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Title: What is inside the jar? Forensically informative nucleotide sequencing (FINS) of a short mitochondrial COI gene fragment reveals a high percentage of mislabeling in jellyfish food products.

Article Type: Research Article

Keywords: DNA sequencing, COI gene, mislabeling, jellyfish, PCR inhibitors, degraded DNA, seafood identification, ethnic food.

Corresponding Author: Dr. Andrea Armani,

Corresponding Author's Institution:

First Author: Andrea Armani

Order of Authors: Andrea Armani; Lara Tinacci; Alice Giusti; Lorenzo Castigliego; Daniela Gianfaldoni; Alessandra Guidi

Abstract: The jellyfish belonging to the Rhizostomeae order are a typical Asian seafood and are nowadays also marketed in the Western Countries. The jellyfish species used for food cannot be identified due to the loss of the morphological characteristics during processing. In this work, a pre-extraction treatment consisting in desalting under running water for 48h, followed by an incubation in 400 mM EDTA solution for 1h was developed for products containing high concentration of salts. Moreover, considering the DNA degradation observed in both classical (CPs) and ready to eat (RE) commercial samples, five sets of primers for the amplification of fragments with different length belonging to the mitochondrial COI gene were designed and used to obtain the sequences from 54 reference specimens and 70 market samples. A phylogenetic analysis was then performed using the Neighbor-joining method. It was found that a fragment of 142bp was enough informative to allow an identification at the species level. The results showed that most of the products were made of N. nomurai (94% of RE and 45.5% of CPs). The analysis permitted to uncover an alarming level of mislabeling which reaches 79% for CPs and 100% for ready-to-eat products.

*Cover Letter

Dear Editor,

We would like to submit the following manuscript for possible publication.

"What is inside the jar? Forensically informative nucleotide sequencing (FINS) of a short mitochondrial *COI* gene fragment reveals a high percentage of mislabeling in jellyfish food products"

Some kinds of ethnic foods, such as jellyfish, in the past available only in certain Asian countries, are nowadays actively exported and commercialized in the Western markets. In a previous work, in which we assessed the labeling conformity of dry-salted jellyfish products sold within the Italian's Chinese communities, we found many incongruences and shortfalls mainly referable to the trade name.

Considering that the inspection of this seafood is made more difficult by both processing, which completely alter the morphological characteristics, and by the presence of cryptic species, in this work, we developed a molecular method, based on FINS, for the identification of the edible jellyfish sold on the European market.

First we set up a pre-extraction treatment that allowed to obtain amplifiable DNA from dry-salted jellyfish and then, by using new degenerated primers, we obtained sequences of different length belonging to the mitochondrial cytochrome c-oxidase subunit I gene (*COI*) both from fresh and from processed jellyfish, whose DNA is heavily degraded. Finally, by a phylogenetical analysis we identify the 96% of the market samples at the species level and discovered an alarming level of mislabeling which reaches 100% for ready-to-eat products.

The proposed method could be applied not only by the Food and Health Authorities to assess the authenticity of imported products and prevent commercial fraud, but also to enforce fishery control.

Best regards,

Andrea Armani

Dear Editor,
we revised the manuscript as suggested by Reviewers 2 and 3 and here below you can find our answers, comments and rebuttals.

Reviewer #1:
Your manuscript is the original research work contributing to the new knowledge in the field with possible application in control of authenticity of seafood products. The manuscript is excellent written, the main idea and message of the manuscript is clear presented and discussed, the methodological improvement and optimization of DNA extraction from products with high concentration of salt is very beneficial for wide audience and readership and presents a step on in molecular identification of seafood.
I suggest accepting the manuscript in Food Research International without further revision.

Reviewer #2: Armani et al. have developed a very interesting approach to solve the authentication and mislabeling of the foodstuff with edible jellyfish based on FINS methodology. They complement the PCR identification method with the proper strategy for obtaining a good quality DNA from samples highly contaminated and degraded.
Four sets of primers were used for the phylogenetic study of the reference samples. The results showed that a fragment of 142 bp allowed the species identification of the samples.

Would it be possible to develop a primer set for amplification on this fragment? Maybe, the study of the melting temperatures of 142 bp fragment could allow a preliminary identification or discrimination among species prior to the identification by sequencing.

As reported in the manuscript a couple of primers able to amplify a fragment of 142bp from the most part of the edible jellyfish have already been developed. The idea to develop a melting analysis based on the fragment has already been discarded based on a few trials, which gave some overlapping melting temperatures. Indeed, the probability that the melting analysis performed on this fragment could produce a melting temperature able to discriminate the different species is very low: these primers have been designed on the most conserved zone of the *COI* gene (by aligning 75 sequences belonging to 14 species of 5 different families of Rhizostomeae and 54 sequences belonging to 11 species of 3 different families of Semaestomeae). A few changes in base pairs in a fragment of the same length often lead to very small or no changes in melting temperature.
For these reason we are planning to develop a multiplex PCR for the rapid identification of the edible jellyfish. However, this could be an undertaking path.

Some specific comments:

1. **The manuscript needs an extensive orthographical revision.**

The manuscript has been revised.
Some examples:
- **Line 44, add full stop behind "Scyphozoa".**

A full stop has been added.
- **Lines 131, 135, and 197, replace full stop instead of comma in "1,25 U", "1,8%", and "2,5 U".**

A full stop has been added as suggested
- **Line 132, add comma behind "Polymerase", and behind "DNA".**

A comma has been added
- **Line 159, add comma behind "Steffens et al."**

A comma has been added

- **Line 187, add full stop behind "Armani et al", etc.**

A full stop has been added.

2. **The reference list is given in a confused way. The references must be arranged by alphabetic order.**

The reference list has been arranged alphabetically.

3. **the caption of figure 3, I think that the tracks of the molecular markers are mixed up.**

In the caption of figure 3 the molecular marker is not mentioned. In the captions of fig. 1 and 2 the tracks of the molecular markers are correct.

4. **The quality of the figure 3 needs to be improved. The bootstrap values and the accession numbers are illegible.**

The figure has been remade. However, it is advisable to click on the link on the pdf page containing this image to see the high resolution image.

5. **In the head of the table 3. Does AL mean amplicon or primer length?**

AL in column n°4 has been replaced with PL (Primer Length). AL (Amplicon Length) has moved to column n°6.

6. **To me, it would be desirable that the authors provide the alignment reports of the sequences in the manuscript or in the supplementary data.**

We agree with the reviewer, in producing and publishing, as supplementary material, the alignment of the sequences used for the projecting of the primers FFDL and FRDL2 and of the internal reverse primers REV514A, REV256A, REV193HS, REV163, REV133. Table 1SM and 2SM have been added to the manuscript.

Reviewer #3: The authors report an interesting study concerning the authentication and labeling of Jellyfish products using DNA sequencing and phylogenetical analysis of a COI gene fragment. The area should be of interest to readers of the journal, especially, with increased sale of this products and the importance of labeling conformity.
It is an interesting manuscript deserving to get published in Food Research International subject to the following corrections:

There are numerous sentences which are very long and confusing. I strongly encourage the authors to carefully go through the entire manuscript and break the long sentences into smaller, easily understandable sentences. These long sentences are rather confusing and quite difficult to understand.

We went through the manuscript again and we asked an opinion to a native English speaker. Some sentences have been split. However, others up to about three lines, difficult to be split without losing the precise meaning and judged to be understandable, have not been changed.

Specific Comments

Abstract

In abstract should be considered that the DNA extraction protocol was already described in previous publications although the pre-treatment used wasn´t and is some novel information.

The sentence “In this work, a DNA extraction protocol was developed” of the first version has been changed with “In this work, a pre-extraction treatment consisting in desalting under running water

for 48h, followed by an incubation in 400 mM EDTA solution for 1h was developed for products containing high concentration of salts" (lines 30-32).

Maybe including more numerical results so that the abstract becomes more concrete would be important for the readers.
Numerical results has been added. This sentence has been added to the abstract "Results showed that most of the products were made of *N. nomurai* (94% of RE and 45.5% of CPs). The analysis permitted to uncover an alarming level of mislabeling which reaches 79% for CPs and 100% for ready-to-eat products" (lines 38-40; lines 39-41 in the file with tracked corrections).

Introduction: The introduction of some more recent references should be evaluated such as:
Cladistic analysis of Medusozoa and cnidarian evolution
Author(s): Marques, AC (Marques, AC); Collins, AG (Collins, AG)
Source: INVERTEBRATE BIOLOGY Volume: 123 Issue: 1 Pages: 23-42 Published: 2004
This reference has been added in the text (line 48; line 50 in the file with tracked corrections).

Cnidarian phylogenetic relationships as revealed by mitogenomics
Author(s): Kayal, E (Kayal, Ehsan)[1,2] ; Roure, B (Roure, Beatrice)[3] ; Philippe, H (Philippe, Herve)[3] ; Collins, AG (Collins, Allen G.)[4] ; Lavrov, DV (Lavrov, Dennis V.)[1]
Source: BMC EVOLUTIONARY BIOLOGY Volume: 13 Article Number: 5 DOI: 10.1186/1471-2148-13-5 Published: JAN 9 2013
This reference has been added in the text (line 110; line 116 in the file with tracked corrections).

Material and Methods
In the materials and methods is necessary to review all the sequential numbering. Example:
Line 111: 2.4 followed by 2.4.2 on line 129 (where is 2.4.2?)
Line 162: 2.4.4 followed by 2.6 on line 166 (where is 2.5?)
The sequential numbering has been corrected.
Section 2.2 and section 2.6.2 may be could be re-organized and merged since are both DNA extraction procedures.
In our opinion, even though both sections describe DNA extraction procedures, they should remain separate. In fact, the section 2.6.2 (now 5.2.2) is more understandable after the information reported in the section 2.6.1 (now 5.2.1) regarding the "Assessment of PCR inhibitors".

Although in the results are presented some data concerning the aluminum content (3.4.1) of the samples the method used is not described in this section.
As reported in the new version of the manuscript the analysis for the determination of the total content of Aluminum were commissioned to a private company (the name of which has been added in the text) and not performed in our labs. For this reason we did not report the specific protocol used for the determination but we only mentioned the method "atomic absorption spectroscopy" (line 138; line 145 in the file with tracked corrections). However, a specific section 2.1.3. pH and Aluminum determination (lines 135-139; lines 142-146 in the file with tracked corrections) has been added in M&M.

Line 94: How was avoided the DNA contamination?
Each samples was minced with different pairs of scissors. After mincing, a thorough washing with soap was done. After that, two cycles of 20 min each of autoclave (120°C) was done.. Furthermore, the pH was measured on the liquid drainage and not on the tissue used for the DNA extraction.

Line 94: "pH was measured" All the equipment need to have the following details: Model #, equipment manufacture's name, city of the manufacturer, country of manufacturer.

The details have been added (line 136; line 143 in the file with tracked corrections).

Line 97: RT "room temperature"
Done (line 142; line 149 in the file with tracked corrections)

Line 127-128: "Finally, Bovine Serum Albumin (BSA) was tested at the following final concentration 200, 100, 50, 25 and 0 ng/<mu>L" for what purpose?
Among the many substances that act as "enhancing agents" in increasing the yield, specificity and consistency of PCR reactions, the Bovine Serum Albumin (BSA) is one of the most used. However, the concentration of this molecule must be accurately assessed because a too high concentration can have an adverse effect on the PCR reaction. For this reason, different concentrations have been tested. The purpose has been added in the manuscript and the following sentence has been added: "to select the most appropriate concentration able to enhance PCR reaction performances." (lines 176-177; lines 183-184 in the file with tracked corrections)

Line 131 - Line 132-Line 135: All the materials need to have the following details: manufacture's name, city of the manufacturer, country of manufacturer. EX: primers, BSA.
Details regarding primers (Lines 165-166; lines 172-173 in the file with tracked corrections) BSA, dNTP and DNase free water have been added (lines 179-184; lines 187-191 in the file with tracked corrections).

Line 144-Line 149: Results section instead of material and methods?
Line 144-149 has been moved to lines 314-318 (378-382 in the file with tracked corrections) in section 3.3.1 "Folmers' fragment"

Line 164-line 165: Results section instead of material and methods?
The sentence has been shortened and moved to lines 335-336 (lines 402-403 in the file with tracked corrections).

