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A Pentaplex PCR as screening assay for jellyfish species identification in food products.

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Abstract

Salted Jellyfish, a traditional food in Asian Countries, is nowadays spreading on the Western markets. In this work, we developed a Pentaplex PCR for the identification of 5 edible species (*Nemopilema nomurai*, *Rhopilema esculentum*, *Rhizostoma pulmo*, *Pelagia noctiluca*, and *Cotylorhiza tuberculata)*, which cannot be identified by a mere visual inspection in jellyfish products sold as food. A common degenerated forward primer and 5 specie-specific reverse primers were designed to amplify *COI* gene regions of different lengths. Another primer pair was targeted to the *28SrRNA* gene and intended as common positive reaction control. Considering the high level of degradation in the DNA extracted from acidified and salted products, the maximum length of the amplicons was set at 200bp. The PCR was developed using 66 reference DNA samples. It gave successful amplifications in 85.4% of 48 Ready to Eat products (RE) and in 60% of 30 Classical salted Products (CP) collected on the market.

Keywords: Multiplex PCR; Jellyfish; Species identification; *COI* gene; seafood mislabeling

Introduction

In the last decades, Jellyfish proliferation in coastal areas, often referred to as "blooming", has become a reason of concern due to damages to aquaculture, clogging of the cooling systems of coastal power plants, and stings of bathers[1]. Moreover, the fact that jellyfish may feed on fish larvae, further decreasing the resilience of fish populations already weakened by over-fishing, is an emerging ecological and economical issue[2].

The Food and Agriculture Organization of the United Nations (FAO) has recently proposed to reduce jellyfish populations by increasing their utilization as medicine or food[3], considering that their biomass could be a valuable source of bioactive compounds and nutrients.

Both Asian and Mediterranean jellyfish are characterized by a number of metabolites with a healthy potential: extracts from *Nemopilema nomurai*, *Rhopilema esculentum*, *Pelagia noctiluca*, and *Cotylorhiza tuberculata* were reported to have hemolytic, anti-tumoral, antioxidant, anti-microbial, and insecticidal activity[4-7]. The collagen of some species has been demonstrated to possess the same characteristics of that of human origin[8] and the collagen of *Rhizostoma pulmo* is considered as a good candidate for replacing the bovine collagen in biomedical applications[9].

Regarding the utilization as food, while in the past the jellyfish was exclusively exploited in the Eastern Asia[10], nowadays, due to the worldwide migration flows from China, the jellyfish based products are increasingly spreading even in the Western Countries[11,12]. Two different kinds of products are available on the market: the Classical Salted Products (CP), which needs a long washing before consumption, due to the high salt concentration, and the new "ready to eat" products (RE), already desalted[10].

Even though more than 15 edible species belonging to the Rhizostomeae order could be used as food, a recent molecular survey performed on the European market[12] has highlighted that the jellyfish based products were essentially prepared using two big Asian species of the Rhizostomatidae family: *N. nomurai* and *R. esculentum*. However, a small percentage of the analyzed products were *P. noctiluca* and *R. pulmo.*

In the European Union, the Regulation (EC) n. 2065/2001[13] lists the information to be provided for labeling of fishery products. These are the commercial and scientific designation of the species, the production method, and the catch area. Furthermore, the recent Regulation (EU) n. 1379/2013[14], which will be applied from the 13th December 2014, established that the category of fishing gear used for the capture must also be reported.

Unfortunately, labelling of the ethnic products often presents incongruences and deficits with respect to the mandatory information requested. D'Amico *et al.*[15] found that a very high percentage of Chinese seafood products imported in Italy do not meet EU requirements on traceability. In a previous survey, it has been found that labels of jellyfish products not only missed an unequivocal specification of ingredients, but were also labelled with a trade name referring to vegetables[11].

One of the main problems associated with the inspection of jellyfish products, related to the loss of their morphological characteristics during processing, is the impossibility to unambiguously identify the species used[12]. For this reason, it is necessary to rely on laboratory methods. Among these, those based on the analysis of nucleic acids are the most used in case of fish and seafood products[16,17].

Polymerase chain reaction (PCR), the technique of choice for species identification, is often coupled with sequencing, which is used to determine the precise order of nucleotides in a DNA molecule[16,18]. FINS (Forensically Informative Nucleotide Sequencing) combines DNA sequencing with a phylogenetic analysis[19]. The development of the DNA Barcoding, an evolution of the FINS method, has determined the increase of use of the cytochrome c oxidase I gene (*COI*) as a genetic marker for species identification[20,21]. In a previous work[12] we showed that a specific pre-DNA extraction treatment, coupled with FINS of a 142bp *COI* gene fragment, allowed the molecular identification of jellyfish species, also in case of salted products, in which the DNA is heavily degraded.

Considering that FINS is often limited by high costs and long-time of execution, in this work an alternative method to be used with screening purposes was developed to minimize expenses and

save time. This new method, a Pentaplex PCR assay, is capable to identify two Asian jellyfish commonly used as food, such as *N. nomurai* and *R. esculentum*, and another 3 species distributed in the Mediterranean Sea, such as *R. pulmo*, *P. noctiluca*, and *C. tuberculata*, which could represent a novel business for the industry. This is possible by using a common forward primer together with 5 specific reverse primers, capable to amplify 5 regions with different size of the *COI* gene. This method can be used as rapid test for checking commercial samples, in which the diagnostic morphological characteristics have been lost.

2. Materials and methods

2.1. Jellyfish reference (RS) and market samples (MS) collection

Nine samples belonging to the species *Aurelia sp., C. tuberculata*, and *P. noctiluca* were kindly provided by the Institute of Sciences of Food Production of the National Research Council of Lecce (Table 1SI).

2.2. DNA extraction

All the jellyfish RS were extracted according to the protocol proposed by Armani *et al.*[22] The amount of DNA was determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) by measuring the absorbance at 260 nm. DNA samples obtained from 57 jellyfish RS (Table 1SI) and 78 MS (Table 1), all of them molecularly identified in a previous work[22], were also used to develop and validate the Pentaplex PCR. Finally, 3 DNA samples of *R. luteum* from the study of Prieto *et al*.[23] and 6 DNA samples of *P. benovici* from the study of Piraino *et al*.[24] were used.

