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Authenticating the origin of different shrimp products on the Tunisian markets by PCR/RFLP method

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This study describes a polymerase chain reaction using restriction fragment length polymorphism (PCR-RFLP) assay based on the 16S rRNA mitochondrial gene to identify commercial food products of wide range of *Penaeidae* and *Pandalidae* shrimp species commercialised in the Tunisian market. Phylogenetic analyses on 16S rRNA mitochondrial gene were used to study the relationships among the considered species. *Penaeidae* shrimp species was easily differentiated and confirmed by direct sequencing, showing a genetic distance of 0.34 with respect to *Pandalidae* species. A rapid and reliable PCR method using restriction fragment length polymorphism (RFLP) with three restriction enzymes (HpyCH4III/ MboI / AluI) was optimized for unambiguous differentiation of shrimp from 19 commercial market samples (raw and processed products). Results showed that the restriction fragment length polymorphism technique can be used to identify Tunisian shrimp species and thus to control not only commercial fraud but also efficient restocking program.

Key words: Shrimps, species identification, 16S rRNA mtDNA, PCR-RFLP.

INTRODUCTION

In recent decades, there has been a tendency towards increased globalisation of the fishery trade and a diversification of seafood in term of species and products, with prawns and shrimps being among the most internationally commercialised aquatic product (FAO, 2012). The shrimps of Tunisia belong mainly to the Penaeidae family; with a minority of Pandalidae of the decapod groups (Santos and Chaouchi, 2002). Penaeidae species, such as the caramote prawn *Penaeus kerathurus* and the deepwater rose *Parapenaeus longirosttris* are highly prized shellfish products with considerable commercial value. For instance *P. kerathurus* gained important market niche in some European countries (unpublished data), at the same time it is an overfished species submitted to fishing regulation (Ben Mariem, 1993). Such controversial

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Table 1. Shrimp species considered in this study, scientific names, commercial name, origin, 3-alpha codes, 16S rRNA sequence GenBank accession numbers.

Scientific name	Commercial name	Origin	FAO species code	16S rRNA accession numbers NCBI
Penaeus kerathurus	Caromate prawn	Tunisia	TGS	JX089981
Parapenaeus longirostris	Deepwater rose shrimp	Tunisia	DPS	JX089984
Metapenaeus monoceros	Speckled shrimp	Tunisia	MPN	JX089983
Plesionika heterocarpus	Shrimp	Tunisia	LKO	JX089982
Plesionika edwardsii	Shrimp	Tunisia	LKW	JX089986
Litopenaeus vannamei	White Leg shrimp	Dubaii	PNV	JX089979
Penaeus monodon	Black Tiger shrimp	Dubaii	GIT	JX089985
Pandalus borealis	Shrimp	Groenland	PRA	JX089980

situation may lead to adulteration to the consumers, but can also reduce the efficiency of management programs that help protecting over exploited species such as the Mediterranean *P. kerathurus*.

Adulteration occurs when the usual identifying characteristics are removed by processing and/or when accurate labeling is missed. It is therefore of prime importance to develop a common system for all fishery trading countries to control frauds when species substitutions occur. Such system should be recognised internationally and should include a data base through method diversification across species and laboratories for two main reasons. Firstly to exchange homologous data and secondly; to develop alternative but reliable method for species identification overcoming the lack of expensive product or consumable needed for such analysis especially in developing countries. Biochemical and molecular tools provide valuable support for the rapid and accurate identification of morphologically indistinct processed species.

Most of the genetic approaches for species identification are based on the amplification of a region of mitochondrial (mtDNA) DNA by polymerase chain reaction (PCR) (Maggioni et al., 2001; Khamnamtong et al., 2005; Calo-Mata et al., 2009; Pascoal et al., 2011; Benedict et al., 2013; Armani et al., 2015). The aim of this study was to develope a method that is quick and reliable molecular tool for the authentication of commercial shrimps in Tunisian markets. Therefore 19 commercial shrimp/prawn products collected from various markets were analyzed using PCR–RFLP of a 400-bp fragment in the mitochondrial 16S rRNA region.

MATERIALS AND METHODS

Commercial food products

The fresh specimens used in this study were caught along the Tunisian coast and identified morphologically by researchers using the FAO species identification keys. Shrimp-type products were purchased at local supermarkets or directly from Tunisian seafood companies. The scientific names are given in Table 1. Frozen products and pre-cooked products (Table 2) were purchased from local supermarkets. All samples (19) were frozen upon arrival in the laboratory.