Line 191: All the MPs samples? Not in accordance with section 2.2.
Line 189-190: "all the samples were amplified under the PCR conditions described in section 2.4.2, choosing the reverse primers according to the degradation pattern of the total DNA" followed by a section 2.8 with de description of the PCR protocol of the same MPs ?. A nice change may be the merge of the information concerning DNA extraction procedures, PCR protocols!
No, "all the samples" means all the 10 samples used for the development of the pre-extraction method. The sentence has been rewritten as follow: "The amount of DNA and the degradation pattern was determined as reported in sections 2.2. 2.3. Then, the DNA obtained from the ten MPs used for the developing of the pre-treatment were amplified under the PCR conditions described in section 2.4.2, choosing the reverse primers according to the degradation pattern of the total DNA" (lines 234-238; lines247-250 in the file with tracked corrections).

Line 206-line209: the same information twice.
The sentence has been corrected.

Line 210: All the software need to have the following details: manufacture's name, city of the manufacturer, country of manufacturer.
The reference for the software has already been reported at lines 170 (Tamura, Peterson, Peterson, Stecher, Nei & Kumar, 2011). See the following link http://www.megasoftware.net/.

Results and Discussion

Line 214 -line 240 and Line 243-260: Since in introduction it should be put in perspective what follows may be it should be considered the rearrange of the text since some of this information could be more adequate on the introduction and not in a section of results.
The information reported from line 214 to line 240 and to line 243 to 260 have been moved and rearranged in the introduction.

Line 314: the internal reverse primers were also tested in the reference samples?
Yes, as reported in the new manuscript "The best reverse primers (REV514A, REV256A, REV193HS, REV163, REV133) (Table 3) were chosen according to the aforesaid criteria, after a test on all the RS DNA, as described in section 2.4. (Databases reference sequence analysis, primer design and optimization of the PCR protocol for the amplification of the Folmer's fragment from the mitochondrial COI gene). See line 203-206 (216-221 in the file with tracked corrections).

Line 344-line 354: Although in the results are presented some data concerning the aluminum content (3.4.1) of the samples the method used is not described in this section.
Please see the previous answer.

Line 344: KAl(SO4)2 replabe by KAl (SO4)2
The original formulation is correct. We do not understand the correction.

Line 393: replace 2010 by (2010)
Done.

Line 418: PCs?
PCs has been changed in CPs (classical dry salted jellyfish products) (line 131; line 138 in the file with tracked corrections)

References
Should be arranged first alphabetically and then further sorted chronologically if necessary.
The reference list has been arranged alphabetically and chronologically.

Fig 1 and Fig 2 Please consider to include in the figure a ruler with the molecular weight of the ladder.
A ruler has been added in both figures.

***Highlights (for review)**

- Cryptic species and processing complicate inspection of jellyfish food products
- A new pre-extraction treatment of dry-salted jellyfish was developed
- New degenerated primers for COI gene allowed specie-identification by FINS
- Analysis of marketed samples showed very high mislabelling levels

What is inside the jar? Forensically informative nucleotide sequencing (FINS) of a short mitochondrial *COI* gene fragment reveals a high percentage of mislabeling in jellyfish food products.

Andrea Armani*, Lara Tinacci, Alice Giusti, Lorenzo Castigliego, Daniela Gianfaldoni, Alessandra Guidi.

FishLab, Department of Veterinary Sciences, University of Pisa, Via delle Piagge 2, 56124, Pisa (Italy).

***corresponding author**:

Postal address: *FishLab, Department of Veterinary Sciences,* University of Pisa, Via delle Piagge 2, 56124, Pisa (Italy)

Tel: +390502210207

Fax: +390502210213

Email: aarmani@vet.unipi.it

Abstract

The jellyfish belonging to the *Rhizostomeae* order are a typical Asian seafood and are nowadays also marketed in the Western Countries. The jellyfish species used for food cannot be identified due to the loss of the morphological characteristics during processing. In this work, a ~~DNA~~ pre-extraction ~~protocol~~treatment consisting in desalting under running water for 48h, followed by an incubation in 400 mM EDTA solution for 1h was developed for products containing high concentration of salts. Moreover, considering the DNA degradation observed in ~~the commercial samples (~~both classical (CPs) and ready to eat~~), four~~ (RE) commercial samples, five sets of primers for the amplification of fragments with different length belonging to the mitochondrial *COI* gene were designed and used to obtain the sequences from 54 reference specimens and 70 market samples. A phylogenetic analysis was then performed using the Neighbor-joining method. It was found that a fragment of 142bp was enough informative to allow an identification at the species level. ~~This~~The results showed that most of the products were made of *N. nomurai* (94% of RE and 45.5% of CPs). The analysis permitted to uncover an alarming level of mislabeling which reaches 79% for CPs and 100% for ready-to-eat products.

Keywords: DNA sequencing, *COI* gene, mislabeling, jellyfish, PCR inhibitors, degraded DNA, seafood identification, ethnic food.

Introduction

The phylum *Cnidaria* comprises four classes: Anthozoa, Cubozoa, Hydrozoa and Scyphozoa The latter, commonly called jellyfish, includes approximately 200 morphospecies, which inhabits seas and oceans worldwide (Mayer, 1910~~).~~

; Marques & Collins, 2004). The jellyfish are famous due to their aggregations and blooming, which negatively affect fisheries, human enterprises and ecosystem (Dong, Liu & Keesing, 2010). However, in some Asian countries, ~~they~~edible jellyfish have been largely caught and exploited as

food for over a thousand years (Hsieh, Leong & Rudloe, 2001; Omori & Nakano, 2001; Dong *et al*., 2010). Due to the organoleptic characteristics and big dimensions, most of the edible jellyfish belong to the *Rhizostomeae* order. In fact, the market price of the processed jellyfish varies according to different characteristics, such as color, consistency, texture, and size: usually, the larger the jellyfish the higher the price (Hsieh *et al*., 2001; Omori & Nakano, 2001). The increased demand for jellyfish, which started from the 70's, together with a reduction in the stock size of the Asian species, has determined the explosive development of fisheries in many other Western countries (Hsieh *et al*., 2001). While the most part of the jellyfish were initially caught in the Pacific and Indian Ocean, starting from 1969, this activity expanded all over the seas and the jellyfish actually enters into commerce from all over the world (Hsieh *et al*., 2001; Omori & Nakano, 2001). Currently, more than 15 species belonging to the *Cepheidae*, *Catostylidae*, *Lobonematidae*, *Rhizostomatidae* and *Stomolophidae* families can be used as food, even though *Rhopilema esculentum* has been the most exploited for human consumption (Omori & Nakano, 2001). Moreover, some jellyfish belonging to the *Semaestomeae* order have been tested for food production (Lu, Dai & Yan, 2003). Finally, we are aware that Chinese people use to collect jellyfish from beaches and to buy jellyfish from fishermen along the Tyrrhenian coast of Italy (author's note).

~~The most important edible jellyfish belong to the *Rhizostomeae*. Even though more than 15 species can be used as food only those of big dimensions, in particular *Rhopilema esculentum*, have been largely exploited for human consumption (Omori & Nakano, 2001).~~

The jellyfish mainly consist of water and protein and ~~their~~its conservation can be achieved only by dehydration. This procedure, traditionally performed with a mixtures of salt and alum, eliminates the compounds responsible for the sting and confers the typical elastic and crispy texture (Hsieh *et al*., 2001). Currently, two different kinds of products are available on the market: the classical one, which, due to the high salt concentration needs a long washing before consumption, and the new "ready to eat" (RE) jellyfish products. While in the past these products were available only in the

Asian countries, nowadays the worldwide spreading of Chinese communities has led to their exportation even towards Western markets.

In a previous work, we assessed the labeling conformity of some dry-salted jellyfish products sold within the Italian's Chinese communities and we found many incongruences and shortfalls mainly referable to the trade name (Armani, D'Amico, Castigliego, Sheng, Gianfaldoni & Guidi, 2012a). The inspection of this kind of seafood is complicated by both processing and the presence of cryptic species complexes that makes difficult the morphological identification also in presence of whole and fresh specimens. ~~For this reason, the molecular approach, such as FINS (Forensically informative nucleotide sequencing) (Bartlett & Davidson, 1992),~~

The power of DNA in discriminating seafood species has been widely demonstrated and, nowadays, the molecular methods are routinely used to verify the traceability information reported on the label. In fact, their flexibility allows to identify a huge number of different species even from processed food products, where the DNA may be partially degraded (Armani, Castigliego, Tinacci, Gianfaldoni & Guidi, 2012b) or contaminated by molecules that can act as PCR inhibitors (Teletchea, 2009). The advances in DNA based analytical methods combined with their falling costs may make it realistic to roll this technology also across Europe's inspection and enforcement agencies as already done by the U.S. Food and Drug Administration (Handy *et al.*, 2011).

The most used techniques for seafood identification are the polymerase chain reaction (PCR) coupled with the restriction enzyme length polymorphism (PCR-RFLP), the PCR with sequence-specific primers (PCR-SSP) and the PCR-sequencing (Teletchea, 2009). The latter method, better known as FINS (Forensically informative nucleotide sequencing), combines a DNA sequencing with phylogenetic analysis, which is performed with a database of reference sequences (Bartlett & Davidson, 1992). FINS often represents the only chance to assess the nature of the product and to verify the information reported on the label, in the light of the EU Commission Implementing Regulation No. 404/2011 (2011) on fish and fishery traceability.

Some genes of the mitochondrial DNA (mtDNA) represent the most targeted molecular markers in this field (Armani, Castigliego & Guidi 2012c). Although the cytochrome b (*cytb*) gene showed a phylogenetic performance comparable to that of *COI* in discriminating closer species (Armani, Castigliego, Tinacci, Gianfaldoni & Guidi, 2011; Armani, Castigliego, Tinacci, Gandini, Gianfaldoni & Guidi, 2012d), the latter has been proposed as the reference gene for a Global Bioidentification System (GBS) for all the living animals. Therefore, thanks to the creation of a dedicated database (http://www.boldsystems.org/), the number of available *COI* sequences has considerably increased, prompting this mitochondrial marker as the most preferred in case of phylogenetical analysis. In fact, the higher the number of available *COI* sequences the higher is the possibility to match unknown sequences with those already deposited. Sufficient levels of *COI* gene variation, indicative of speciation, have been shown for scyphozoan species and many studies have used this "barcode region" to assess their phylogeny (Dawson, 2005; Ramsak, Stopar & Malej, 2012~~-~~; Kayal, Roure, Philippe, Collins & Lavrov, 2013). For all these reasons, in this work the COI gene was selected as target to develop a molecular method aimed to identify the species contained in dry-salted jellyfish products sold on the European market.

Unfortunately, every food sample has a different composition ~~that~~, which implies the potential presence of different contaminants in the extracted DNA. Therefore, particular substrates often require the development of new extraction methods, since the isolation of high quality DNA is a prerequisite for the application of all the molecular techniques.

In this work, we developed a pre-extraction treatment that ~~allow~~allows to obtain amplifiable DNA from dry-salted jellyfish ~~and~~. We also developed a PCR protocol with new degenerated primers for the amplification of fragments with different length, belonging to the mitochondrial cytochrome c-oxidase subunit I gene (*COI*). We then performed a phylogenetic analysis of the selected molecular marker, with the aim to identify the jellyfish species contained in 70 products sold on the European markets and to evaluate the level of mislabeling.

2. Materials and Methods

2.1 Jellyfish sample collection, pH and Aluminum determination.

2.1.1 Reference samples (RS). Fresh, ethanol-preserved, dried, or lyophilized tissue samples from fifty-four jellyfish specimens belonging to *Rhizostomeae* and *Semaeostomeae* orders, identified at the genus or species level by morphological analysis, were kindly provided by Research Institutes or directly collected (Table 1).

2.1.2. Market jellyfish products (MPs). Seventy jellyfish products were purchased in retail food markets and in restaurants within the largest Chinese communities of Italy (in the cities of Prato/Florence, Rome and Milan). Of these, twenty-two were classical dry salted jellyfish products (CPs) and forty-eight were ready to eat products (RE) (Table 2). ~~Others eight~~Eight more samples were purchased in China (Table 2). Each product was brought to our laboratory, labeled with an internal code, ~~then finely minced. pH was measured on the liquid drainage, before being~~and stored at -20°C before the molecular analysis.