2.3. Amplification of COI and 28SrRNA genes

2.3.1. COI. All the DNA reference samples extracted in this study from jellyfish were initially amplified using the primers FFDL and FRDL2[12] to obtain the first 655bp of the *mtCOI* gene. The samples that failed the amplification were subsequently amplified using internal reverse primers[12] in combination with the same forward primer. The DNA was amplified according to the following PCR protocol: 20µL of reaction volume containing 2µL of 10× buffer (5Prime, Gaithersburg,

USA), 200 µM of each dNTP (dNTPmix, Euroclone S.p.A-Life Sciences Division, Pavia, Italy), 300 nM of primers, 25 ng/µL of BSA (Purified BSA 100×, New England BIOLABS® Inc. Ipswich, MA, USA), 1.25 U of PerfectTaq DNA Polymerase (5Prime, Gaithersburg, USA), 100 ng of DNA and DNase free water (Water Mol. Bio. Grade, DNase–RNase and Protease free, 5Prime GmbH, Hamburg, Germany), with the following cycling program: denaturation at 94 °C for 3 min; 40 cycles at 94 °C for 30 s, 51 °C for 30 s, and 72 °C for 35 s; final extension at 72 °C for 5 min. Five µL of PCR products were checked by gel electrophoresis on a 2% agarose gel and the presence of fragments of the expected length was assessed by a comparison with the standard marker SharpMass™50-DNA ladder (Euroclone, Wetherby, UK). Double-stranded PCR products were purified with the Illustra ExoProStar 1-Step Enzimatic PCR and Sequencing Clean-Up (GE Healthcare UK Limited, Amersham Place Little Chalfont Buckinghamshire, UK) following the manufacturer's instruction and then sequenced by GATC Biotech (Germany). The obtained sequences were analyzed using ClustalW in MEGA 5.0[25]. Fine adjustments were manually made after visual inspection. All the sequences were used to run a BLAST analysis on GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and analyzed using the Identification System (IDS) on BOLD (Species Level Barcode Records) (http://www.boldsystems.org/index.php/IDS_OpenIdEngine). Finally, the sequences were deposited on GenBank via EBI (Table 1SI).

2.3.2. 28S rDNA. The *28SrRNA* gene sequences belonging to the Order Rhizostomeae and Semaestomeae produced in the work of Bayha *et al.*[26] were downloaded from GenBank and aligned using Clustal W in MEGA 5.0. The overall mean distance was calculated using the Kimura 2-parameter model[27] on a fragment of 1480bp. Then, a new couple of primers (F28S and R28S) (Table 2) were designed for the amplification of a fragment of ~1600bp from the DNA of *N. nomurai* using the same PCR mix described for the *COI* gene, with the following cycling program: denaturation at 94 °C for 3 min; 40 cycles at 94 °C for 30 s, 51 °C for 30 s, and 72 °C for 35 s; final

extension at 72 °C for 10 min. The sequences were analyzed as reported in section 2.3.1. and were deposited on GenBank via EBI (Table 1SI).

2.4 Primer Design for the Pentaplex PCR Assay.

2.4.1. Specie-specific primers targeting the COI gene. Six hundred sixty six *mtCOI* sequences belonging to 24 species (Table 3) were aligned with Clustal W in MEGA 5.0. Then, two hypothetical common forward primers were designed (FJ1 and FJ2, Table 2) by modifying the primer FFDL designed in a previous work[12]. The reverse primers were designed on highly polymorphic regions for the amplification of fragments of different length in the range of 50-200bp. Primers score were calculated with the software Beacon designer 7.0 (Premier Biosoft International, Palo Alto, CA) and with the software available on the Eurofins MWG Operon (Ebersberg, Germany) page (http://www.eurofinsgenomics.eu/). The number of mismatches between the reverse primers and the annealing region of the no-target species (Table 2SI) was also taken into consideration as criterion to select specie-specific primers. Totally 11 primers, 2 forward and 9 reverse, were synthetized by the Eurofins MWG Operon.

2.4.2. Control PCR primers targeting the 28SrRNA gene

With the aim of amplifying in all the species, a control fragment of 55bp together with the species-specific fragment, two primers (28sfor55 and 28srev55) (Table 2) were designed on highly conserved regions using the software Beacon designer 7.0 after aligning the *28SrRNA* gene sequences (Order Rhizostomeae and Semaestomeae) produced in the work of Bayha *et al.*[26] with the sequences of *N. nomurai* produced in this study.

2.5. PCR reaction optimization

The two forward primers were tested on the DNA of all the reference species in single reaction with each of the reverse primers designed (Table 1SI). The primers capable to amplify the target sequence without producing unspecific bands were selected (Table 2).

All the primers were tested in combination in the same PCR reaction mix using different concentrations (from 100 to 400nM), in presence of increasing concentration of $MgCl_2$ (1; 2; and 5

mM) and nucleotides (from 200 to 600 μM). We also tested different amount of total DNA (from 1 to 200 ng) and of Taq polymerase (1X and 2X). The optimal annealing temperature was selected after trials in the range of 47°C to 51°C, on a PeqStar 96 Universal Gradient, PEQLAB BiotechnologieGmgH (Euroclone, Wetherby, UK). The control PCR primers were initially tested on all the reference DNA samples and then included in the multiplex reaction. The reaction protocol that showed the best amplification performances was tested on 10 samples of not degraded DNA extracted from 5CP and 5RE to verify the output of the Pentaplex method in case of degraded and low quality DNA.