DNA extraction

Total genomic DNA was individually extracted from muscle of shrimp and was performed following optimisation of the method (unpublished data): For this study, the optimisation concerned the concentrations of the Tris–HCl, the EDTA and the SDS, the duration of the tissue incubation and DNA precipitation. The optimal conditions were used and the result, in term of DNA concentration and purity, were compared to seven published methods including the phenol–chloroform-based approaches, the Triton and CTAB methods (with various modifications) and the use of commercial kit such as Chelex, kit fasta DNA, DNA extraction by DNeasy Tissue Kit. The tissue was chopped into small pieces and homogenised in an appropriate volume of the extraction solution (10 mM Tris–HCl, pH 8, 100 mM EDTA, 1% SDS, and 5 mg/ml proteinase K). The homogenate was incubated at 55°C overnight. DNA was extracted twice using phenol/chloroform/isoamyl alcohol (25:24:1) and recovered by ethanol precipitation. DNA concentrations were spectrophotometrically determined at using a NanoDrop; 1000 Spectrophotometer at the absorbance of 260 (A260) and 280 nm (A280). The purity of extracted DNA was determined by using A260/A280 ratio and later tested by PCR amplification.

Primer design

Using Mega software, a set of primers were originally designed for this study and were used for the amplification of mitochondrial 16S rRNA gene. Fifty crustacean shrimp 16S rRNA gene sequences were obtained from GenBank; nucleotide sequence alignment in the shrimp species help in identifying the specific fragments of the 16S rRNA mitochondrial region that lead to the design of the primers. In addition, 16S rRNA gene sequences of shrimp species purchased for this study were determined by a direct sequencing analysis of the amplified products using the sequencing primer pairs, namely:

PNF1 (forward primer 5'-GCGGTATTTTGACCGTGCGAAGG-3')
PNF2 (forward primer 5'-GCGAAGGTAGCATAATCATTAGTC-3')
PNR1reverse primer 5'-CAAAGAAGATTACGCTGTTATCCC-3')
PNR2 reverse primer 5'-GATTACGCTGTTACCCTAAAG-3')
PNR3 reverse primer 5'-CCTTAATTCAACATCGAGGTCGC-3').

Six set primer pairs based on the highly conserved sequence among the target species, PNF1/PNF2 and PNR1/PNR2 /PNR3 for detecting shrimp, were designed to amplify the 16S rRNA gene. For

Table 2. Authenticity determination of prawn and shrimp species in commercial frozen, pre-cooked products.

Sample	16S rRNA Accession numbers NCBI	Product type	Processing	Declared species	Detected species
FP1	JX196945	Frozen shrimps	Freezing	*P. kerathurus*	*P. kerathurus*
FP2	JX196948	Frozen shrimps	Freezing	*Par. longirostris*	*P. longirostris*
FP3	JX196950	Frozen shrimps	Freezing	*Par. longirostris*	*P. heterocarpus*
FP4	JX196951	Frozen shrimps	Freezing	*Par. longirostris*	*P. edwardsii*
FP5	JX196952	Frozen shrimps	Freezing	*M. monoceros*	*M. monoceros*
PP1	JX196946	Peeled shrimps	Peeling + Freezing	*P. kerathurus*	*P. kerathurus*
PP2	JX196947	Peeled shrimps	Peeling + Freezing	*P. kerathurus*	*P. kerathurus*
PP3	JX196949	Peeled shrimps	Peeling + Freezing	*P. kerathurus*	*P. longirostris*
PP4	JX089985	Cooked shrimps	Peeling + Boiling + Freezing	Black tiger	*P. monodon*
PP5	JX089979	Premium peeled shrimps	Peeling + Freezing	Shrimp	*L. vannamei*
PP6	JX196955	Breaded shrimps	Peeling + Frying + Freezing	Shrimp	*L. vannamei*
PP7	JX089980	Cooked shrimps	Peeling + Boiling + Freezing	Northern Shrimp	*P. borealis*
PP8	JX196953	Cooked shrimps	Peeling + Boiling + Freezing	Shrimp	*M. monoceros*
PP9	JX196954	Cooked shrimps	Peeling + Boiling + Freezing	Shrimp	*M. monoceros*

FP, Frozen products; PP, Pre-cooked product.

all species, one set of primer pairs PNF1/PNR3 were used in the PCR amplifications to obtain 400 bp DNA fragments of 16S rRNA.