2.1.3. *pH and Aluminum determination.* Before the DNA extraction, pH was measured on the liquid drainage obtained after mincing the product, using a Eutech 700 pH meter (Eutech Instruments Pte Ltd, Singapore). Ten samples (five CPs and five RE) were send to a private company (Archa Laboratories S.r.l., Pisa) for the determination of the total Aluminum by atomic absorption spectroscopy.

2.2. Total DNA extraction with standard protocol

The ethanol-preserved and lyophilized RS were washed/re-hydrated in 100 mM TRIS-base, pH 7.8 for 30 min at room temperature (RT) on a thermoshaker~~,~~ (T-Shaker ambient, Euroclone, Siziano, Pavia, Italy), while the MPs were washed in running water for 2h. Total DNA extraction was performed starting from at least 20 mg of tissue following the procedure described by Armani~~, Castigliego, Tinacci, Gianfaldoni & Guidi~~, *et al.*, (2011) reducing the digestion step to 1 h. In case of MPs, five different aliquots ~~have been~~were collected form the same sample. The amount of DNA was determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies,

Wilmington, DE, US), by measuring the absorbance at 260 nm. The purity of the DNA was evaluated by the ratio of absorbance at 260/280 nm and at 260/230 nm.

2.3 Evaluation of DNA fragmentation by gel electrophoresis

One thousand nanograms of the total DNA extracted from the MPs and from the fresh samples of *Rhizostoma pulmo* (positive control) was electrophoresed on 1% agarose gel GellyPhorLE (Euroclone, Wetherby, UK), stained with GelRed™ Nucleid Acid Gel Stain (Biotium, Hayward, CA, USA), and visualized via ultraviolet transillumination. DNA fragment size was estimated by comparison with the standard marker SharpMass™50-DNA ladder and SharpMass™1-DNA ladder (Euroclone~~, Wetherby, UK~~ S.p.A-Life Sciences Division, Pavia, Italy).

2.4. Databases reference sequence analysis, primer design and optimization of the PCR protocol for the amplification of the Folmer's fragment from the mitochondrial COI gene

COI gene reference sequences downloaded from the GenBank (~~JN700940;~~ JN700934; JN700936; ~~JN700988;~~ JN700937; JN700940; JN700941; JN700949; JN700988; GQ120101; HQ694729~~),~~) were aligned with the universal primers LCO1490 and HC02198 designed by Folmer, Black, Hoeh, Lutz & Vrijenhoek, (1994) (Table 3), using Clustal W in Molecular Evolutionary Genetics Analysis (MEGA) version 5.10 (Tamura, Peterson, Peterson, Stecher, Nei & Kumar, 2011) (Table 1SM). On the basis of the transitions and transversions detected, new primers were produced with degenerated nucleotides at different positions (Table 3). ~~These~~All the oligonucleotides used in this work have been synthesized by the company Eurofins MWG Operon (Ebersberg, Germany). The new primers and the original Folmer's primers were tested for their amplification performance on all the RS samples by coupling them in all the possible combinations and in different concentrations, on the basis of their degeneration degree. The best results in terms of amplification yield (evaluated by visualization in UV light), specificity (no aspecific amplification products), absence of inter-oligos reaction (no amplification in the blank) and percentage of successfully amplified RS were obtained using the primers FFDL and FRDL2 (Table 3). The optimal annealing temperature (T_a) was determined using the temperature gradient function

on the peqSTAR 96 Universal Gradient thermocycler (Euroclone, Milan, Italy). The selected primers were then added with the oligonucleotide-tails proposed by Steffens, Sutter & Roemer, (1993) (Table 3). Finally, Bovine Serum Albumin (BSA) was tested at the ~~following~~ final concentration of 200, 100, 50, 25 and 0 ng/µL~~.~~ to select the most appropriate concentration able to enhance the PCR reaction performances.

2.4.~~2~~1. PCR protocols, sequencing and sequences analysis of RS. All the DNA from RS were amplified using the following PCR protocol: 20 µl reaction volume containing 2 µl of a 10x buffer (5Prime, Gaithersburg, USA), 200 µM of each dNTP~~,~~ (dnTPmix, Euroclone S.p.A-Life Sciences Division, Pavia, Italy), 300 nM primers, 25 ng/µL of BSA~~,~~ (Purified BSA 100X, Bew England BIOLABS® Inc. Ipswich, MA, USA), 1~~,~~.25 U 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, 72 °C for 35 s; final extension at 72 °C for 10 min. The PCR products (5 µL) were checked by gel electrophoresis on a 1~~,~~.8% 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 Agencourt® AMPure® XP PCR Purification kit (Beckman Coulter, Beverly, Massachusetts, USA) following the procedure proposed by the manufacturer. Purified products were sequenced by BMR Genomics (Padova, Italy). The obtained sequences were analyzed using Clustal W in MEGA version 5.10. Fine adjustments were manually made after visual inspection. All the sequences were checked for nuclear mitochondrial pseudogenes (numts) following the quality control proposed by Song, Buhay, Whiting & Crandall, (2008). The comparison with other sequences available on the databases were performed running a BLAST analysis both on ~~GenBank and on the Barcode of Life Database (Table 1). A total of fifty four partial *COI* gene sequences of 658bp belonging to fourteen species of *Rhizostomeae* and *Semaeostomeae* were deposited in GenBank (Table 1).~~ the GenBank and on the Barcode of Life Database (Table 1). ~~The sequences of~~

~~*R. luteum* were retrieved from a previous work (under review) (Table 1SM). This is the first time, as far as we are aware, that *COI* gene sequences of *Cotylorhiza tuberculata*, *Lychnorhiza lucerna* and *Cyanea lamarckii* has been obtained.~~

2.4.~~3~~2. Designing of internal reverse primers for the amplification of short COI gene fragments. Taking in consideration the degradation pattern highlighted by the gel electrophoresis analysis we decide to design internal reverse primers for the amplification of shorter fragments of the *COI* gene in combination with the forward FFDL. For this reason, the sequences produced in this study were aligned with those retrieved from public databases~~,~~: 75 sequences belonging to 14 species of 5 different families of *Rhizostomeae* and 54 sequences belonging to 11 species of 3 different families of *Semaestomeae*~~, and used to design~~. Alignment was aimed at designing internal reverse primers~~.~~ (Table 2SM). The best reverse primers (REV514A, REV256A, REV193HS, REV163, REV133) (Table 3) were chosen according to the aforesaid criteria, after a test on all the RS DNA, as described in section 2.4. The selected primers were also linked to the Steffen's tails (Steffens ~~et al.,~~ Sutter & Roemer, 1993). The amplification protocols for the ~~fragment less~~fragments shorter than 500bp differed from those optimized for the Folmer's fragment in the annealing temperature and the extension time, which were 50°C and 20 s, respectively.

2.4.~~4~~3. Amplification of MPs DNA. The developed protocols were used for the amplification of the DNA belonging to MPs according to the fragmentation pattern highlighted by gel electrophoresis. ~~Even though increasing concentration of DNA were used (from 100 to 500ng) the amplicon was obtained only from five RE DNA samples.~~

2.~~6~~5. Assessment of PCR inhibitors and development of a method for the DNA extraction from MPs

2.~~6~~5.1. Assessment of PCR inhibitors. The DNA of three *N. nomurai* (positive control) plus three RE and three CP samples, whose amplification was negative, was used to evaluate the presence of PCR inhibitors co-extracted with DNA. In particular we performed:

Serial dilution test: three different DNA dilutions (10, 1, 0~~,~~.1 ng/µL) of each sample were prepared in DNase free water.

Spiking test: the DNAs from *N. nomurai* were diluted with different percentage of DNA extracts form the selected RE and CP (10%, 50% and 100%) and DNase free water to reach the working concentration of 100 ng/µL.

Extract purification: the DNA obtained with the standard extraction method was purified with the Agencourt® AMPure® XP PCR Purification kit (Beckman Coulter, Beverly, ~~Massachusetts~~MA, USA), according to the manufacturer's instruction and, after the quantification, a working solution of 100 ng/µL of DNA was prepared.

All the samples prepared were then amplified under the PCR conditions previously described, according to the degradation level of the DNA.

2.~~6~~5.2. Method for the DNA extraction from MPs. Some pre-treatment procedures were tested on ten MPs (five RE and five CPs). The samples were grossly chopped with scissors and then differently processed according to the following scheme: 1) desalted in running water at RT for 24, 48 or 72h; 2) incubated in four different solution of EDTA (50, 100, 200, 400 mM) at RT for 1h on a thermoshaker; 3) procedure 1 and 2 together. The total DNA extraction was then performed according Armani *et al*~~,~~. (2011) starting from ~ 250 mg of tissue. The amount of DNA and the degradation pattern was determined as reported in ~~section 2.3 and, finally, all the samples~~sections 2.2. 2.3. Then, the DNA obtained from the ten MPs used for the developing of the pre-treatment were amplified under the PCR conditions described in section 2.4.2, choosing the reverse primers according to the degradation pattern of the total DNA.

The samples pretreatment n°3 was chosen for the analysis of the MPs, with 24-48h of desalting in running water followed by an an incubation in 400 mM EDTA solution for 1h at RT.

2.~~8~~6. Final PCR protocol sequencing and sequence analysis of the MPs.

The final protocol for the amplification of the MPs was optimized as follow: 20 µl reaction volume containing 2 µl of a 10x PCR buffer (5Prime, Gaithersburg, USA), 200 µM of each dNTP,

300 nM primers, 25 ng/µL of BSA, 100 ng of DNA and DNase free water. The concentration of the PerfectTaq DNA Polymerase was ~~augmented~~increased to 2~~.~~.5 U, while the cycling program was increased to 45 cycles.

All the MPs were amplified under the aforesaid conditions and checked by gel electrophoresis, purified, sequenced and analyzed as reported is section 2.4.~~2.~~ A total of 82 partial *COI* gene sequences of variable length (from 145 to 658) were deposited in the GenBank (Table 2).

2.~~10~~7. Phylogenetic analysis

Fifty-four sequences obtained in this study from RS (Table 1) and 78 sequences obtained from the MPs (Table 2) were aligned with 60 sequences of jellyfish belonging to *Rhizostomeae* and *Semaeostomeae* Orders retrieved from the GenBank (Table ~~1SM~~3SM), using Clustal W in MEGA version 5.10. When possible, 5 different sequences per each species were retrieved from the databases. Only in the case of *Aurelia spp*, of which a recent study demonstrated that there are at least 14 species, more than 5 sequences ~~have been Only in the case of Aurelia spp, of which at least 14 species have been reported, more than 5 sequences have been used (Table 1SM).~~were used (Table 3SM). A fragment of 142bp belonging to the *COI* gene was selected. The neighbour-joining (NJ) dendograms were obtained using MEGA version 5.10~~,~~ and distances were computed using the Kimura 2-parameter model with 2000 bootstraps re-samplings (Saitou & Nei, 1987).

3. Results and Discussion

~~The power of DNA sequences in discriminating seafood species has been widely demonstrated and, nowadays, the molecular methods are routinely used to verify the traceability information reported on the label and to foil fishing frauds. In fact, their flexibility allows to identify a huge number of different species even from processed food products, where the DNA may be partially degraded (Armani, Castigliego, Tinacci, Gianfaldoni & Guidi, 2012b) or contaminated by molecules that can act as PCR inhibitors (Teletchea, 2009). The advances in DNA based analytical methods combined with their falling costs may make it realistic to roll this technology also across~~

Europe's inspection and enforcement agencies as already performed by the U.S. Food and Drug Administration (Handy et al., 2011).

The most used techniques for seafood identification are the polymerase chain reaction (PCR) coupled with the restriction enzyme length polymorphism (PCR-RFLP), the PCR with sequence-specific primers (PCR-SSP) and the PCR-sequencing (Teletchea, 2009). The latter method, better known as FINS, combines a DNA sequencing with phylogenetic analysis, which is performed with a database of reference sequences, allowing to identified the species contained in the unknown food samples (Bartlett & David, 1992).