2.6. Final Pentaplex PCR protocol and analysis of commercial samples

The following final protocol: 10μL of reaction volume containing 1μL of 10× buffer (Fisher Molecular Biology, Trevose PA, USA), 400 μM of each dNTP (dNTPmix, Euroclone S.p.A-Life Sciences Division, Pavia, Italy), 300 nM of FJ2, 200 nM COT2, 200 nM RHOP1-1, 225 nM NEMO1, 200 nM PEL1 and 100 nM RHIZ1-1reverse primers, 100 nM of 28sfor55 and 28srev55 control primers, 25 ng/μL of BSA (Purified BSA 100×, New England BIOLABS® Inc. Ipswich, MA, USA), 1 U of SubTherm TAQ Polymerase (Fisher Molecular Biology, Trevose PA, USA), 3 mM of $MgCl_2$, 50 ng of DNA and DNase free water with the following cycling conditions: denaturation at 95 °C for 3 min; 40 cycles at 95 °C for 25 s, 49 °C for 5 s, and 72 °C for 5 s; final extension at 72 °C for 5 min. The electrophoresis was performed using a 4% agarose gel. In case of MS DNA, the length of the specific bands obtained with the multiplex PCR was compared to the results of the BLAST analysis performed on the same samples in a previous article[12] (Table 1), with the aim to assess the efficiency and specificity of the assay in case of low quality DNA.

3. Results and Discussion

Seafood species identification has gained increasing importance in the current global market context. In fact, many studies, carried out in different geographical areas, have highlighted that almost one third of the commercialized seafood is made of a different species with respect to what

declared on the label[20,21]. In some cases, the substitution rate can reach alarming levels, as in the case of jellyfish products[12].

In this light, the diagnostic techniques, particularly those based on DNA analysis, may assist both private companies and official agencies in ensuring a fair commerce in the light of the traceability regulations. Among the available methods, the multiplex PCR has the advantage to allow considerable saving of time and labor. This methods have been successfully used for several different purposes of identification, fish species included[28,29].

3.1 Selection of the species and of the DNA target

3.1.1 Species selection. R. esculentum and *N. nomurai* are the Asian species most exploited for human consumption[10,12].

As for the other species selected as target for the Pentaplex PCR, the *R. pulmo* was chosen due to the fact that it is one of the largest and most abundant jellyfish exclusively distributed in the Mediterranean Sea and adjacent areas[30]. The utilization of this Mediterranean jellyfish for food preparation was suggested in the work of Muhammed and Sultana[31] and by the fact that an Italian company has recently started to process *R. pulmo* for exportation to the Japanese market (author's note). Finally, Chinese people use to collect jellyfish aground on beaches and to buy jellyfish from fishermen along the Tyrrhenian coast of Italy[12]. *C. tuberculata* and *P. noctiluca* are also widely distributed in the Mediterranean Sea[32,33]. They may likely be used as food products owing to their anatomical features and wide distribution (http://www.lagazzettadelmezzogiorno.it/notizie-nascoste/meduse-buone-anche-in-cucina-e-contro-l-ipertensione-no619007/). As support of this, the molecular analyzes carried out in a previous work[12] have identified the presence of the species *P. noctiluca* in some commercial products.

3.1.2 DNA target selection. The selection of a mitochondrial molecular marker is based on its several advantages with respect to the nuclear markers, such as the higher number of copies and the greater resistance to degradation induced by chemical-physical treatments. Moreover, the higher degree of interspecific variability is particularly useful for discrimination of species very close from

a phylogenetic point of view[16,18]. Among the mitochondrial markers most used for seafood authentication (*COI*, *16srDNA*, and *cytb*), in this study, the *COI* gene was chosen due the availability of a higher number of reference sequences (Table 3). In fact, the creation of the Barcode of Life Data Systems database (BOLD) (http://www.boldsystems.org/) has gained the attention of many researchers worldwide making the first 650bp of the *COI* region one the most targeted mtDNA region for species identification[20,21,34,]. Furthermore, the *COI* gene was found to be enough informative to allow the identification of edible jellyfish species[12].

3.2. Primer design

3.2.1. Specie-specific primers targeting the COI gene. One of the main points to take into consideration during primers designing is the nature of the sample to be analyzed. In fact, a net difference exists between fresh and processed products: technological treatments, such as heating, acidification pressurization or their combination may negatively influence the integrity of DNA and hence the PCR outputs[18]. In particular, heat treatment and sharp shifts of pH were identified as the main DNA degrading factors in food[35].

In a previous work[12], we found that jellyfish products were characterized by very low pH values: ~4 and ~6 in CP and RE, respectively. Despite a certain level of DNA degradation, we were able to amplify bands of about 700bp in the 75% of the DNA extracted from RE samples and in the 44% of the DNA extracted from CP samples. However, in 100% of the DNA extracted from both kind of products, it was possible to obtain amplicons of ~ 200bp. For this reason, in this work the primers were designed in such a way as to produce amplicons with a maximum length of 200bp (Table 2).

With the aim to decrease the probability of template-independent primer interactions during amplification, which is greater in a multiplex PCR than in a uniplex, we decided to design a common forward primer (FJ2). This approach was successfully used to develop other methods based on multiplex PCR[28,29]. Considering the high variability of the *COI* gene, which makes difficult to find a common conserved region among 5 different species, we decided to design a degenerated primer. This was designed by modifying the forward primers proposed in a previous

work[12], while the reverse primers were designed on a polymorphic region of the *COI* gene. Once tested in single and multiplex reactions, the reverse primers PEL1, NEMO1 showed a high specificity for their target species (*P. noctiluca* and *N. nomurai*), producing the expected amplicon of 165 and 143 bp, respectively. No bands were obtained in case of DNA samples belonging to other jellyfish species of the Rhizostomeae and Semaestomeae order (Table 1SI). Interestingly, the primer PEL1 was able to amplify the DNA of *P. noctiluca* but not the DNA of *P. benovici*, a new *Pelagia* species recently described by Piraino *et al*.[24] For these reasons the reverse PEL1, NEMO1 were selected without modifications, which, on the contrary, were necessary for RHIZ1, RHOP1, and COT1.