PCR amplification

PCR amplifications were carried out using Bio-Rad MyCycler (Bio-Rad). The reactions were set up in volumes of 50 µL containing PCR buffer (75 mM Tris-HCl, pH 9.0; 50 mM KCl; 20 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$); 400 µM dNTP mix; 0.2 µM each primer; 2.5 units of UptiTherm DNA polymerase (Uptima- Interchim); and 100 ng of template DNA (PCI extracts). Amplification conditions were as follows: a previous an initial denaturation step of 94°C for 3 min, followed by 35 cycles consisting of denaturation (30 s at 9°C), annealing (30 s at 55°C) and extension (40 s at 72°C), and the final extension for 5 min at 72°C was also included.

Cleanup and sequencing of PCR products

Before sequencing, double-stranded PCR products were purified by filtration through a DNA Clean and Concentrator-25 of ZYMO RESEARCH CORP according to the manufacturer's protocol.

PCR fragments were used for direct cycle sequencing with the dye terminator cycle sequencing kit (Beckman). Sequencing analysis was performed with a Beckman Coulter CEQ 8000 DNA sequencer in both directions with the primers used for PCR amplification.

Sequence alignment, genetic distances, and phylogenetic analyses

The DNA sequences were edited with BioEdit software (Hall, 1999). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 5.1. (Tamura et al., 2011). Nucleotide divergences were computed using the Tamura-Nei model, (Tamura et al., 1993) which takes substitutional rate biases and the inequality of base frequencies into account. Phylogenetic trees were constructed using the neighbor-joining (NJ) method, (Saitou and Nei, 1987) and the robustness of topology nodes was tested by the bootstrap method with 1500 replicates. The new sequences were deposited in the NCBI GenBank.

RFLP analysis

The sequences of 400 bp fragments of the tested species from GenBank database were aligned using the Mega software to detect the presence of one or more restriction sites that could be characteristic of each species. On the basis of such analysis some restriction enzymes were selected for carrying out the RFLP analysis: HpyCH4III, MboI and AluI. (Fermentas Int., Burlington, Ontario, Canada).

The endonucleases with the position of restriction sites and the length of expected fragments in each of the tested species are reported in (Table 4). For each sample, 5-10 µL of the PCR reaction containing amplified DNA was digested overnight at 37°C with 2 U of each endonuclease in a final volume of 20 µL. The resulting fragments were separated by electrophoresis on a 2% agarose gel, the sizes of the resulting DNA fragments were estimated by comparison with a commercial 100-bp ladder (Protech Technology Enterprise Co., Taipei, Taiwan).

RESULTS AND DISCUSSION

PCR amplification of prawn and shrimp mtDNA from commercial food products

Although DNA exhibits fairly high thermal stability, it is well known that intense heat may cause severe DNA degradation. A direct relationship between heat treatment and the intensity of DNA fragmentation has been reported (Chapela et al., 2007; Besbes et al., 2011). Other processing conditions, including overpressure conditions and the presence of additives that may inhibit DNA polymerase have also been reported as important cause for the lack of amplification (Pascoal et al., 2008;

2011; Besbes et al., 2011). For PCR amplification, the melting temperature (Tm) correlates with how easily a double stranded DNA complex is formed. The Tm is affected by a number of factors such as concentration of DNA, concentration of ions in the solution (specially Mg^+ and K^+), nucleotide sequences (that is, nucleotide pair 'A-T' has a weaker bond than the nucleotide pair 'G-C') and the length of the DNA fragment (Liedl and Simmel, 2007).

Several studies revealed the usefulness of a 1.38 kb mitochondrial region that comprises fragments of the 16S rRNA and 12S rRNA genes and the entire tRNAVal region for phylogenetic analysis of penaeid shrimps (Gutiérrez-Millan et al., 2002; Rasmussen et al., 2008). Moreover, the mitochondrial gene coding for 16S rRNA has been reported to be a good candidate for authentication of fish and seafood due to its acceptable length, mutation rate, and availability of sequence information in databases. Other studies described Pascoal et al., 2011). In this study, we amplified and sequenced a mitochondrial marker corresponding to 16S rRNA gene in an effort to improve fraud detection of shrimp for the first time in Tunisia, and for its validation in commercial products.