Some genes of the mitochondrial DNA (mtDNA) represent the most targeted molecular markers in this field (Armani, Castigliego & Guidi 2012c). Although the cytochrome b (*cytb*) gene showed a phylogenetic performance comparable to that of *COI* in discriminating closer species (Armani, et al., 2011; Armani, Castigliego, Tinacci, Gandini, Gianfaldoni & Guidi, 2012d), the latter has been proposed as the reference gene for a Global Bioidentification System (GBS) for all the living animals. Therefore, thanks to the creation of a dedicated database (http://www.boldsystems.org/), the number of available *COI* sequences has considerably increased, prompting this mitochondrial marker as the most preferred in case of phylogenetical analysis. In fact, the higher the number of available *COI* sequences the higher is the possibility to match unknown sequences with those already deposited. Sufficient levels of *COI* gene variation, indicative of speciation, have been shown for scyphozoan species and many studies have used this "barcode region" to assess their phylogeny (Dawson, 2005; Ramsak, Stopar & Malej, 2012). For all these reasons, in this work the *COI* gene was selected as target to develop a molecular method aimed to identify the species contained in dry-salted jellyfish products sold on the European market.

3.1 Jellyfish sample collection

For over a thousand years, edible jellyfish belonging to *Rhizostomeae* order have been exploited along the coasts of China. Starting from the 70's, with an increasing demand on the Japanese market, jellyfish fishing has become very popular not only in Southeast Asia, but also in other

countries. While the most part of the jellyfish were initially caught in the Pacific and Indian Ocean, starting from 1969, this activity expanded all over the seas and the jellyfish actually enters into commerce from all over the world (Hsieh *et al.*, 2001; Omori & Nakano, 2001)

Due to the organoleptic characteristics and big dimensions most of the edible jellyfish belong to the Rhizostomeae order. In fact, the market price of the processed jellyfish varies according to different characteristics, such as color, consistency, texture, and size: usually, the larger the jellyfish the higher the price (Hsieh *et al.*, 2001; Omori & Nakano, 2001).

Even though, until 1996, the FAO has referred all the statistical data of jellyfish catches to the unique species *R. esculentum*, more than 15 species belonging to the *Cepheidae*, *Catostylidae*, *Lobonematidae*, *Rhizostomatidae* and *Stomolophidae* families can be used as food (Omori et al., 2001). Moreover, some jellyfish belonging to the *Semaestomeae* order have been tested for food production (Lu, Dai & Yan, 2003). Finally, we are aware that Chinese people use to collect jellyfish from beaches and to buy jellyfish from fishermen along the Tyrrhenian coast of Italy (author's note).

Thus, with the aim to test the ability of the new primers developed in this study to amplify the most part of the jellyfish exploitable for food preparation, we collected 54 reference samples belonging to 5 families of *Rhizostomeae* and to 3 families of *Semaestomeae* (Table 1).

3.2. Total DNA extraction with standard protocol and evaluation of DNA fragmentation by gel electrophoresis.

DNA amplificability depends not only on the quality (i.e. absence of inhibitors), but also on the integrity of the target sequence, which can be degraded during processing. In fact, while the DNA extracted from fresh samples generally presents large fragments (>1kb) (Teletchea, 2009), thosethe DNA obtained from processed seafood, is often severely degraded (Armani *et al.*, 2012b). In particular, besides temperature, the low pH is responsible for DNA fragmentation (Teletchea, 2009). DNA is very sensitive to acid agents, which, by enhancing depurination, represents one of

the most important factor responsible for strand breaks, with effects that can be worse than those provoked by boiling (Armani *et al*., 2012b).

Considering that one of the main ingredients in jellyfish preparation, the Potassium Alum $KAl(SO_4)_2$, is essential for the pH lowering (Omori & Nakano, 2001), we checked the pH of the MPs. As expected, the CPs had a pH values considerably lower than RE, which are desalted before entering the market. In particular, CPs had a pH of 3.84 ± 0,28 while REs had a pH 5.~~9~~90 ± 0,82. In previous works it was found that the extent of DNA degradation under acid conditions is strictly dependent on exposure time and temperature (Bauer, Weller, Hammes & Hertel, 2003; Armani *et al*., 2012b). The long shelf life of jellyfish products (12 months for the RE and 24 for the CPs) and the fact that all the samples were collected from the sale counter at room temperature, although the indications on storing modalities usually suggest refrigeration (Hsieh *et al*., 2001; Armani *et al*., 2012a), could explain the variability in the DNA fragmentation patterns ~~here~~ observed.

The gel electrophoresis analysis clearly shows that most of the total DNA samples extracted from CPs presented fragments between 50 and 250bp, while only few samples showed DNA fragments of 500-600bp (Fig. 1). On the contrary, the total DNA obtained from RE products contained fragments as long as 1kbp in most of the samples, even though, in some cases, the degradation pattern was similar to those of CPs (Fig. 1).

3.~~3~~2. Primers design for the amplification of the mitCOI gene

3.~~3~~2.1 Folmers' fragment. Most of the studies based on the molecular characterization of the jellyfish *mitCOI* gene have relied on the primers designed by Folmer *et al*. (1994) for the amplification of the first ~700bp (Table 3). These primers have given good results with species such as *Rhizostoma pulmo* (Ramsak *et al*., 2012~~)~~), while for others, such as *Catostylus mosaicus* (Dawson, 2005), some nucleotide modifications on their sequence were necessary. Surprisingly, to our knowledge, the use of degenerated primers has not been reported in the literature, even though such expedient may increase the PCR performances by reducing the number of mismatches responsible for the annealing failure. Moreover, the possibility to use the same pair of primers for

the contemporary amplification of the same fragment from different DNA samples would simplify the analysis procedures.

With the aim to design primers capable to amplify the *COI* Folmer's fragment from the DNA of most of the edible jellyfish belonging to *Rhizostomeae* and *Semaeostomeae* orders, all the sequences referable to this portion have been downloaded from the databases and analyzed. The target zone was recovered only for nine species (5 *Rhizostomeae* and 4 *Semaeostomeae*) ~~on~~in the GenBank, while no sequences were found ~~on~~in BOLD. This could be explained considering that the aim of ~~the~~ BOLD is to create a DNA barcoding system based exclusively on the first part of the *COI* gene. Thus, due to the fact that sequencing leaves out the primer annealing regions, all the deposited sequence have a length of ~ 650bp or less. The unavailability of the complete sequence of the *COI* gene can represent a limit, especially when primers have to be modified to improve the efficiency of amplification.

The primer couple FFDL and FRDL2 were chosen considering their ability in producing the expected amplicon in all the reference sample DNA. On this regard, it has to be highlighted that the utilization of the oligonucleotide-tails proposed by Steffen *et al* ~~(~~.,(1993) allowed to obtain sequences of 658bp from all the RS analyzed. Therefore, with the aim to test the ability of the new primers developed in this study to amplify the most part of the jellyfish exploitable for food preparation, we collected 54 reference samples belonging to 5 families of *Rhizostomeae* and to 3 families of *Semaestomeae* (Table 1).

A total of fifty-four partial *COI* gene sequences of 658bp belonging to 14 species of *Rhizostomeae* and *Semaeostomeae* were deposited in the GenBank (Table 1). The sequences of *R. luteum* were retrieved from a previous work (Prieto, Armani & Macias, 2013) (Table 3SM). This is the first time, as far as we are aware, that *COI* gene sequences of *Cotylorhiza tuberculata*, *Lychnorhiza lucerna* and *Cyanea lamarckii* has been obtained.

3.~~3~~2.2. Internal reverse primers for the amplification of a short COI gene fragment. Considering the degradation pattern ~~obtained by~~observed with the electrophoresis analysis, new internal reverse

primers for the amplification of shorter fragments of the *COI* gene in combination with the forward FFDL were designed and tested for their amplification performance (Table 3). ~~Those~~The primers selected were used for the amplification of the MPs DNA samples. Only for few MPs DNA samples ~~gave~~ the expected amplicon was obtained, while for most ~~failed~~of them the amplification ~~or showed bands too feeble for sequencing~~failed. In particular, we found that the "inhibitory" effects affected more the longest fragments than the shortest ones (Fig. 2). In fact, even though some samples presented a DNA smear that exceeded 700bp, it was impossible to obtain amplicons longer than 256bp.

3.~~4~~3. Assessment of PCR inhibitors in DNA MPs samples

The extraction of DNA contaminated by inhibitors is one of the main obstacle associated with PCR application in complex matrices, such as food. In fact, the inhibitors can interfere with the reaction, leading to a decrease or even to a complete inhibition of the amplification capability of PCR (Teletchea, 2009). Even though the PCR inhibition mechanism can be categorized into two main forms, which are process and template inhibition, the activity of many molecules remain unclear. ~~Considering that the characterization of PCR inhibitors represents an important step in the development of efficient extraction method and in the light~~An investigation was performed on this issue, as a consequence of the low percentage of successful amplifications obtained from the DNA of MPs extracted with the standard protocol~~, an investigation was performed on this issue.~~ (only five samples in RE products) and considering that the characterization of PCR inhibitors represents an important step in the development of efficient extraction methods.

Serial dilution represents a simple expedient, which permits the amplification of DNA contaminated samples. In fact, the sensitivity of PCR allows to amplify the target DNA that is still present when inhibitors have been diluted out. Usually, a 100/fold dilution of the starting template may be enough to reduce the inhibitory effect. In different trials, the expected amplicons could not be obtained even starting from diluted DNA solutions (10, 1, 0,1 ng/µL). Therefore, the DNA of *N. nomurai* (positive control) was spiked with increasing concentration of some MPs DNA solutions

that ~~previously~~ could not be amplified in previous trials. The positive amplification of the spiked samples revealed the absence of PCR inhibitors free in the DNA solutions. Finally, the amplification failed in all the samples even after the use of the Agencourt® AMPure® XP PCR Purification kit (Beckman Coulter, Beverly, Massachusetts, USA). This suggests that PCR inhibition is somehow directly associated to the DNA condition.

3.~~4~~3.1. Aluminum. The Potassium Alum $KAl(SO_4)_2$, which is essential for the jellyfish processing, accumulates in the tissues and reaches a high concentration in the final product: Wong, Chung, Kwong, Yin Ho, & Xiao, (2010) found a mean value of 1200 mg/kg in RE samples collected from the Japan market. In this work we commissioned to a private company an analysis of 10 samples for the determination of the total content of Aluminum ~~by atomic absorption spectroscopy~~ and we found values ranging from 484 mg/kg to 1450 mg/kg. These differences in the concentration could be explained considering that processing time, temperature and amount of alum used affect the retention of aluminum in jellyfish tissues (Hsieh *et al*., 2001).

Al^{3+}, capable to form complexes with DNA molecules (Wu, Du, Zhang, Khan, Chen & Liang, 2005), can inhibit the PCR reaction (Wadowsky, Laus, Libert, States & Ehrlich, 1994). Therefore, the presence of Al^{3+} ions could contribute to the overall DNA amplification failure for certain MPs.

3.~~5~~4. DNA extraction method and final protocol for the amplification of the MPs

The selection of a proper extraction method is determinant for a successful and valid PCR analysis. Even though several extraction techniques have been proposed, either based on commercial kit or on classical procedures, it seems unlikely that a single method could be suitable for all kind of samples.

Considering that the 2h of pre-washing in running water, performed before the classical extraction, did not allow to obtain good quality DNA (i.e. free of contaminants), different kind of procedures were tested. Besides the washing of the samples under slowly running water for a variable time, different amounts of Ethylenediaminetetraacetic acid (EDTA) were used for its

chelating properties, with the purpose of reducing the Aluminum concentration in the final DNA solution.

The best results in terms of DNA yield and quality (no inhibition during PCR amplification), for both the CPs and for the RE, were obtained with a washing of 24-48h. Desalting for 72h did not showed any improvements during PCR amplifications, while drastically reduced the DNA recovery. The additional incubation in a 400 mM EDTA solution for 1 hour at RT was able to increase the amplification performances, especially in case of the 709bp fragment (Fig. 2). Thus, considering all the variable that can affect the final composition of the products and that, in some cases, different aliquots collected from different portions of the same product showed different PCR results, a washing of 48h and an addition of EDTA to increase the amplicon yield before extraction were adopted in the final sample pre-treatment protocol. Once extracted with the proposed method, all the DNA MPs samples were successfully amplified and sequenced (Table 2). In particular, in case of RE we obtained: 20 sequences (~42%) of 658bp, 16 (~33%) of 206, and 12 (~25%) of 145bp. In case of CPs: 6 sequences (~17.5%) of 658bp, 9 (~26.5%) of 206, and 19 (~56%) of 145bp. These results testify a synergic negative influence of DNA degradation and of inhibition by contaminants on the amplification reaction.