The original RHIZ1 reverse primer gave the expected amplicon of 203bp on all the species belonging to the genus *Rhizostoma*, which, according to Russell[30] is composed of *R. pulmo*, *R. octopus* and *R. luteum*. While a recent study of Prieto *et al.*[23], on the basis of morphological and molecular results, confirmed that *R. luteum* is a distinct species with respect to the other two, the status of *R. octopus* is still controversial. In fact, Kramp[36] considered *R. luteum* as a variant of *R. pulmo* and Holst *et al*.[37] advocated that detailed morphological studies and molecular analyses are needed to better define the relationships among *Rhizostoma* species. For these reason, the pairwise Kimura 2-parameter molecular divergence between *R. pulmo* and *R. octopus COI* sequences (Table 3) was calculated. The low values found (6%) suggest that *R. pulmo* and *R. octopus* cannot be unquestionably considered as distinct species by a molecular point of view, supporting the theory of Kramp[36]. In fact, the literature suggests that 10–20 % *COI* sequence divergence may be a suitable benchmark value to define species[38].

In the light of this considerations, the reverse primer RHIZ1 was improved: two new primers were produced (Table 2) by introducing single (RHIZ1-1) and multiple (RHIZ1-2) base modification at different positions, with the aim to create additional mismatches on the annealing region of *R. luteum COI* sequence. RHIZ1-2 was discarded due to its inability to amplify the DNA

283 of both *Rhizostoma* species, while the reverse RHIZ1-1 was selected for its specificity in uniquely
284 amplifying the DNA of *R. pulmo*.

285 The primer RHOP1 produced the 101bp amplicon from the DNA of *P. benovici*, other than of *R.*
286 *esculentum*, due to the presence of only one difference on the annealing sequence of the two
287 species. For these reason, the primer RHOP1 was modified (RHOP1-1) near to the 3'-end. In fact,
288 mismatch at or near the terminal 3′ base of a primer affects PCR more dramatically than those
289 single mismatches located internally or at 5′ end[39]. The new primer RHOP1-1 gave the expected
290 amplicon only in presence of DNA of *R. esculentum*. Likely, selectivity of amplification using these
291 primers has been increased by the stringent conditions adopted for the amplification protocol (see
292 section 2.6). In fact, to avoid unspecific amplification we used an annealing time of only 5 sec.

293 Finally, even though specific, the reverse primers COT1 was re-designed (COT2) due to its low
294 amplification performance.

295 *3.2.2. Control PCR primers targeting 28SrRNA gene*

296 The extraction of DNA contaminated by inhibitors is one of the main obstacles associated with
297 PCR application in complex matrices, such as food. In fact, the inhibitors can interfere with the
298 reaction, even leading to a complete amplification inhibition[18]. In our previous study[12], we found
299 that Al^{3+} ions may contribute to the overall DNA amplification failure. In fact, the initial
300 concentration of the aluminum in jellyfish food products is extremely different[12,40] and, even though
301 the pre-extraction procedure can reduce its concentration, it is impossible to predict the residual
302 amount in different DNA samples. Therefore, considering that inhibition may be the cause of false-
303 negative reactions, we decided to introduce a reaction control. This consideration, together with the
304 high degradation level of the DNA extracted from MS, led us to design control primers for the
305 amplification of a short DNA fragment of 55bp.

306 Ribosomal genes, such as the *16sRNA* gene, are characterized by a low mutation rate[41]. In this
307 study, the overall mean distance calculated on a fragment of 1480bp belonging to the *28S rDNA*
308 gene sequences (those produced by Bayha *et al*.[26] together with the sequence of *N. nomurai*

produced in this study) was 2%. In the light of these results and taking into consideration that in a previous study[28] the high degree of conservation has played a decisive role for designing primers to be used for the amplification of many species at once, the *28SrRNA* gene was selected as target for designing the control primers.

3.3 Pentaplex PCR assay: development and final protocol

The optimization of a multiplex PCR can imply several difficulties. In fact, the presence of more than one primer pair in the reaction increases the chance of obtaining spurious amplification products and may increase the possibility to obtain the formation of primer dimers. Increasing the concentration of PCR reagents during the optimization step can increase the likelihood of mispriming, with subsequent production of nonspecific amplification[42]. However, optimization of these components is usually beneficial. For the aforesaid reasons the final reaction protocol was selected through a process in which different concentration of primers, $MgCl_2$, nucelotides, and Taq polymerase were tested. In the meanwhile, the best cycling conditions were selected by testing different temperatures and times.

3.3.1. Primers and *DNA template amount.* The primers were used at the same concentration during the first trials (100 nM). Subsequently, the concentrations were modified with the aim to obtain bands of similar intensity. In general, due to the good efficiency of the reverse primers, the concentration was maintained in the range of 100-200 nM. Only the degenerated forward primer was used at a higher concentration (300 nM). Overall, primers' concentrations were lower than those at which it was observed the formation of considerable amount of primer-dimer (~ 500 nM)[42]. We found that the introduction of the control primers (28sfor55; 28srev55) reduced the amplification efficiency of some specific primers (PEL1 and NEMO1), even when used at low concentration (50, 100 nM). For this reason, both nucleotides and Taq polymerase concentration were increased (see section 3.3.3).

Another fundamental step for the obtaining of a specific amplification, strictly connected with primers concentration, is the selection of an adequate DNA template concentration. In fact, if the

primer-to-template ratio is too low, specific products will not accumulate exponentially while primer dimers may be amplified more efficiently than the desired target[42,43]. In this study, the utilization of total DNA quantities between 50 and 200 ng per reaction did not bring any significant difference regarding bands intensity. On the contrary, DNA quantities over 100 ng negatively affected the background of the gel after electrophoresis, especially in case of DNA extracted from market samples. In fact, when degradation of DNA occurs, small DNA fragments may unspecifically be involved in the reaction, producing spurious amplification products. For this reason 50 ng were used as working concentration.

3.3.2. $MgCl_2$, nucelotides and Taq polymerase. To test the influence of $MgCl_2$ different trials were performed keeping constant the dNTP concentration and gradually increasing magnesium chloride from 1 to 5 mM. Using the standard concentration of dNTP (200 μM), the amplification became more specific and the products acquired comparable intensities at 3 mM: further increase showed a detrimental effect. Then, considering that in a multiplex PCR more amplicons are simultaneously amplified and the pool of enzymes and nucleotides becomes a limiting factor[44], and in the light of the results obtained after the addition to the reaction mixture of the control primers, we decided to test doubled concentration of these reagents (400 μM of dNTP and 1U of TAQ Polymerase). The reaction efficiency was sensibly improved and, even though dNTPs directly chelate a proportional number of Mg^{2+} ions, we did not find any advantage using higher concentration of $MgCl_2$.