The analysis of the sequences obtained revealed that most of the fragments are very conserved between the species studied, with very low interspecific and intraspecific variability found in 16s rDNA located in the mtDNA. Although a lower evolution rate has been described for this fragment, this rate depends on the species groups, with some reports showing its usefulness for population studies (Chapela et al., 2002).

In this study a total of 19 different commercial food products containing or consisting of shrimps were subjected to DNA extraction and purification. The primers were designed to anneal the 16S rDNA region; both primers have equal length (23 nucleotides), GC content (56.52 and 47.82%) and Tm (72°C and 68°C), low self-annealing, and low complementarities between them. DNA amplification was successful in all primers and for all samples analyzed, showing that the designed primers are useful to amplify mitochondrial DNA, not only in the species whose sequences were employed to design them, but also in other species of crustaceans belonging to families *Penaeidae* and *Pandalidae*.

Accordingly, the size of the molecular target (400 bp) proved to be accurate to achieve PCR amplification even in processed products subjected to a variety of technological treatments such as boiling, frying or freezing. Such procedure was also effective in food including shrimp or prawn as ingredients.

Species identification of commercial shrimp food product

It is well recognized that, identification of unknown sample can be performed if the unknown sequence is introduced in the estimation of genetic distance among a set of reference sequences. The unknown sequence will show the lowest distance value with the species groups to which it belongs (Chapela et al., 2002). In this study, the correct labelling for the different processed shrimp-products found in the local Tunisian market was only for species with the common name deepwater pink shrimp or Caromate shrimp. The others imported products were labelled shrimp or did not have any genera specification on their labels, and were phenotypically indistinguishable due to processing. In six of the processed products, the included Decapoda crustacean species were identified as ''prawns" or ''shrimps" (Table 2). Consequently, the DNA amplification products were sequenced and aligned against the 16S rRNA sequences of shrimp obtained from the NCBI GenBank (Table 1). The new sequences, which were equally deposited in the NCBI GenBank, were aligned against the other *Penaeidae* and *Pandalidae* species sequences allowing the construction of a neighbour-joining tree (NJ) (Figure 1). Thus the constructed phylogram showed two distinct family clades, for the two families *Penaeidae* and *Pandalidae*, each associated with each reference species. The genus *Penaeus*, *Litopenaeus*, *Parapenaeus* and *Metapenaeus* are grouped in the clade *Penaeidae* and the species *Plesionika* and *Pandalus* are grouped in the clade *Pandalidae*.

The species exhibiting the highest values of intra-specific homology were *P. kerathurus* (100%), *M. monoceros* (100%), *P. longirostris* (97%), *L. vannamei* (96%), *P. monodon* (75%), *P. heterocarpus* (100%), *P. edwardsii* (100%) and *P. borealis* (99%).

The nucleotide sequences determined for the prawn specimens present in the commercial peeled and frozen products were compared (Figure 1). Fresh *P. kerathurus* (F), *P. kerathurus* food products (FP1, PP1, PP2) and the *P. kerathurus* reference were grouped in the same cluster with a bootstrap value at the nodes of 100%. The shrimp species names in commercial pre-cooked products (PP6, PP5) were not indicated on packages. The genetic identification revealed that species were grouped in the same cluster of *Litopenaeus vannamei* with a bootstrap values at the nodes of 96 and 91% respectively. The commercial pre-cooked products (PP8, PP9), which were not indicated on labels, were grouped in the same cluster of *Metapenaeus monoceros* with a bootstrap values at the nodes of 100%.

The clade of *Pandalidae* grouped PP7 in the same cluster of *Pandalus borealis* with a bootstrap value at the nodes of 99%, and (FP3, FP4) were grouped of *Plesionika heterocarpus* and *P.edwardsii* respectively with a bootstrap values at the nodes of 100%. DNA sequencing and nucleotide analysis confirmed that sample PP5, PP6 (not declared species) were *L. vannamei*, samples PP8, PP9 were *M.monoceros*, and samples FP3, FP4 were *plesionika* species, thus confirming mislabeling of these samples.