3.~~6~~5. Phylogenetical analysis and mislabelling

The possibility to identify the species to which a seafood product belongs is the main issue of the Official control and the FINS analysis of a partial mitochondrial gene has been widely used for this purpose (Armani *et al*., 2011; Pepe, Trotta ~~M.,~~ Di Marco, Anastasio, Bautista & Cortesi, 2007). Even though most of the studies have used sequence fragments longer than 350bp, Hajibabaei, Smith, Janzen, Rodriguez, Whitfield & Hebert, (2006) ~~has~~have demonstrated that a short *COI* gene fragment (~100bp) can be used for the identification of many animal species.

In this study, considering that the majority of the MPs presented a highly degraded DNA, we decided to rely on a fragment of 145bp. Moreover, considering that most of the deposited sequences

~~misses~~miss some bases at the 5', a short fragment of 142bp ~~has been~~was finally selected and a NJ tree with 2000 replicates ~~has been~~was produced with 192 sequences (Fig. 3).

Even though the inter-familiar relationships obtained with our analysis are not well resolved, with the exception of the family *Cepheidae*, *Mastigidae*, and *Cassiopeidae*, our tree agrees with those produced by Bayha, Dawson, Collins, Barbeitos & Haddock, (2010 ~~using~~), who used two nuclear genes. In particular, the species of *Semaestomeae* order formed different clusters according to the family *Pelagidae*, *Ulmaridae*, *Cyaneidae*, and *Drymonematidae*. The species of the order *Rhizostomeae* were grouped according to the suborder, with the exception of *Lychnorhiza lucerna*. In fact, all the species of the suborder *Kolpophorae* (family *Cepheidae*, *Mastigidae*, and *Cassiopeidae*) were clustered together and separately from the species belonging to the suborder *Daktyliophorae* (*Rhizostomatidae* and *Catostylidae*).

The 142bp *COI* fragment gene used in our analysis allowed to unequivocally identify almost the totality of the jellyfish at the species level, with a mean bootstrap value of 98%. Only the samples PC20-21-22, those labeled as Asian jellyfish, were not identified at the species level~~, even though~~. However, they were placed in the *Rhizostomeae* order.

3.~~6~~5.1 Mislabelling

From the 1st January 2012, according to the Art. 68 of the EU Commission Implementing Regulation No 404/2011 (2011), the scientific name, together with the commercial designation, the catch area, and the production method~~,~~ had to be also indicated, on the label or appropriate mark, at the retail level. Jellyfish, which at the time of our previous survey (Armani~~,~~ *et al.*, 2012a) lacked of a trade name, has been recently included in the Italian official seafood list. The provisional commercial denomination of "Asian jellyfish" has only to be used for ~~only~~ the species *R. esculentum.*

Among the 45 market samples labeled as jellyfish, only three products reported the new official denomination of Asian jellyfish. ~~The~~ 98% of these reported the scientific denomination of *R.*

esculentum, while ~~the~~ 2% did not reported the scientific name. Overall, 7 samples were sold without the commercial and the scientific name, while 14 with names referable to vegetables (Table 2).

Regarding the RE samples (market and restaurants), 94% was identified as *N. nomurai*, while only 6 % as *R. esculentum*. In particular, *R. esculentum* was only found in samples collected in the restaurants (Table 2). Thus, considering only the RE samples collected from the markets, the extent of mislabelling is quite impressive, reaching 100%. With regard of ~~PCs~~CPs from the Italian market, 91% was labeled as jellyfish and, among ~~this~~these, 95% reported the scientific denomination of *R. esculentum.* In this case, only two samples were sold without the commercial and the scientific name. The molecular analysis allowed to identify this products as *R. esculentum* (22.7%), *Nemopilema nomurai* (~~36.4~~45.5%), *Rhizostoma pulmo* (9.1%), and *Pelagia noctiluca* (9.1%). The *P. noctiluca* species, which has been never reported as edible, ~~has been~~were also found ~~also~~ in some ~~PCs~~CPs among those collected in the Chinese markets. Interestingly, the products labeled as Asian jellyfish are the only three samples that were not identified at the species level. They probably belong to the *Rhizostomidae* and *Catostylidae* species that have not been still analyzed. Mislabeling ~~of PCs~~in CPs, despite lower than in RE samples, was 79%.

The production of *R. esculentum*, the species traditionally exploited for food production, significantly declined over the last decade and stock enhancement was conducted in 2005 and 2006, with the aim to save the fishery of this valuable jellyfish (Dong *et al*., ~~2008~~2009). On the other hand, the blooming forming jellyfish *Aurelia aurita*, *N. nomurai*, *Cyanea capillata*, and *Cyanea nozakii*~~,~~ increased gradually in the Chinese seas and were regarded as the main cause for the decline of the *R. esculentum* (Dong~~,~~ *et al.*, 2010). The jellyfish products made of *N. nomurai*, a species that can reach a bell diameter of 2 m and a weight of 200 kg, have been reported to have an undesirable taste, which made them cheap and unpopular. In spite of this, its utilization could be explained considering that, in front of an increasing request for jellyfish food and a drastic reduction of *R. esculentum*, massive blooms of *N. nomurai* were reported in the northern part of East China, in the Yellow Sea and the Liaodong Bay, where the annual catch increased 250% from 2000 to 2003

(Dong~~,~~ *et al.*, 2010). Finally, considering the worldwide presence of Chinese communities and the low cost of jellyfish processing (Hsieh~~,~~ *et al.*, 2001), it is plausible that some never-exploited or ~~underused~~little used species are now being ~~used~~considered for food production. In fact, the preparation of the shredded RE products has eliminated the problems related to the size of the jellyfish and increased the utilization of their oral arms~~ ~~~~,~~ which, even though not regarded by the Japanese brokers, are requested by the Chinese ones (Hsieh~~,~~ *et al.*, 2001). In fact, we found some samples, collected both from Chinese and from Italian markets, belonging to *P. noctiluca*. This species has a wide distribution and, even though typical of warm waters, can be also found in the north Atlantic and in the north Pacific (Mariottini, Giacco & Pane, 2008). In particular, *P. noctiluca* is one of the most common jellyfish species responsible for stinging cases in the popular coastal areas of China (Dong *et al.*, 2010).

The possibility of replacing valuable species with cheaper species makes mislabelling a convenient practice, above all when the processing completely alter the original morphological characteristics~~, makes mislabelling a convenient practice~~. This principle has been once more confirmed in this study, where *R. esculentum*, the most expensive among all the edible jellyfish, with a price that could be about 7-fold higher than that of other species (Omori ~~et al.,~~& Nakano, 2001), has been frequently replaced with the *N. nomurai*.

Finally, this study confirm that the extent of label non-conformities reach high levels in ethnic markets, as already highlighted by previous surveys (Armani~~, 2012d~~ *et al.*, 2012d; D'Amico, Armani, Castigliego, Sheng, Gianfaldoni & Guidi, 2014).

Conclusions

The pre-extraction treatment developed in this work, coupled with DNA sequencing and phylogenetical analysis of a *COI* gene fragment, allowed the molecular identification at the species level ~~96%~~ of the marketed jellyfish, even in case of classical salted products, where the DNA is heavily degraded. The possibility to apply this kind of analysis permits to overcome the problems

related to the visual inspection of this unusual seafood products, which is currently commercialized on the European markets.

This method, which ~~allowed~~permitted to highlight a high percentage of label non-conformities in ethnic products, could be applied to assess the authenticity of imported species in the light of the European provisions and represent a valid tool for protection of the consumer rights and for fishery control.

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References

1. ~~Mayer A. G. (1910). Medusae of the world III – the Scyphomedusae. Washington, DC: Carnegie Institute.~~
2. ~~Dong, D., Liu, D., & Keesing, J. K. (2010). Jellyfish blooms in China: Dominant species, causes and consequences, *Marine Pollution Bulletin, 60 (7)*, 954–963~~

~~1. Hsieh, H-Y.P., Leong, F-M., & Rudloe, J. (2001). Jellyfish as food. *Hydrobiologia, 451*, 11–17~~

~~2. Omori, M. & Nakano, E. (2001). Jellyfish fisheries in southeast Asia. *Hydrobiologia, 451(1-3)*, 19–26~~

~~3. Dong, J., Jiang, L-X., Tan, K-F., Liu, H.Y., Purcell, J. E., Li, P-j., Ye, C. C. (2009). Stock enhancement of the edible jellyfish (Rhopilema esculentum Kishinouye) in Liaodong Bay, China: a review. *Hydrobiologia, 616*,113–118~~

3. Armani, A., Castigliego, L., Tinacci, L., Gianfaldoni, D., & Guidi, A. (2011). Molecular characterization of icefish, (Salangidae family), using direct sequencing of mitochondrial cytochrome b gene. *Food Control*, *22*, 888-895.

~~3.~~4. Armani, A., D'Amico, P., Castigliego, L., Sheng, G., Gianfaldoni, D., & Guidi A. (2012a) Mislabeling of an "unlabelable" seafood sold on the European market: The jellyfish. *Food Control, 26* , 247-251~~.~~.

5. Armani, A., Castigliego, L., Tinacci, L., Gianfaldoni, D., & Guidi A. (2012b). Multiplex conventional and real-time PCR for fish species identification of Bianchetto (juvenile form of *Sardina pilchardus*), Rossetto (*Aphia minuta*), and Icefish in fresh, marinated and cooked products". *Food Chemistry, 133 (1)*, 184-192.

~~4.~~6. Armani, A., Castigliego, L., & Guidi, A. (2012c). Fish frauds: the DNA challenge. Animal Science Reviews 2012, 227.

7. Armani, A., Castigliego, L., Tinacci, L., Gandini G., Gianfaldoni, D., & Guidi, A. (2012d). A rapid PCR–RFLP method for the identifcation of Lophius species. *European Food Research and Technologies, 235,* 253–263.

~~5.~~8. Bartlett, S. E., & Davidson, W. S. (1992) FINS (forensically informative nucleotide sequencing): a procedure for identifying the animal origin of biological specimens. *Biotechniques. 12(3),* 408-411.

9. Bauer, T., Weller, P., Hammes, W. P. & Hertel, C. (2003). The effect of processing parameters on DNA degradation in food. *European Food Research and Technology, 217,* 338–343.

~~6.~~10. Bayha K. M., Dawson M., Collins A. G., Barbeitos M. S. & Haddock S. H. (2010). Evolutionary relationship among Scyphozoan jellyfish families based on complete taxon sampling and phylogenetic analyses of 18s and 28s ribosomal DNA. *Integrative and Comparative Ecology, 50 (3),* 436-455.

~~4.~~ Commission Implementing Regulation No. 404/2011 of 8 April 2011 laying down detailed rules for the implementation of Council Regulation (EC) No 1224/2009 establishing a Community control system for ensuring compliance with the rules of the Common Fisheries Policy. *Official Journal of the European Communities, L112,* 1-153~~Commission Implementing Regulation No. 404/2011 of 8 April 2011 laying down detailed rules for the implementation of Council Regulation (EC) No 1224/2009 establishing a Community control system for ensuring compliance with the rules of the Common Fisheries Policy. *Official Journal of the European Communities, L112,* 1-153~~

11. .

12. D'Amico P., Armani~~,~~ A., Castigliego~~,~~ L., ~~Tinacci, L~~Sheng G., Gianfaldoni~~,~~ D~~.~~,. & Guidi~~, A. (2011). Molecular characterization of icefish, (Salangidae family), using direct sequencing~~ A. (2014). Seafood traceability issues in Chinese food business activities in the light of ~~mitochondrial cytochrome b gene.~~the european provisions. *Food Control, 35 (1),* 7-13.

~~7.~~13. Dawson M. N. (2005). Incipient speciation of Catostylus mosaicus (Scyphozoa, Rhizostomeae, Catostylidae), comparative phylogeography and biogeography in south-east *Australia Journal of Biogeography, 32,* 515–533

~~8.~~14. Dong, D., Liu, D., & Keesing, J. K. (2010). Jellyfish blooms in China: Dominant species, causes and consequences, *Marine Pollution Bulletin, 60 (7),* 954–963

~~9.~~15. Dong, J., Jiang, L-X., Tan, K-F., Liu, H.Y., Purcell, J. E., Li, P-j., Ye, C. C. (2009). Stock enhancement of the edible jellyfish (Rhopilema esculentum Kishinouye) in Liaodong Bay, China: a review. *Hydrobiologia, 616,*113–118~~22, 888-895~~.