3.3.3 Cycling condition. All the primer pairs used in a multiplex PCR should enable similar amplification efficiencies for their respective targets. This may be achieved through the utilization of primers with very similar optimum annealing temperatures. In this work, the average melting temperature of the species-specific reverse primers was 50.5°C , while the degenerated reverse has a melting temperature of 53.7°C. Even though our primers produced the expected amplicon in the range of 47-51°C, an annealing temperature of 49°C was selected. This temperature facilitate the annealing of the degenerated FJ2 primer and increased the specificity of the reverse primers. This

probably has enhanced the specificity of our Pentaplex method allowing to avoid unspecific amplification even in case of close species.

3.4 Validation of the Pentaplex PCR assay using market samples DNA

Once optimized on DNA extracted from RS and part of the MS (Figure 1), the final Pentaplex PCR protocol was applied to all the DNA samples extracted from MS identified by FINS in a previous work[12]. In particular, 48 RE and 30 CP DNA samples were tested. The results of the amplification are reported in Table 1, while the percentages of successful amplification are summarized in Table 4. Overall, in the case of RE, the Pentaplex PCR was capable to specifically amplify 85.4% of DNA. The higher percentage of amplification (95%) was obtained using those DNA samples from which a sequence of 658bp (amplicon including primers of 709bp) was previously amplified, while lower percentages were obtained in case of more degraded DNA samples (Table 4). In case of DNA extracted from CP, the Pentaplex PCR was less effective. Overall, it was able to correctly amplify ~60% of tested samples. Moreover, the absence of specific amplicons in 3 CP sample that have not been identified at the species level by the phylogenetical analysis (identity value of 86% with *N. nomadica* and 85% with *C. stuhlmanni*) can be considered as a further demonstration of the specificity of the proposed method.

The lower rate of success in case of CP with respect to RE could be attributed to the presence of strongly degraded DNA extracted from products characterized by lower pH values (see section 3.2.1), or other than to the action of inhibitors, such as aluminum. In fact, in all the DNA from which the specie-specific band was not obtained, the 55bp control amplicon was always visible (Figure 1). Furthermore, in a previous study[12] we found that the inhibitory effects affected more the longest than the shortest ones.

This results might seem to be in contrast with those obtained in our previous study[12], in which an amplicon of 145bp was amplified even from the most degraded DNA from market samples. One possible explanation could be related to the more stringent cycling conditions adopted to increase specificity. In PCR protocols using degenerated primers, the difference between the melting (MT)

and the annealing temperature (AT) should be in between 10 and 20°C[45]. In the previous work, in which only degenerated primers were used, AT was 9°C lower than the mean MT of the primers. In this Pentaplex PCR, the low difference (4.7°C) between MT and AT of primers could have led to a more difficult annealing. Finally, multiplex PCRs are proven to be less sensitive than single PCR reactions[46].

3.5. Jellyfish labeling: issues and outlooks

All the jellyfish recorded in FAO statistics belong to the order Rhizostomeae and in particular to the genus *Rhopilema*[47]. These statistics are quite questionable considering that at least 11 species in 5 families are known to be exploited worldwide.

Most of the Asian jellyfish are exported to Japan and the remainder are sold to South Korea, Taiwan, Singapore, Hong Kong, USA[10,48], and Europe[11,12]. While in the Eastern Asian Countries the jellyfish food products are usually categorized in 8 types based on the color, form, texture, and size of the semi-dried products[48], the Western Countries have tried to regulate the commercialization of these products by establishing a specific labeling.

In USA , the acceptable market name of jellyfish has been associated to all the species belonging to the genus *Rhopilema* (http://www.accessdata.fda.gov/scripts/fdcc/?set=seafoodlist&id=%3Ci%3ERhopilema%3C/i%3E%20spp.&sort=SLSN&order=ASC&startrow=1&type=basic&search=jellyfish). In Italy, the Ministry of Agriculture Food and Forestry Policies (MIPAFF) has adopted the provisional commercial denomination of "Asian jellyfish" for the species *R. esculentum*. At present, to the best of our knowledge, no other EU member Countries has defined a commercial denomination for jellyfish species.

We think that, due to the impossibility to identify the species in the commodity by a visual inspection, some traceability information, as for example the commercial and scientific denomination, have been deduced at official level from the importation documents. In fact, animal products from Third Countries outside the EU must be imported via a port or an airport with a

Border Inspection Post (BIP), the official office authorized by the Ministry of Health in performing the control according to the European legislation. In fact, the official controls shall include at least a systematic documentary check, a random identity check and, where appropriate, a physical check. However, physical check, which could include checks on the means of transport, on the packaging, labelling and temperature, the sampling for analysis and laboratory testing and any other check necessary to verify compliance with the food laws are performed with a frequency depending on: the risks associated with different food; the history of compliances with the requirements for the product concerned of the third Country; the controls that the food business operator importing the product has carried out and, finally, the guarantees that the competent authority of the third Country of origin has given[49].

In the US, the absence of adequate documentation of fish products entering the market is a serious impediment to establishing the legal origin[50]. Moreover, the incredible rate of adulteration of fish products found in the US market reflected not only the weak collaboration between FDA (Food and Drug Administration) and CBP (Customs and Border Protection) and NMFS (National Marine Fisheries Service), but also the inadequate attention on this issue from FDA[51]. Therefore, new food products coming on the market may be not properly identified.

The hypothesis that, in Italy, the commercial name adopted for Asian jellyfish is not correct seems to be confirmed by one molecular survey previously carried out in our laboratory[12], which highlighted how the most part of jellyfish food products sold in Italy were *N. nomurai* instead of *R. esculentum*. Moreover, the identification of other species such as *R. pulmo* and *P. noctiluca* put in evidence the need of a revision of the commercial denomination proposed for these products. For example, the denomination of "Asian jellyfish" should be extended also to the species *N. nomurai* and a new denomination for the Mediterranean species *R. pulmo* should be defined.