Species divergence is an another tool to evaluate

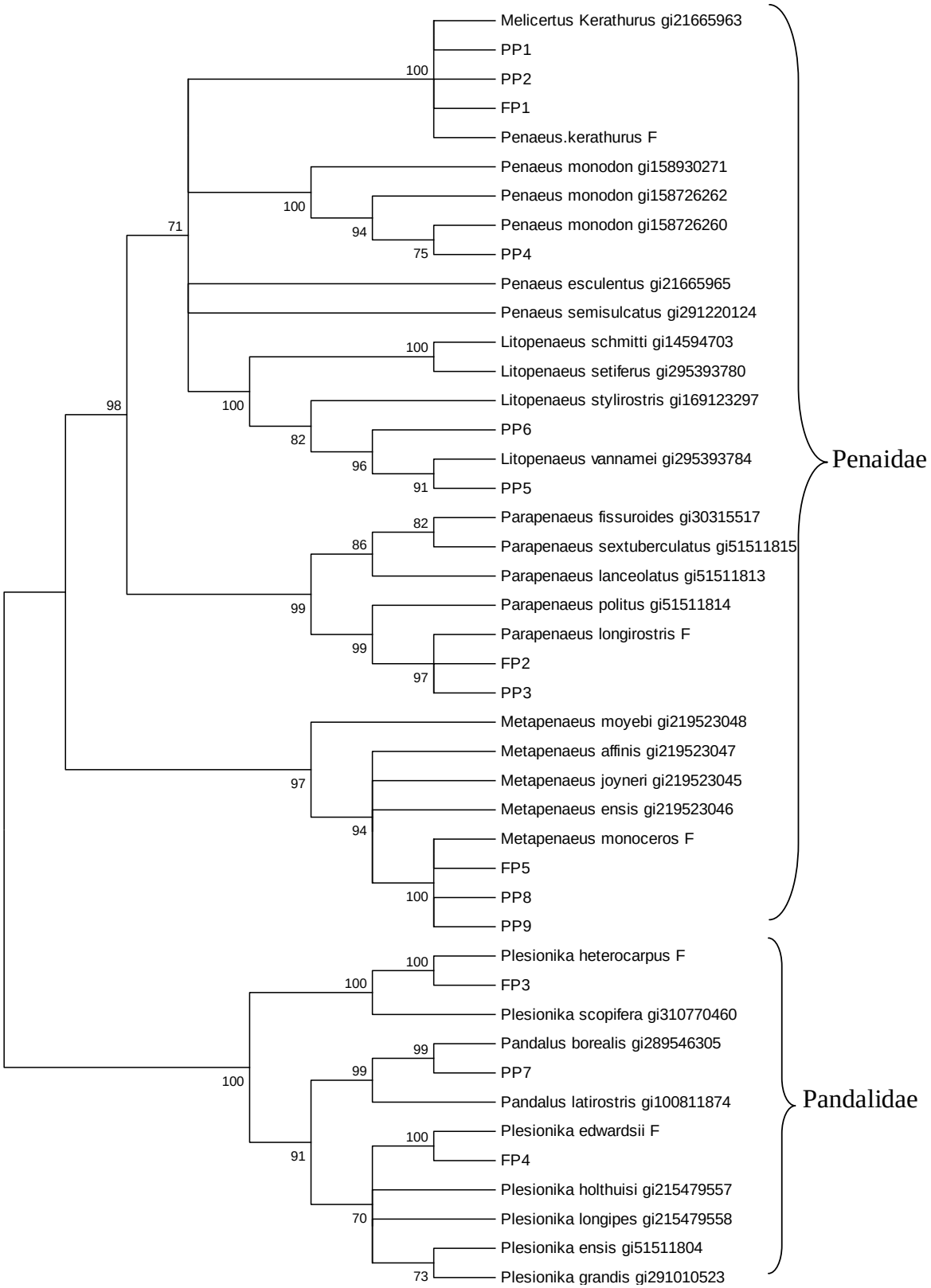

Figure 1. Phylogenetic tree obtained from sequence divergence of a 16S rRNA mitochondrial gene for Nineteen sequences of Penaidae and Pandalidae Shrimp Species. NJ tree inferred from TN distances between sequences of partial 16S rRNA gene in 16 samples of Penaidae and 3 Panadalidae species and the sequenced gene referenced in the NCBI GenBank; Numbers denote bootstrap percentages based on 1500 replications from neighbor-joining analysis.

Table 3. Pairwise sequence divergences for the 16S rRNA mitochondrial gene for *Penaeidae* and *Pandalidae* shrimp estimated by the Tamura and Nei method.