~~10.~~16. Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhoek, R., (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase I subunit from diverse metazoan invertebrates. *Molecular Marine Biology Biotechnology*, *3*, 294–299.

~~11.~~17. Hajibabaei, M., Smith, M., Janzen, D. H., Rodriguez, J. J., Whitfield, J. B., & Hebert, P. D. (2006). A minimalist barcode can identify a specimen whose DNA is degraded. *Molecular Ecology Notes, 6(4)*, 959-964.

~~12.~~18. ~~Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular. Biology Evolution, 28(10)*, 2731–2739.~~

~~13.~~19. ~~Steffens, D. L., Sutter, S. L., & Roemer S. C. (1993). An alternate universal forward primer for improved automated DNA sequencing of M13. *Biotechniques, 15(4)*, 580-582.~~

~~14.~~20. ~~Song, H., Buhay J. E., Whiting, M. F., & Crandall, K.A. (2008). Many species in one: DNA barcoding overestimates the number of species when nuclear mitochondrial pseudogenes are coamplified. *Proceedings of the National Academy of Sciences, 105*, 13486–91.~~

~~5. Saitou, N., & Nei M (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology Evolution, 4*, 406–425~~

~~6. Teletchea, F. (2009). Molecular identification methods of fish species: reassessment and possible applications. *Fish Biology and Fisheries, 19(3)*,265–93~~

~~7. Armani, A., Castigliego, L., Tinacci, L., Gianfaldoni, D., & Guidi A. (2012b). Multiplex conventional and real-time PCR for fish species identification of Bianchetto (juvenile form of *Sardina pilchardus*), Rossetto (*Aphia minuta*), and Icefish in fresh, marinated and cooked products". *Food Chemistry, 133 (1)*, 184-192~~

~~15.~~21. Handy, S. M. , Deeds, J. R., Ivanova, N. V., Hebert, P. D. N., Hanner, R. H., Ormos, A., Weigt, L. A., Moore, M. M., & Yancy, H. F. (2011). A single-laboratory validated method for the generation of DNA barcodes for the identification of fish for regulatory compliance. *Journal of AOAC International, 94 (1)*, 201-210.

22. Hsieh, H-Y.P., Leong, F-M., & Rudloe, J. (2001). Jellyfish as food. *Hydrobiologia, 451*, 11–17~~Armani, A., Castigliego, L., & Guidi, A. (2013).~~.

23. Kayal E., Roure B., Philippe H., Collins A. G. & Lavrov D. V. (2013). Cnidarian phylogenetic relationship as revealed by mitogenomics. *BMC Evolutionary Biology, 13 (5)*,

~~16.~~24. Lu, Z., Dai, Q. & Yan, Y. (2003). Fishery biology of Cyanea nozakii resources in the waters of Dongshan Island. Chinese *Journal of Applied Ecology, 14*, 973–976 (in Chinese).

25. Mariottini G. L., Giacco E. & Pane L. (2008) The Mauve Stinger *Pelagia noctiluca* (Forsskål, 1775). Distribution, Ecology, Toxicity and Epidemiology of Stings. A Review. *Marine drugs, 6,* 496-513.

~~17.~~26. Mayer A. G. (1910). Medusae of the world III – the Scyphomedusae. Washington, DC: Carnegie Institute.

27. Marques A. C. & Collins A. G. (2004). Cladistic analysis of Medusozoa and cnidaria evolution. *Invertebrate Biology, 123 (1),* 23-42;

28. Omori, M. & Nakano, E. (2001). Jellyfish fisheries in southeast Asia. *Hydrobiologia, 451(1-3),* 19–26.

29. Pepe T., Trotta M., Di Marco I., Anastasio A., Bautista J. M. & Cortesi L. (2007). Fish species identification in surimi-based products. *Journal of Agricultural and Food Chemistry, 55 (9),* 3681-3685.

30. Prieto L., Armani A. & Macias D. (2013) Recent strandings of the giant jellyfish *Rhizostoma luteum* Quoy and Gaimard, 1827 (Cnidaria: Scyphozoa: Rhizostomeae) on the Atlantic and Mediterranean coasts. *Marine Biology*, DOI 10.1007/s00227-013-2293-6.

31. Ramšak, A., Stopar, K., & Malej. A. (2012) Comparative phylogeography of meroplanktonic species, Aurelia spp. And Rhizostoma pulmo (Cnidaria: Scyphozoa) in European Seas. *Hydrobiologia , 690*, 69-80.

32. Saitou, N., & Nei M (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology Evolution, 4,* 406–425.

~~18.~~33. Song, H., Buhay J. E., Whiting, M. F., & Crandall, K.A. (2008). Many species in one: DNA barcoding overestimates the number of species when nuclear mitochondrial pseudogenes are coamplified. *Proceedings of the National Academy of Sciences, 105,* 13486–91.

~~19.~~34. Steffens, D. L., Sutter, S. L., & Roemer S. C. (1993). An alternate universal forward primer for improved automated DNA sequencing of M13. *Biotechniques, 15(4),* 580-582.

~~20.~~35. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular. Biology Evolution, 28(10),* 2731–2739.

36. Teletchea, F. (2009). Molecular identification methods of fish species: reassessment and possible applications. *Fish Biology and Fisheries, 19(3),*265–93.

~~21.~~37. Wadowsky R. M., Laus S., Libert T., States S. J. & Ehrlich G. E. (1994). Inhibition of PCR-based assay for *Bordetella pertussis* by using calcium alginate fiber and aluminum

shaft components of a nasopharyngeal swab. *Journal of Clinical Microbiology, 32 (4),* 1054-1057.

~~22.~~38. ~~Fish frauds: the DNA challenge. Animal Science Reviews 2012, 227.~~

~~8. Armani, A., Castigliego, L., Tinacci, L., Gandini G., Gianfaldoni, D., & Guidi, A. (2012d). A rapid PCR–RFLP method for the identifcation of Lophius species. *European Food Research and Technologies, 235,* 253–263~~

~~9. Ramšak, A., Stopar, K., & Malej. A. (2012) Comparative phylogeography of meroplanktonic species, Aurelia spp. And Rhizostoma pulmo (Cnidaria: Scyphozoa) in European Seas. *Hydrobiologia , 690,* 69-80~~

~~23.~~39. ~~Lu, Z., Dai, Q. & Yan, Y. (2003). Fishery biology of Cyanea nozakii resources in the waters of Dongshan Island. Chinese *Journal of Applied Ecology, 14,* 973–976 (in Chinese).~~

~~10. Bauer, T., Weller, P., Hammes, W. P. & Hertel, C. (2003). The effect of processing parameters on DNA degradation in food. *European Food Research and Technology, 217,* 338–343~~

~~24.~~40. ~~Dawson M. N. (2005). Incipient speciation of Catostylus mosaicus (Scyphozoa, Rhizostomeae, Catostylidae), comparative phylogeography and biogeography in south-east *Australia Journal of Biogeography, 32,* 515–533~~

~~25.~~41. Wong, W. W. K., Chung, S. W. C., Kwong, K. P., Yin Ho, Y., & Xiao, Y. (2010). Dietary exposure to aluminium of the Hong Kong population. *Food Additives & Contaminants, 27(4),* 457- 463.

~~11.~~ Wu J., Du F., Zhang P., Khan, I. A., Chen J., & Liang Y. (2005). Thermodynamics of the interaction of aluminum ions with DNA: Implications for the biological function of aluminum. *Journal of inorganic biochemistry 99*, 1145-1154.

~~26.~~42. ~~Wadowsky R. M., Laus S., Libert T., States S. J. & Ehrlich G. E. (1994). Inhibition of PCR-based assay for *Bordetella pertussis* by using calcium alginate fiber and aluminum shaft components of a nasopharyngeal swab. *Journal of Clinical Microbiology, 32 (4),* 1054-1057.~~

~~12. Pepe T., Trotta M., Di Marco I., Anastasio A., Bautista J. M. & Cortesi L. (2007). Fish species identification in surimi-based products. *Journal of Agricultural and Food Chemistry, 55 (9),* 3681-3685.~~

~~27.~~43. ~~Hajibabaei, M., Smith, M., Janzen, D. H., Rodriguez, J. J., Whitfield, J. B., & Hebert, P. D. (2006). A minimalist barcode can identify a specimen whose DNA is degraded. *Molecular Ecology Notes, 6(4),* 959-964.~~

~~28.~~44. ~~Bayha K. M., Dawson M., Collins A. G., Barbeitos M. S. & Haddock S. H. (2010). Evolutionary relationship among Scyphozoan jellyfish families based on complete taxon~~

sampling and phylogenetic analyses of 18s and 28s ribosomal DNA. *Integrative and Comparative Ecology, 50 (3),* 436-455.

29.45. Mariottini G. L., Giacco E. & Pane L. (2008) The Mauve Stinger *Pelagia noctiluca* (Forsskål, 1775). Distribution, Ecology, Toxicity and Epidemiology of Stings. A Review. *Marine drugs, 6,* 496-513.

Figure

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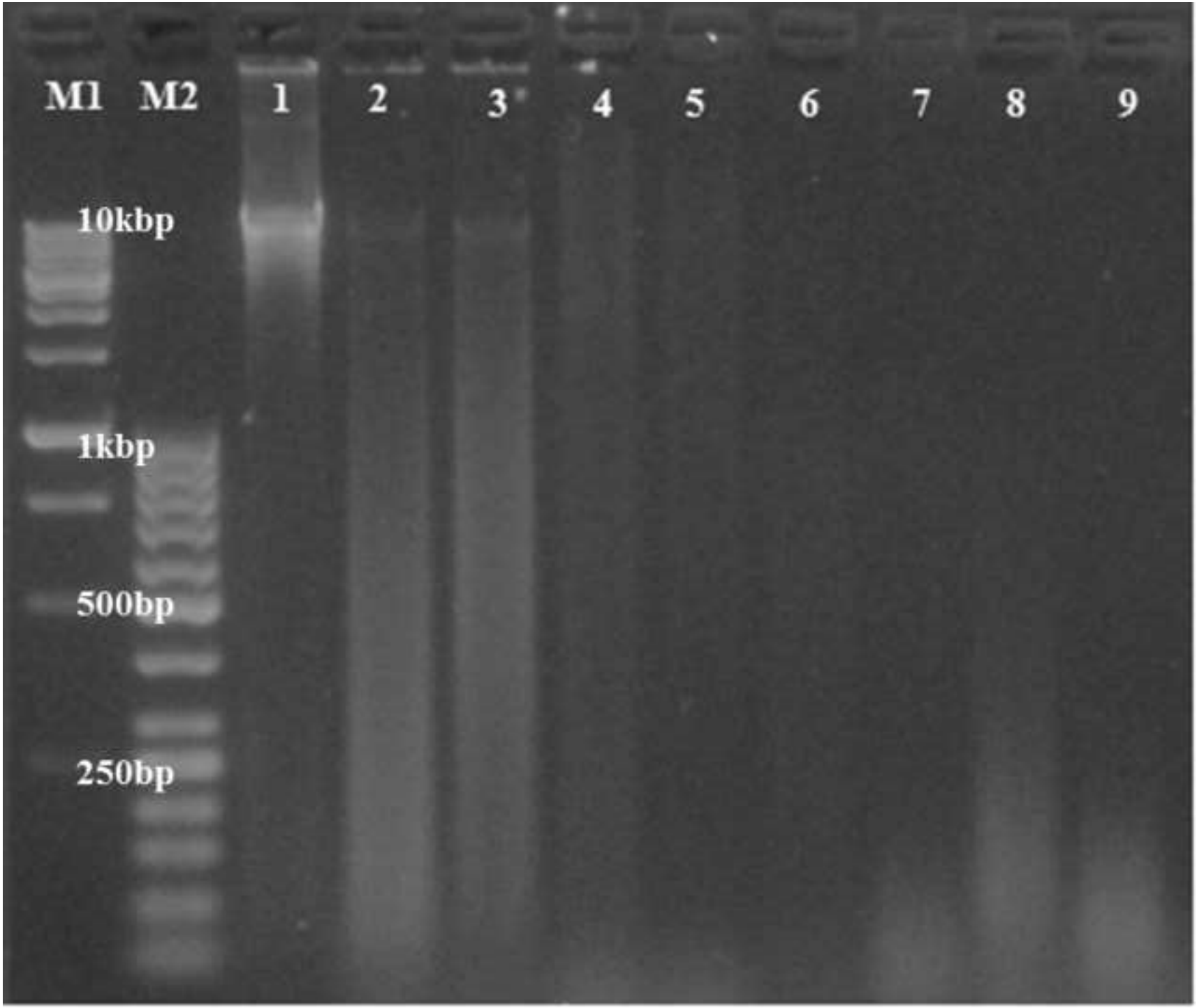