It follows that, as specified by the Regulation (EU) N. 1379/2013[14]: "*the available technologies, including DNA-testing, should be used to protect the consumer and in order to deter operators from falsely labeling catches*". In particular, considering the strategic position of the BIPs in the

international commerce framework, it could be very important, especially in case of new products (species), to establish a standardized checking procedure to be used during the inspection, to correctly verify the labeling information. This could allow not only to avoid the commercialization of mislabeled products, but to also clearly assess the seafood product characteristics before proposing new official commercial denominations. In fact, in the case of Italy, the number of these denominations has already overcome the nine hundreds[21].

Jellyfish populations appear to be increasing in the majority of the world's coastal ecosystems and seas. Due to the fact that jellyfish populations can have an important impact on human activities and marine ecosystems, but, at the same time, they also represent a valuable source of food, it is very important to increase the knowledge regarding the edible species actually exploited worldwide. Although the DNA sequencing appears to be the most commonly applied approach for fish species identification across European laboratories[17], this procedure needs expensive equipment and long-times of execution. On the countrary, the Multiplex PCR assay developed in this work represents an useful test for the screening of samples and allows to speed up the analysis flow and to reduce costs. Finally, this method represents a useful tool to enforce the law on traceability and to prevent mislabeling in the seafood chain.

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Supporting Information description

Table 1SI. Reference samples obtained in this study (highlighted in grey) and in previous studies (Armani et al., 2013; Pietro et al., 2013) used for the development of the Pentaplex PCR assay.

Table 2SI. Mismatches between the reverse specie-specific primers selected for the target jellyfish species and the sequences of other species.

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Figure caption

Fig. 1 Electrophoresis on agarose gel (4%) of the five species of jellyfish selected as target after the Pentaplex PCR. Line 1) C. tuberculata (72bp) ; 2) R. esculentum (101bp); 3) N. nomurai (143bp); 4) P. noctiluca (165bp); 5) R. pulmo (203bp); 6) DNA from RE product; 7) DNA from CP product; 8) H2O. DNA ladder band size: 1000 bp, 900 bp, 800 bp, 700 bp, 600 bp, 500 bp, 400 bp, 300 bp, 250 bp, 200 bp, 150 bp, 100 bp, 50.

615
616
617 **Tables**

Samples	City and place of collection	Sequence length and code	BLAST (Max identity)		Pentaplex PCR results
			Bold	GenBank	
RE-2	Prato / M	658 – HF930536	100% *N. nomurai*		✓ 143pb
RE 3	Prato / M	658 - HF930537	100% *N. nomurai*		✓ 143pb
RE-5	Milan / M	658 - HF930538	100% *N. nomurai*		✓ 143pb
RE-6	Milan / M	658 - HF930539	99% *N. nomurai*		✓ 143pb
RE-8	Prato / M	658 - HF930540	100% *N. nomurai*		✓ 143pb
RE-9	Prato / M	658 - HF930541	99% *N. nomurai*		✓ 143pb
RE-10	Florence / M	658 - HF930542	99% *N. nomurai*		✓ 143pb
RE-11	Rome / M	658 - HF930543	100% *N. nomurai*		✓ 143pb
RE-12	Rome / M	658 - HF930544	100% *N. nomurai*		✓ 143pb
RE-13	Rome / M	658 - HF930545	100% *N. nomurai*		✓ 143p
RE-14	Rome / R	658 - HF930546	NM	100% *R. esculentum*	✓ 101pb
RE-15	Rome / M	658 - HF930547	100% *N. nomurai*		✓ 143pb
RE-19	Rome / M	658 - HF930548	99% *N. nomurai*		✓ 143p
RE-21	Florence / M	658 - HF930549	100% *N. nomurai*		✓ 143pb
RE-24	Florence / R	658- HF930550	NM	100% *R. esculentum*	✓ 101pb
RE-27	Milan / M	658 - HF930551	99% *N. nomurai*		✓ 143pb
RE-40	Prato / M	658 - HF937280	99% *N. nomurai*		✓ 143pb
RE-43	Prato / R	658 - HF937281	99% *N. nomurai*		NB
RE-44	Prato / R	658 - HF937282	NM	100% *R. esculentum*	✓ 101pb
RE-48	Florence/M	658 - HF937283	100% *N. nomurai*		✓ 143pb
RE-28	Milan / M	206 - HF937294	100% *N. nomurai*		✓ 143pb
RE-29	Milan / M	206 - HF937295	100% *N. nomurai*		✓ 143pb
RE-34	Prato / M	206 - HF937296	100% *N. nomurai*		✓ 143pb
RE-38	Prato / M	206 - HF937297	100% *N. nomurai*		✓ 143pb
RE-39	Prato / M	206 - HF937298	100% *N. nomurai*		✓ 143pb
RE-41	Prato / M	206 -	100% *N. nomurai*		✓ 143pb