Genera	F1	F2	F3	F4	F5	PP1	PP2	FP1	FP2	PP3	PP8	FP5	PP9	PP5	PP6	FP3	FP4	PP4
P. kerathurus-F1																		
Par. longirostris-F2	0.11																	
Met. monoceros-F3	0.17	0.13																
Pl. heterocarpus-F4	0.34	0.31	0.33															
Pl. edwardsii-F5	0.33	0.32	0.29	0.31														
P. kerathurus-PP1	0.01	0.11	0.17	0.34	0.33													
P. kerathurus-PP2	0.01	0.11	0.17	0.34	0.33	0.01												
P. keratherus-FP1	0.01	0.11	0.17	0.34	0.33	0.01	0.01											
Par. longirostris-FP2	0.11	0.00	0.13	0.31	0.32	0.11	0.11	0.11										
Par. longirostris-PP3	0.11	0.00	0.13	0.31	0.32	0.11	0.11	0.11	0.01									
Met. monoceros-PP8	0.17	0.13	0.01	0.33	0.29	0.17	0.17	0.17	0.13	0.13								
Met. monoceros-FP5	0.17	0.13	0.01	0.33	0.29	0.17	0.17	0.17	0.13	0.13	0.01							
Met. monoceros-PP9	0.17	0.13	0.01	0.33	0.29	0.17	0.17	0.17	0.13	0.13	0.01	0.01						
Lit. vannamei-PP5	0.14	0.12	0.17	0.40	0.33	0.14	0.14	0.14	0.12	0.12	0.17	0.17	0.17					
Lit. vannamei-PP6	0.13	0.12	0.17	0.40	0.33	0.13	0.13	0.13	0.12	0.12	0.17	0.17	0.17	0.01				
Pl. heterocarpus-FP3	0.34	0.31	0.33	0.01	0.31	0.34	0.34	0.34	0.31	0.31	0.33	0.33	0.33	0.40	0.40			
Pl. edwardsii-FP4	0.33	0.32	0.29	0.31	0.01	0.33	0.33	0.33	0.32	0.32	0.29	0.29	0.29	0.33	0.33	0.31		
P. monodon-PP4	0.11	0.13	0.18	0.37	0.31	0.11	0.11	0.11	0.13	0.13	0.18	0.18	0.18	0.13	0.13	0.37	0.31	
Pd. borealis-PP7	0.32	0.27	0.28	0.31	0.23	0.32	0.32	0.32	0.27	0.27	0.28	0.28	0.28	0.34	0.33	0.31	0.23	0.30

similarity of an unknown sequence with a pool of reference samples. In this study, the genetic distances of *Penaeidae* was easily differentiated from the studied *Pandalidae* species (0.34) (Table 3). The genetic distance measured between *P. kerathurus* and the *P. kerathurus* processed product was 0.01 and with *P. longirostris* species was 0.11 and with *M. Monoceros* was 0.17 and with Plesionika species was 0.34.

The identification of the shrimp species and the different processed food products could be easily achieved by PCR-RFLP analysis. The differentiation of the shrimp could be achieved by cleaving the different species on two groups (Pink and White shrimp) according to morphological similarity (and to the fact that their industrial processing often removes their external shell). On one hand the Pink shrimps groups composed of *Parapenaeus longirostris*, *Pandalus borealis*, *Plesionika edwardsii* and *Plesionika heterocarpus* was discriminated and cleaved with HpyCH4III (Table 4 and Figure 2A). The white prawn groups composed of *Litopenaeus vannamei*, *Penaeus kerathurus*, *Metapenaeus monoceros* and *Penaeus monodon* were discriminated and cleaved with MboI (Figure 2B). However, *Litopenaeus vannamei*, *P. kerathurus* displayed the same profile, and were discriminated with AluI (Figure 2C). Species identification by sequencing and PCR-RFLP revealed that among the food products analysed, three were not correctly labelled (Table 2).

Concerning species substitution, some instances were likely unintentional probably because of phenotypic similarities. This is the case of *P. longirostris* which was confounded with *P. ldwardsii* and *P. leterocarpus* species (distributed in Tunisia waters) because they are of very similar phenotype and caught together in mixed fisheries. Moreover, endonuclease HpyCH4III also allowed the differentiation of *P. longirostris* species from *Plesionika* species, since the 400 bp PCR product exhibits a sequence recognized by HpyCH4III in the case of *P. longirostris* thus producing five restriction fragments of (380 bp, 261 bp, 176 bp, 127 bp and 61 bp) (Table 4). Other detected cases of incorrect labelling involved the complete substitution of *P. keratherus* by the pink prawn *P. longirostris* PP3 (Table 3). *P. longirostris* of lower commercial value than *P. keratherus* was easily distinguished with the restriction enzymes HpyCH4III (Table 4).