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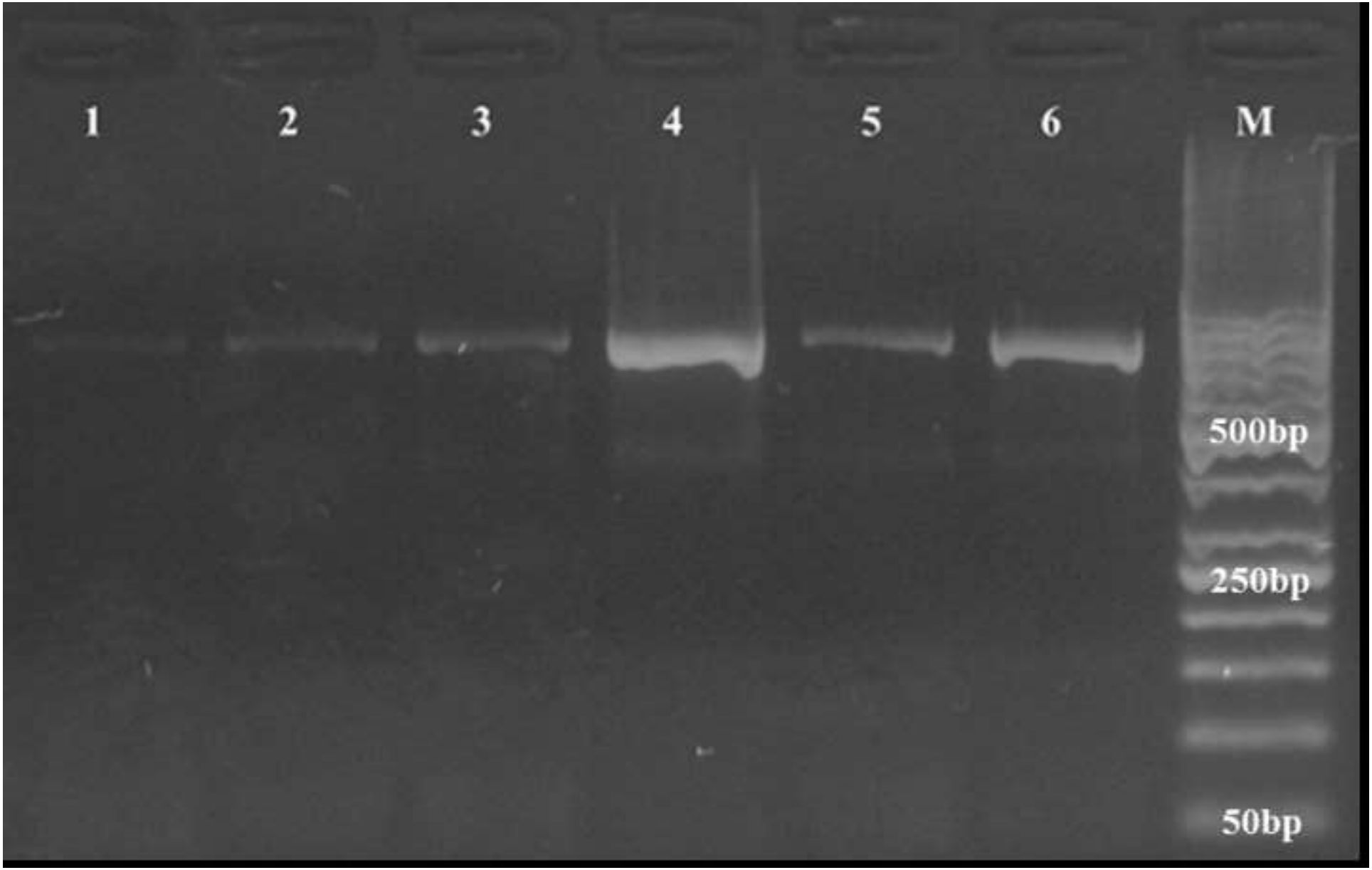

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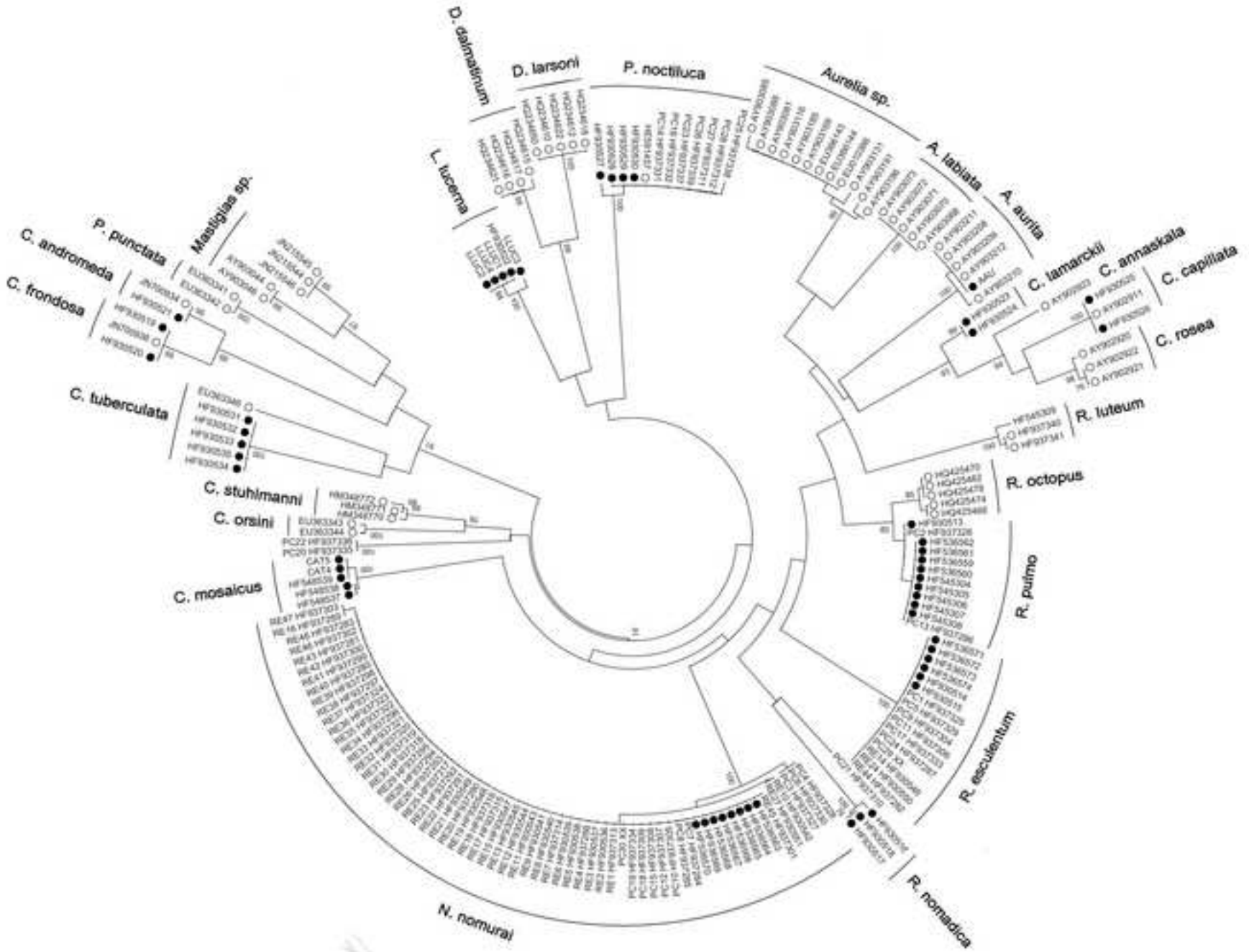

Figures captions

Fig. 1: Electrophoretic analysis of total DNA extracts on 1% agarose gel from: Lane 1: fresh jellyfish (positive control); Lane 2-5: RE products; Lane 6-9: CPs. M1: MW marker, SharpMass™50-DNA ladder (MW sizes of 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 bp). M2: MW marker SharpMass™1-DNA ladder (MW sizes of 10000 bp, 8000 bp, 6000 bp, 5000 bp, 4000 bp, 3000 bp, 2500 bp, 2000 bp, 1500 bp, 1000 bp, 750 bp, 500 bp, 250 bp).

Fig. 2: Effect of different pre-extraction treatments on DNA amplification of *COI* gene with primers FFDL and FRDL2 (709bp). Lane 1: 2h of washing; Lane 2: 2h of washing + EDTA; Lane 3: 24h of washing; Lane 4: 24h of washing + EDTA; Lane 5: 48h of washing; Lane 6: 48h of washing + EDTA. M: Ladder. M1: MW marker, SharpMass™50-DNA ladder (MW sizes of 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 bp). The samples have been electrophoresed on a 2% agarose gel.

Fig. 3: Neighbour-joining (NJ) tree obtained using 192 *COI* gene sequences (142 bp) of market samples, jellyfish reference specimens obtained in this study (black dot) and retrieved from the GenBank (white dot). Accession numbers of market samples are prefixed with voucher identifiers. Bootstrap values > 70%, obtained from 2000 replications using Kimura two parameter genetic distances, are reported in the tree.

Table

Order	Family	Genus/Species	Research Institute	Collection area	Sequence code	BLAST (Max identity)	
						BOLD	GenBank
Rihizostomeae	Catostylidae	*Catostylus mosaicus*	Australian Museum Sydney Australia	Pacific, Southwest FAO 81	HF548537	100% *C. mosaicus*	
					HF548538		
				Unknown	HF548539		
			Australian Rivers Institute Griffith School of Environment Gold Coast Campus	Pacific, Western Central FAO 71	HF968746		
					HF968747		
	Cassiopeidae	*Cassiopea spp.*	Department of Chemistry, Biology and Marine Science, Faculty of Science, University of the Ryukyus, Okinawa, Japan	Pacific, Northwest FAO 61	HF930519	100% *C. frondosa*	
			Stiftung Tieraerztliche Hochschule Hannover ITZ, Ecology and Evolution Buenteweg, Hannover Germany	Unknown	HF930520		
		Cassiopea andromeda	National Center for Mariculture Israel Oceanographic and Limnological Research Eilat, Israel	Indian Ocean, Western, FAO 51.1	HF930521	100% *C. andromeda*	
	Cepheidae	*Cotylorhiza tubercolata*	Instituto de Ciencias Marinas de Andalucia, ICMAN (CSIC) Puerto Real, Cadiz	Western Mediterranean FAO 37-1.1	HF930531	NS	
					HF930532		
					HF930533		
					HF930534		
			Stiftung Tieraerztliche Hochschule Hannover ITZ, Ecology and Evolution Buenteweg, Hannover Germany	Unknown	HF930535		
	Lychnorhizidae	*Lychnorhiza lucerna*	Departamento de Zoologia, Instituto de Biociências, Universidade de São Paulo, Brasil	Atlantic, Southwest FAO 41 2.1	HF930522	NS	
					HF968748		
					HF968749		
					HF968750		
					HF968751		
	Rhizostomatidae	*Rhizostoma pulmo*	This study	Western Mediterranean FAO 37-1.3	HF536559	NS	99% *R. pulmo*
					HF536560		
					HF536561		
					HF536562		
			Instituto de Ciencias Marinas de Andalucia, ICMAN (CSIC) Puerto	Western Mediterranean	HF545304		
					HF545305		

			Real, Cadiz	FAO 37-1.1	HF545306		
					HF545307		
					HF545308		
					HF930513		
		Nemopilema nomurai	Japan Sea National Fisheries Research Institute, Fisheries Research Agency, Niigata, Japan	Pacific, Northwest FAO 61	HF536563	99-100% *N. nomurai*	
					HF536564		
					HF536565		
					HF536566		
					HF536567		
					HF536568		
					HF536569		
					HF536570		
		Rhopilema esculentum	Ocean University of China. Fisheries of College. Qingdao, China	Pacific, Northwest FAO 61	HF536571	NS	99-100% *R. esculentum*
					HF536572		
			This study		HF536573		
					HF536574		
			Graduate School of Biosphere Science, Hiroshima University, Higashi-Hiroshima, Japan		HF930514		
					HF930515		
		Rhopilema nomadica	Israel Oceanographic and Limnological Research National Center for Mariculture Eilat, Israel	Western Mediterranean FAO 37-3.2	HF930516	NS	99% *R. nomadica*
			Department of Marine Biology, University of Haifa, Israel		HF930517		
					HF930518		
Semaeostomeae	Cyaneidae	*Cyanea lamarckii*	Institute of Coastal Research Marine Bioanalytical Chemistry Geesthacht, Germany	Atlantic, Northeast FAO 27 Div. IVa	HF930523	100% C.*lamarckii*	NS
					HF930524		
		Cyanea capillata			HF930525	99-100% *C.capillata*	
					HF930526		
	Pelagidae	*Pelagia noctiluca*	Dipartimento di Biologia, Università di Genova, Genova	Western Mediterranean FAO 37-1.3	HF930527	NS	100% *P. noctiluca*
					HF930528		
			This study		HF930529		
					HF930530		
	Ulmaridae	*Aurelia aurita*	Stiftung Tieraerztliche Hochschule Hannover ITZ, Ecology and Evolution Buenteweg, Hannover Germany	Pacific, Northwest FAO 61	HF968752	99% *A. aurita*	

Table 1. Reference samples analyzed in this study. NS: no sequences available.