		HF937299			
RE-42	Prato / M	206 - HF937300	100% *N. nomurai*		NB
RE-45	Florence/M	206 - HF937301	100% *N. nomurai*		NB
RE-46	Florence/M	206 - HF937302	100% *N. nomurai*		✓ 143pb
RE-47	Florence/M	206 - HF937303	99% *N. nomurai*		✓ 143pb
RE-4	Prato / M	206 - HF937288	100% *N. nomurai*		NB
RE-16	Rome / M	206 - HF937289	100% *N. nomurai*		✓ 143pb
RE-20	Florence / M	206 - HF937290	100% *N. nomurai*		✓ 143pb
RE-23	Florence / M	206 - HF937292	100% *N. nomurai*		✓ 143pb
RE-22	Florence / M	206 - HF937291	100% *N. nomurai*		✓ 143pb
RE-26	Prato / M	206 - HF937293	100% *N. nomurai*		✓ 143pb
RE-30	Milan / M	145 - HF937318	100% *N. nomurai*		✓ 143pb
RE-31	Milan / M	145 - HF937319	100% *N. nomurai*		✓ 143pb
RE-32	Prato / M	145- HF937320	100% *N. nomurai*		✓ 143pb
RE-33	Prato / M	145 - HF937321	100% *N. nomurai*		✓ 143pb
RE-35	Prato / M	145 - HF937322	100% *N. nomurai*		NB
RE-36	Prato / M	145 - HF937323	100% *N. nomurai*		✓ 143pb
RE-37	Prato / M	145 - HF937324	100% *N. nomurai*		✓ 143pb
RE-1	Prato / M	145 – HF937313	100% *N. nomurai*		NB
RE-7	Milan / M	145 – HF937314	100% *N. nomurai*		NB
RE-17	Rome / M	145 – HF937315	100% *N. nomurai*		✓ 143
RE-18	Rome / M	145 - HF937316	100% *N. nomurai*		✓ 143
RE-25	Prato / M	145 - HF937317	100% *N. nomurai*		✓ 143
CP-7	Prato / M	658 - HF937284	99% *N. nomurai*		NB
CP-8	Prato / M	658 - HF937285	100% *N. nomurai*		143
CP-24	Zhejiang (PRC) / M	658 - HF937287	NM	100% *R. esculentum*	101
CP-13	Rome / M	658 - HF937286	NM	99% *R. pulmo*	203
CP-30	Jangsu (PRC) / M	658 - XX	99% *N. nomurai*		143
CP-10	Florence/M	206 - HF937305	100% *N. nomurai*		NB

CP-9	Prato / M	206 - HF937304	NM	100% *R. esculentum*	NB
CP-11	Rome / M	206 - HF937306	NM	100% *R. esculentum*	✓ 101
CP-15	Rome / M	206 - HF937308	100% *N. nomurai*		✓ 143
CP-12	Rome / M	206 - HF937307	100% *N. nomurai*		✓ 143
CP-18	Florence/M	206 - HF937309	99% *N. nomurai*		✓ 143
CP-21	Prato / M	206 - HF937310	NM	86% *R. nomadica*	✓ NB
CP-27	Zhejiang (PRC) / M	206 - HF937311	NM	100% *P. noctiluca*	✓ 165
CP-28	Zhejiang (PRC) / M	206 - HF937312	NM	100% *P. noctiluca*	NB
CP-1	Prato / M	145 - HF937325	NM	100% *R. esculentum*	✓ 101
CP-2	Prato / M	145 - HF937326	NM	100% *R. pulmo*	✓ 203
CP-3	Prato / M	145 - HF937327	99% *N. nomurai*		NB
CP-4	Prato / M	145 - HF937328	99% *N. nomurai*		✓ 143
CP-5	Prato / M	145 - HF937329	NM	100% *R. esculentum*	NB
CP-6	Prato / M	145 - HF937330	100% *N. nomurai*		✓ 143
CP-14	Rome / M	145 - HF937331	NM	100% *P. noctiluca*	NB
CP-16	Rome / M	145 - HF937332	NM	100% *P. noctiluca*	NB
CP-17	Rome / M	145 - HF937333	NM	100% *R. esculentum*	✓ 101
CP-19	Prato / M	145 - HF937334	100% *N. nomurai*		✓ 143
CP-20	Prato / M	145 - HF937335	NM	85% *C. stuhlmanni*	✓ NB
CP-22	Prato / M	145 - HF937336	NM	85% *C. stuhlmanni*	✓ NB
CP-23	Zhejiang (PRC) / M	145 - HF937337	NM	100% *P. noctiluca*	NB
CP-25	Zhejiang (PRC) / M	145 - HF937338	NM	100% *P. noctiluca*	✓ 165
CP-26	Zhejiang (PRC) / M	145 - HF937339	NM	100% *P. noctiluca*	NB
CP-29	Jangsu (PRC) / M	145 - XX	NM	100% *R. esculentum*	✓ 101

618

619 Table 1. DNA market jellyfish products identified in a previous study (Armani et al., 2013) and used in this
620 study for the validation of the Pentaplex PCR assay. RE: ready-to-eat, CP: classical products. NB: No bands.

621

Primers code	Sequence 5'-3'	bp	MT	AL
FJ1	CAAACCATAAAGAYATWGG	19	49.1	
FJ2	CAAACCATAAAGAYATTGGWAC	22	53.7	
RHIZ1	ACCTCCTATCAACACTG	17	50.4	203
RHIZ1-1	ACGTCCTATCAACACTG	17	50.4	
RHIZ1-2	ACGTCGTATCAACACTG	17	50.4	
PEL1	TTATTAGGGCGTGAGC	16	49.2	165
NEMO1	AACTACGTTATAAAGTTGG	19	50.2	143
RHOP1	ACCTGATAGTTCTAATCTA	19	48	101
RHOP1-1	ACGCGATAGTTCTAATCTA	19	50.2	
COT1	GCCGGCACCTATAATT	16	49.2	68
COT2	GGCACCTATAATTCCG	16	49.2	72
28sfor55	TGGTTACAACGGGTGA	16	49.2	55
28srev55	TCTCAGGCTCCCTCT	15	50.6	
F28s	ACTGTGAAACTGCGAATG	18	52.4	1598
R28s	CCATTCAATCGGTAGTAGC	19	51.1	

622

623 Table 2. Primers designed for setting up the Pentaplex PCR assay. In grey primers finally selected. bp= base
624 pair; MT= Melting Temperature; AL= Amplicon length.