The risk of miss-identification would be the similarity in external morphological feature such as the case of the shrimp species having the same colors of the carapace (*P. longirostris*, *Pandalus borealis*, *P. edwardsii* and *P. heterocarpus*). In this study, such species were easily distinguished with the restriction enzymes HpyCH4III. Other cases of incorrect labelling involved the possibility of substitution of *P. keratherus* with imported species such as *L. vannamei* and *P. monodon*. These species were also, easily distinguished with the restriction enzymes MboI. Hence, the molecular method used in this work was revealed as a valuable tool to circumvent these problems, and was successful for the identification of a large number of crustacean products found in the local market.

Table 4. RFLP specific patterns (bp) for the *Penaeidae* and *Pandalidae* shrimp species considered as references in this study.

Shrimp species	Restriction enzyme		
	HpyCH4III	**MboI**	**AluI**
P.longirostris-F2	380+261+176+127+61		
P.longirostris-FP2	380+261+176+127+61		
P.longirostris-PP3	380+261+176+127+61		
P.borealis-PP7	235+121		
P.heterocarpus-F4	209+86+61		
P.heterocarpus-FP3	209+86+61		
P.edwardsii-F5	147+121+87		
P.edwardsii-FP4	147+121+87		
P.kerathurus-F1		276+86	222+81+69
P.keratherus-FP1		276+86	222+81+69
P.kerathurus-PP1		276+86	222+81+69
P.kerathurus-PP2		276+86	222+81+69
M.monoceros-F3		167+110+86	
M.monoceros-FP5		167+110+86	
M.monoceros-PP8		167+110+86	
M.monoceros-PP9		167+110+86	
L.vannamei-PP5		277+72+14	223+140
L.vannamei-PP6		277+72+14	223+140
P.monodon-PP4		183+93+86	

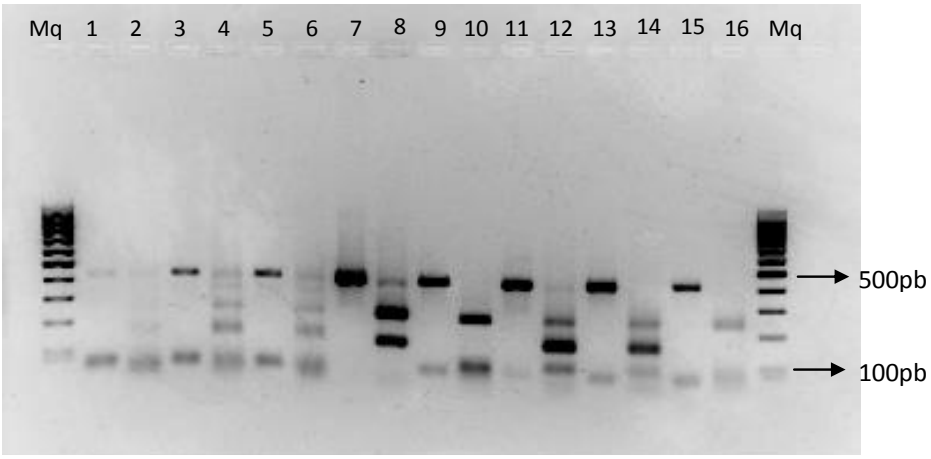

Figure 2A. Example of PCR-RFLP identification on 2% agarose gel of pink prawn fresh and food product with the restriction by HpyCH4III of 16S rRNA mitochondrial gene **(A)** from left to right, Mq:molecular weight marker (100pb DNA ladder, Protech Technology), DNA fragment products obtained with *P.longirostris* F2, FP2, PP3 (lane 2, 4, 6), *P.borealis*-PP7(lane 8), *P.heterocarpus*-F4 (lane 10), *P. edwardsii*-F5 (lane 12), *P.edwardsii*-FP4(lane 14), *P.heterocarpus*-FP3 (lane 16), and Ctrl: Non-digested fragment *P. longirostris* F2, FP2, PP3 (lane 1, 3, 5), *P.borealis*-PP7(lane 7), *P. heterocarpus*-F4 (lane 9), *P.edwardsii*-F5 (lane 11), *P.edwardsii*-FP4(lane 13), *P.heterocarpus*-FP3 (lane 15).

