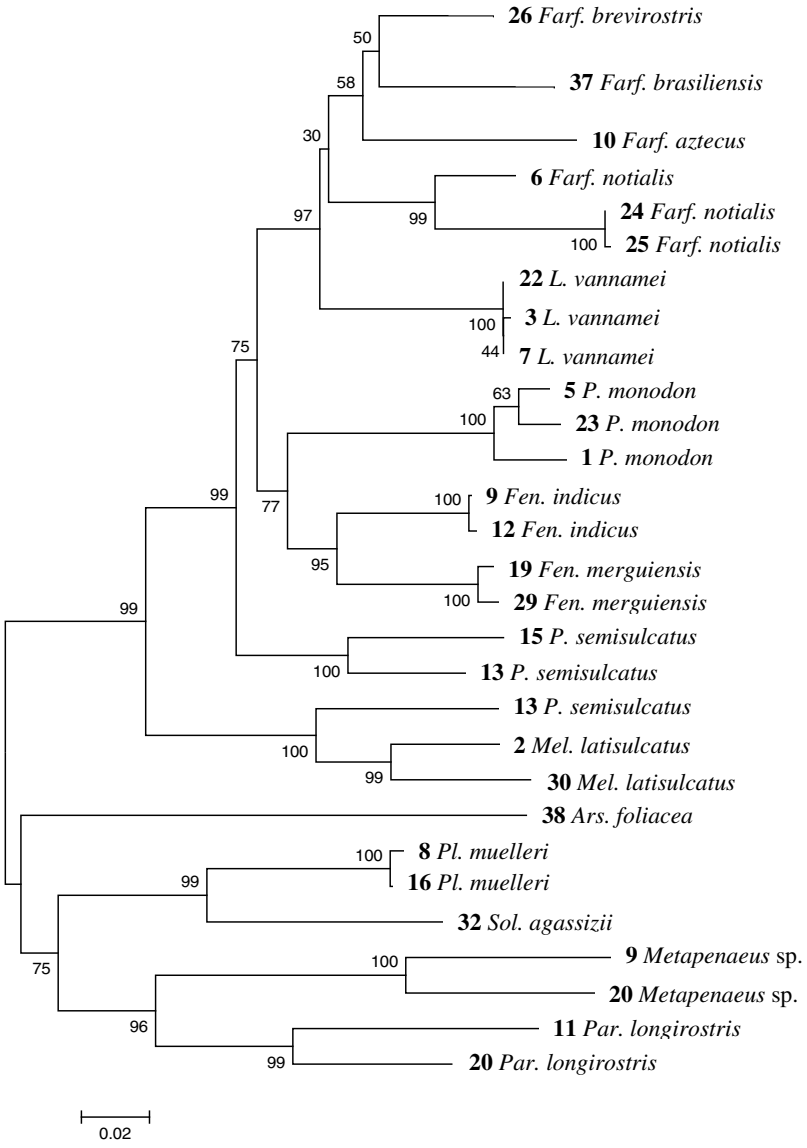

Fig. 2. Topologies resulting from the phylogenetic analysis of the nucleotide sequences of the 16S rRNA/tRNA^{Val} mitochondrial region in the commercial prawn and shrimp species investigated, by means of the neighbor-joining method. Numbers above and below branches indicate bootstrap values from neighbour-joining analysis. Numbers in bold are the samples identification. Sample 18 is not shown.

Table 4
Authenticity of prawn and shrimp species in commercial pre-cooked products

Sample	Product type	Processing	Declared species	Detected species
8P	Prawns with garlic	Peeling + Cooking + Freezing	Prawns	*Metapenaeus* sp.
10P	Spinach with prawns	Peeling + Cooking + Freezing	Prawns	*Sol. agassizii*
12P	Peeled prawns	Peeling + Freezing	*Solenocera* sp.	*Solenocera* sp.
13P	Peeled prawns	Peeling + Freezing	*Parapenaeus* sp. *Solenocera* sp. *Trachypenaeus* sp.	*Metapenaeus* sp.
14P	Peeled prawns	Peeling + Freezing	Prawns	*Sol. agassizii Pl. muelleri*
15P	Peeled prawns	Peeling + Freezing	*Parapenaeopsis* sp.	*Pl. muelleri Par. longirostris*
16P	Tail-on prawns	Peeling + Frying + Freezing	Prawns	*Fen. indicus*
17P	Prawns rolled in breadcrumbs	Peeling + Frying + Freezing	Prawns	*L. vannamei*
18P	Shrimp wraps	Peeling + Extruding + Freezing	Shrimps	*L. vannamei Solenocera* sp.

species level, but included on their labels the names of the genera for such species. Of these three cases, only one (sample 12P) proved to be correctly labelled. In contrast, sample 15P reflected a complete replacement of *Parapenaeopsis* sp. by *Pl. muelleri*, while sample 13P did not contain any *Parapenaeus* sp., *Solenocera* sp. or *Trachypenaeus* sp., such species being completely replaced by *Metapenaeus* sp.

4. Conclusions

This study demonstrates that the heterogeneity of the prawn and penaeid shrimp species commercialized is great and mislabelling frequent, thus underlining the need for molecular methods that may provide the control authorities and the industrial processors with the tools to fulfil and comply with labelling at species level. Regarding this issue, the PCR-RFLP method, aimed at the molecular analysis of a *ca.* 530 bp mtDNA fragment on the basis of the use of novel 16ScruC4/16ScruC3 primers targeted to two well-conserved regions of the 16S rRNA and $tRNA^{Val}$ genes, confirmed the presence of up to 17 different prawn and shrimp species in the 41 commercial products analyzed. The labelling indicated the presence of the Pacific white shrimp *L. vannamei* and the Argentine red shrimp *Pl. muelleri* in two food products, but these species were the most frequent species identified, being present in seven and five food products, respectively. The proposed method may represent an advance with respect to previous reported methods targeted to other regions of the mtDNA and limited to the identification of three – *Farf. californiensis, L. vannamei* and *L. stylirostris* (Gutiérrez-Millan et al., 2002) – or five – *P. monodon, P. semisulcatus*, *L. vannamei*, *Fen. merguiensis* and *Mars. japonicus* (Khamnamtong et al., 2005) – Decapoda crustacean species, respectively. The main advantages of the method evaluated here with respect to previous studies are: (i) it has been specifically designed for the identification of shrimp and prawn species of commercial interest to the food industry, (ii) a large number of species – up to seventeen – have been successfully identified, and (iii) the method works well with processed foods subjected to a variety of technological treatments.

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