625

Species	Sequences	
	Number	Accession Code
Aurelia aurita	40	AY903093 – 95; AY903117 – 18 AY903208 – 12; **HF968752** JQ353726 – 28; JQ623914 JX995329; KC311384 – 85 KC440127; KC789074-93
Cassiopea andromeda	24	AB563739-40; AY319448-62 AY319471-73; AY331593-95; **HF930521**
Cassiopea frondosa	5	AY319467; AY319469-70 HF930515; HF930520
Cassiopea xamachana	5	AY319463-66; AY319468
Catosylus mosaicus	70	AY319476; AY737184– 247 **HF548537 – 39; HF968746 – 47**
Cotylorhiza tuberculata	5	**HF930531 - 35**
Crambionella orsini	2	EU363343-44
Cyanea annaskala	7	AY902912-17; AY902923
Cyanea capillata	21	AY902911; AY902924; HF930525-26; JX995329-46
Cyanea lamarckii	14	JX995347-57; JX995360-62
Cyanea rosea	5	AY902918-22
Drymonema dalmatinum	5	HQ234614-17; HQ234621
Drymonema larsoni	9	HQ234610-13; HQ234618-20 HQ234622; HQ234650
Lychnorhiza lucerna	5	**HF930522; HF968748 - 51**
Mastigias sp.	132	AY902925-99; AY903000-51 EU363340; JN215543-46
Nemopilema nomurai	15	AB243416; EU373728; GU145135 **HF536563 – 70;** HM045296 JX845348; JQ353746 – 47
Pelagia noctiluca	183	GQ120093 – 96; GQ375903 - 6025 HE591457; **HF930527 – 30** HM358383- 403; JQ697960 - 74 JX235428 - 42
Phyllorhiza punctata	3	EU363341-42; GQ120101
Rhizostoma luteum	3	**HF937340-41; HF545309**
Rhizostoma octopus	63	HQ425417-79
Rhizostoma pulmo	24	GQ999568 – 71; **HF536559 - 62 HF545304 – 08; HF930513;** HG931669; HQ902114 - 22
Rhopilema esculentum	12	EU373722 – 24; **HF536571 - 74 HF930514 – 15;** JQ353756 – 57 JX845347
Rhopilema nomadica	5	JN378391; HG931668; HF930516-18

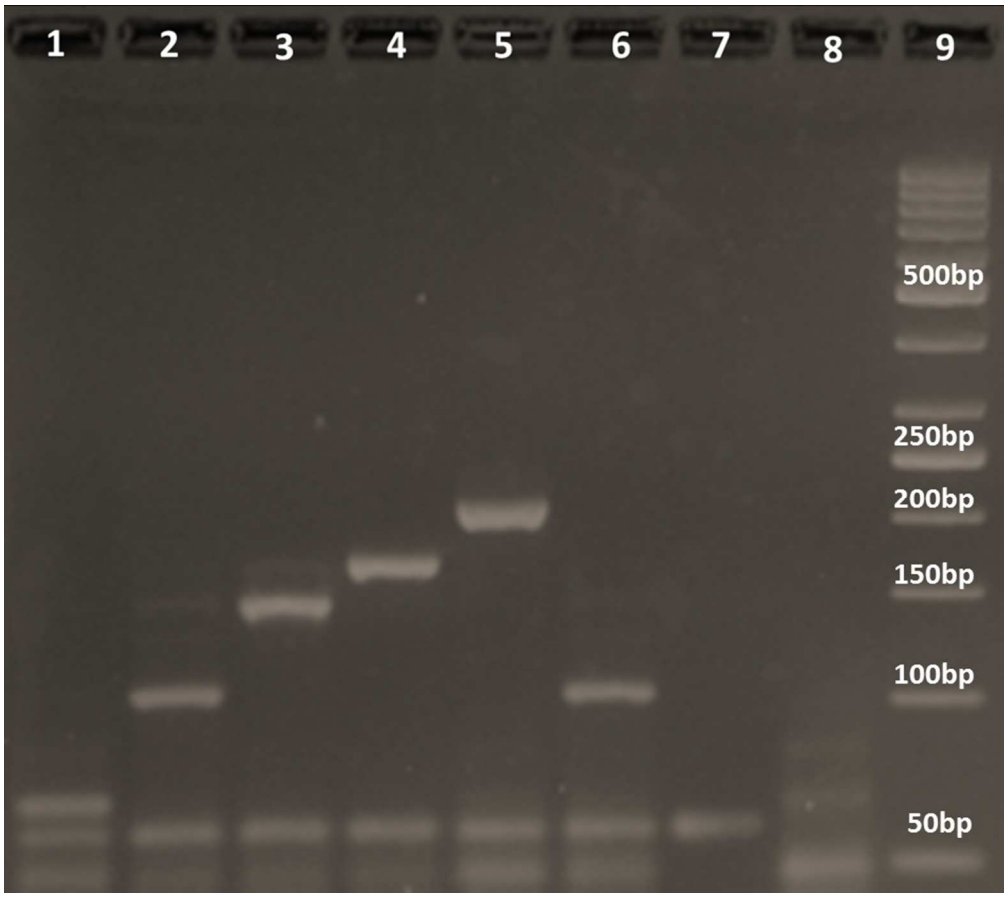
626

627 Table 3. Reference sequences used in this work for the projecting of the primers for the Pentaplex PCR
628 assay. Sequences in bold have been produced in this or previous studies (Armani et al., 2013; Prieto et al,
629 2013).

Sequence length (with primers)	709bp		256bp		193bp		Total	
	RE	CP	RE	CP	RE	CP	RE	CP
Tested samples	20	5	16	9	12	16	48	30
Positive samples	19	4	13	5	9	8	41	17
Percentage of positive samples	95%	80%	81%	55.5%	75%	37.5%	85.4%	57%

630

631 Table 4. Amplification rate of DNA extracted from market samples using the Pentaplex PCR method. RE:
632 Ready to Eat products, CP: Classical Products.



Electrophoresis on agarose gel (4%) of the five species of jellyfish selected as target after the Pentaplex PCR. Line 1) C. tuberculata (72bp) ; 2) R. esculentum (101bp); 3) N. nomurai (143bp); 4) P. noctiluca (165bp); 5) R. pulmo (203bp); 6) DNA from RE product; 7) DNA from CP product; 8) H2O. DNA ladder band size: 1000 bp, 900 bp, 800 bp, 700 bp, 600 bp, 500 bp, 400 bp, 300 bp, 250 bp, 200 bp, 150 bp, 100 bp, 50.
160x142mm (150 x 150 DPI)