This study demonstrates that PCR-RFLP method, aimed at the molecular analysis of a 400 bp mtDNA fragment on the basis of the use of novel PNF1/PNR3 primers targeted to conserved regions of the 16S rRNA

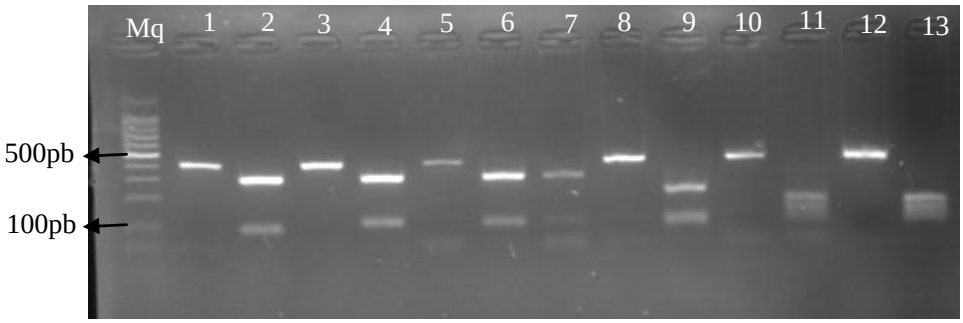

Figure 2B. white shrimp fresh and food product with the restriction by MboI of 16S rRNA mitochondrial gene from left to right, Mq : molecular weight marker (100pb DNA ladder, Protech Technology), DNA fragment products obtained with *L.vannamei*-PP5 (lane 2), *P.kerathurus*-F1, PP1, PP2 (lane 4,6,7), *P.monodon*-PP4 (lane 9), *M.monoceros*-F3, PP8 (lane 11,13), and Ctrl: Non-digested fragment *L.vannamei*-PP5 (lane 1), *P.kerathurus*-F1, PP1, PP2 (lane 3, 5), *P.monodon*-PP4 (lane 8), *M.monoceros*-F3, PP8 (lane 10,12).

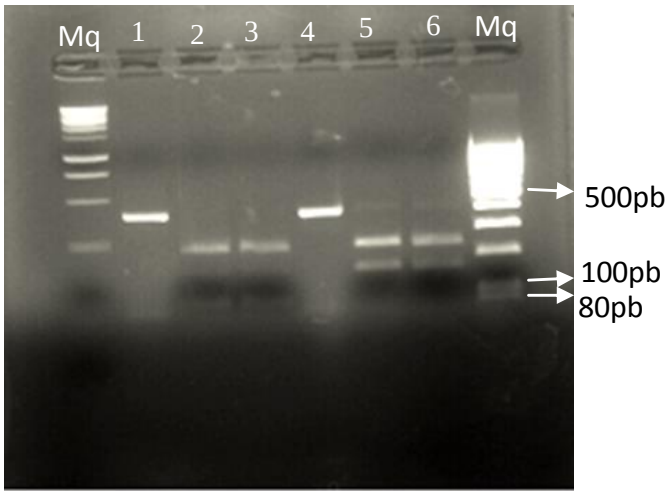

Figure 2C. shrimp fresh and food product with the restriction by AluI of 16S rRNA mitochondrial gene from left to right, Mq (1): molecular weight marker (1 Kb DNA ladder), Mq (2): molecular weight marker (100 pb DNA ladder, Protech Technology) DNA fragment products obtained with *P.kerathurus*-F1, PP1(lane 2,3), *L.vannamei*-PP5, PP6 (lane 5,6), and Ctrl: Non-digested fragment *P.kerathurus* (lane 1), *L.vannamei* (lane 4), (n=3 in each case).

represents an additional data to previous reported methods targeting other regions of the mtDNA. The diversification of method is necessary as molecular tools are not always favourable to adaptation by multiples sectors or countries; these tools are however valuable to the authorities for the rapid and accurate identification of morphologically indistinct species of commercial interest. Such need is more obvious with the increased international shrimp/ prawns trade where control should be reinforced to mitigate imports of product with lower unit value such as cultured shrimps, but also exports of overexploited species such as *P. kerathurus* outside the fishing seasons.

Conflict of interests

The author(s) did not declare any conflict of interest.

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