Samples	City and place of collection	Label information		Sequence length and code	BLAST (Max identity)	
		Commercial name	Scientific name		Bold	GenBank
RE-1	Prato / M	Bamboo	---	145 – HF937313	100% *N. nomurai*	
RE-2	Prato / M	Bamboo	---	658 – HF930536	100% *N. nomurai*	
RE-3	Prato / M	Jellyfish	*R. esculentum*	658 - HF930537	100% *N. nomurai*	
RE-4	Prato / M	Jellyfish	*R. esculentum*	206 - HF937288	100% *N. nomurai*	
RE-5	Milan / M	Jellyfish	*R. esculentum*	658 - HF930538	100% *N. nomurai*	
RE-6	Milan / M	Jellyfish	*R. esculentum*	658 - HF930539	99% *N. nomurai*	
RE-7	Milan / M	Jellyfish	*R. esculentum*	145 – HF937314	100% *N. nomurai*	
RE-8	Prato / M	Mustard tuber	---	658 - HF930540	100% *N. nomurai*	
RE-9	Prato / M	Mustard tuber	---	658 - HF930541	99% *N. nomurai*	
RE-10	Florence / M	Mustard tuber	---	658 - HF930542	99% *N. nomurai*	
RE-11	Rome / M	Mustard tuber	---	658 - HF930543	100% *N. nomurai*	
RE-12	Rome / M	Jellyfish	*R. esculentum*	658 - HF930544	100% *N. nomurai*	
RE-13	Rome / M	Jellyfish	*R. esculentum*	658 - HF930545	100% *N. nomurai*	
RE-14	Rome / R	Jellyfish	---	658 - HF930546	NM	100% *R. esculentum*
RE-15	Rome / M	Jellyfish	*R. esculentum*	658 - HF930547	100% *N. nomurai*	
RE-16	Rome / M	Jellyfish	*R. esculentum*	206 - HF937289	100% *N. nomurai*	
RE-17	Rome / M	Mustard tuber	---	145 – HF937315	100% *N. nomurai*	
RE-18	Rome / M	Mustard tuber	---	145 - HF937316	100% *N. nomurai*	
RE-19	Rome / M	Jellyfish	*R. esculentum*	658 - HF930548	99% *N. nomurai*	
RE-20	Florence / M	Jellyfish	*R. esculentum*	206 - HF937290	100% *N. nomurai*	

RE-21	Florence / M	Jellyfish	*R. esculentum*	658 - HF930549	100% *N. nomurai*	
RE-22	Florence / M	Jellyfish	*R. esculentum*	206 - HF937291	100% *N. nomurai*	
RE-23	Florence / M	Jellyfish	*R. esculentum*	206 - HF937292	100% *N. nomurai*	
RE-24	Florence / R	Jellyfish	---	658- HF930550	NM	100% *R. esculentum*
RE-25	Prato / M	Jellyfish	*R. esculentum*	145 - HF937317	100% *N. nomurai*	
RE-26	Prato / M	Jellyfish	*R. esculentum*	206 - HF937293	100% *N. nomurai*	
RE-27	Milan / M	Jellyfish	*R. esculentum*	658 - HF930551	99% *N. nomurai*	
RE-28	Milan / M	---	---	206 - HF937294	100% *N. nomurai*	
RE-29	Milan / M	---	---	206 - HF937295	100% *N. nomurai*	
RE-30	Milan / M	---	---	145 - HF937318	100% *N. nomurai*	
RE-31	Milan / M	---	---	145 - HF937319	100% *N. nomurai*	
RE-32	Prato / M	Jellyfish	*R. esculentum*	145- HF937320	100% *N. nomurai*	
RE-33	Prato / M	Jellyfish	*R. esculentum*	145 - HF937321	100% *N. nomurai*	
RE-34	Prato / M	Jellyfish	*R. esculentum*	206 - HF937296	100% *N. nomurai*	
RE-35	Prato / M	Jellyfish	*R. esculentum*	145 - HF937322	100% *N. nomurai*	
RE-36	Prato / M	---	---	145 - HF937323	100% *N. nomurai*	
RE-37	Prato / M	Mustard tuber	---	145 - HF937324	100% *N. nomurai*	
RE-38	Prato / M	Lily flower	*Hemerocallis fulva*	206 - HF937297	100% *N. nomurai*	
RE-39	Prato / M	Lily flower	*Hemerocallis fulva*	206 - HF937298	100% *N. nomurai*	
RE-40	Prato / M	Lily flower	*Hemerocallis fulva*	658 - HF937280	99% *N. nomurai*	
RE-41	Prato / M	Jellyfish	*R. esculentum*	206 - HF937299	100% *N. nomurai*	
RE-42	Prato / M	Jellyfish	*R. esculentum*	206 - HF937300	100% *N. nomurai*	
RE-43	Prato / R	Jellyfish	---	658 - HF937281	99% *N. nomurai*	

RE-44	Prato / R	Jellyfish	---	658 - HF937282	NM	100% *R. esculentum*
RE-45	Florence/M	Jellyfish	*R. esculentum*	206 - HF937301	100% *N. nomurai*	
RE-46	Florence/M	Jellyfish	*R. esculentum*	206 - HF937302	100% *N. nomurai*	
RE-47	Florence/M	Mustard tuber	---	206 - HF937303	99% *N. nomurai*	
RE-48	Florence/M	Mustard tuber	---	658 - HF937283	100% *N. nomurai*	
CP-1	Prato / M	Jellyfish	*R. esculentum*	145 - HF937325	NM	100% *R. esculentum*
CP-2	Prato / M	Jellyfish	*R. esculentum*	145 - HF937326	NM	100% *R. pulmo*
CP-3	Prato / M	Jellyfish	*R. esculentum*	145 - HF937327	99% *N. nomurai*	
CP-4	Prato / M	Jellyfish	*R. esculentum*	145 - HF937328	99% *N. nomurai*	
CP-5	Prato / M	Jellyfish	*R. esculentum*	145 - HF937329	NM	100% *R. esculentum*
CP-6	Prato / M	Jellyfish	*R. esculentum*	145 - HF937330	100% *N. nomurai*	
CP-7	Prato / M	Jellyfish	*R. esculentum*	658 - HF937284	99% *N. nomurai*	
CP-8	Prato / M	Jellyfish	*R. esculentum*	658 - HF937285	100% *N. nomurai*	
CP-9	Prato / M	Jellyfish	*R. esculentum*	206 - HF937304	NM	100% *R. esculentum*
CP-10	Florence/M	Jellyfish	*R. esculentum*	206 - HF937305	100% *N. nomurai*	
CP-11	Rome / M	Jellyfish	---	206 - HF937306		100% *R. esculentum*
CP-12	Rome / M	---	---	206 - HF937307	100% *N. nomurai*	
CP-13	Rome / M	Jellyfish	*R. esculentum*	658 - HF937286	NM	99% *R. pulmo*
CP-14	Rome / M	Jellyfish	*R. esculentum*	145 - HF937331	NM	100% *P. noctiluca*
CP-15	Rome / M	Jellyfish	*R. esculentum*	206 - HF937308	100% *N. nomurai*	
CP-16	Rome / M	Jellyfish	*R. esculentum*	145 - HF937332	NM	100% *P. noctiluca*

CP-17	Rome / M	Jellyfish	*R. esculentum*	145 - HF937333	NM	100% *R. esculentum*
CP-18	Florence/M	---	---	206 - HF937309	99% *N. nomurai*	
CP-19	Prato / M	Jellyfish	*R. esculentum*	145 - HF937334	100% *N. nomurai*	
CP-20	Prato / M	Asiatic jellyfish	*R. esculentum*	145 - HF937335	NM	85% *C. stuhlmanni*
CP-21	Prato / M	Asiatic jellyfish	*R. esculentum*	206 - HF937310	NM	86% *R. nomadica*
CP-22	Prato / M	Asiatic jellyfish	*R. esculentum*	145 - HF937336	NM	85% *C. stuhlmanni*
CP-23	Zhejiang (PRC) / M	Jellyfish	---	145 - HF937337	NM	100% *P. noctiluca*
CP-24	Zhejiang (PRC) / M	Jellyfish	---	658 - HF937287	NM	100% *R. esculentum*
CP-25	Zhejiang (PRC) / M	Jellyfish	---	145 - HF937338	NM	100% *P. noctiluca*
CP-26	Zhejiang (PRC) / M	Jellyfish	---	145 - HF937339	NM	100% *P. noctiluca*
CP-27	Zhejiang (PRC) / M	Jellyfish	---	206 - HF937311	NM	100% *P. noctiluca*
CP-28	Zhejiang (PRC) / M	Jellyfish	---	206 - HF937312	NM	100% *P. noctiluca*
CP-29	Jangsu (PRC) / M	Jellyfish	---	145 - XX	NM	100% *R. esculentum*
CP-30	Jangsu (PRC) / M	Jellyfish	---	658 - XX	99% *N. nomurai*	

Table 2. Market jellyfish products analyzed in this study. RE: ready-to-eat, CP: classical products, M: Market; R: Restaurant; NM: no match.

Primer code	Reference	Primer sequence (5'→3')	PL (bp)	TM (°C)	AL with and without primers
LCO1490	Folmer, 1994	GGTCAACAAATCATAAAGATATTGG	25	56,4	709/658
HCO2198		TAAACTTCAGGGTGACCAAAAAATCA	26	58,5	
FDF	THIS STUDY	TCAACAAACCAYAAAGATATWGG	23	54,4	
FDR		CTTCTGGGTGNCCRAARAAYCA	22	60,2	
FRDL		TAAACTTCTGGGTGNCCRAARAAYCA	26	61,6	
FFDL		TTTCAACTAACCAYAAAGAYATWGG	25	56,4	
FRDL2		TANACTTCWGGRTGNCCRAAGAATCA	26	61,6	
REV514		CCATAGTCATTCCDGGDGC	19	58,1	514/469
REV514N		CCATNGTCATWCCDGGDG	18	56,3	
REV514A		TCCATNGTCATWCCTGGRGC	20	59,3	
REV514B		TCCATNGTCATWCCTGGRGCYCTC	24	65,3	
REV256A		GCCATRTCYGGDGCTCCTATATATA	25	61,9	256/206
REV256B		GCCATATCNGGACTACCAATATATA	25	58,9	
REV193W		GGCATWACRAAGAARAATATCATTAT	26	55.3	193/145
REV193H		GGCATHACRAAGAARAATATCATTAT	26	55,9	
REV193HS		GGCATHACRAAGAARAAWATCAT	23	54,1	
REV163		GCRTGDGCTGTBACAACCAC	20	60.4	163/118
REV133		TGRTCRTCWCCTARCATAGATCC	23	59.8	133/85
M13F	Steffen et al 1993	CACGACGTTGTAAAACGAC	19		
M13R		GGATAACAATTTCACACAGG	20		

Table 3. Primers designed for setting up the FINS methods. In grey primers finally selected. PL: primer length. TM: melting temperature. AL: amplicon length. The length of the amplicon has been calculated on the sequence of *R. pulmo* (JN700988).

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