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Universal primers used for species identification of foodstuff of animal origin: effects of oligonucleotide tails on PCR amplification and sequencing performance

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Universal primers used for species identification of foodstuff of animal origin: effects of oligonucleotide tails on PCR amplification and sequencing performance

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Abstract

M13 universal non-homologous oligonucleotide tails incorporated to universal primers have been shown to improve amplification and sequencing performance. However, few protocols use these tails in the field of food inspection. In this study, two types of M13 tails (by Steffens and Messing) were selected to assess their benefits using universal Cytochrome oxidase subunit I (*COI*) and 16S ribosomal RNA gene (*16SrRNA*) primers in standard procedures. The primers characteristics were tested *in silico*. Then, using 20 DNA samples of edible species (birds, fish and mammals), their performance during PCR amplification (bands recovery and intensity) and sequencing (sequences' recovery, length and Phred score) were assessed and compared. While *16SrRNA* tailed and not-tailed primers performed similarly, differences were found for *COI* primers. Messing's tails negatively affected the reactions outputs, while Steffens' tails significantly improved the band intensity and the length of the final contigs based on the individual bidirectional reads sequence. This different performance could be related to a destabilization effect of certain tails on primers with unfavorable mismatches on the annealing region. Even though our results cannot be generalized because the tails performances are strictly dependent on laboratory conditions, they show that appropriate tails can improve the overall throughput of the analysis, supporting food traceability.

Keywords

Universal primers, M13 oligonucleotide tails, tailed primers, species identification, amplification, sequencing

Introduction

In the last decades, molecular methods based on PCR amplification of target genes have been developed and widely applied for species identification in foodstuff of animal origin (Armani et al. 2012; Galimberti et al. 2013; Teletchea 2009). These techniques may rely on species-specific primers, designed *ad hoc* in order to anneal only to DNA of a given species (Lockley and Bardsley 2000), or on universal primers, matching regions of DNA conserved across species (Carrera et al. 2000).

Although universal primers are able to bind to a wide variety of DNA templates, they cannot assure DNA amplification of all kind of organisms due to the presence of mutations which cause primer-sequence mismatches. Thus, even though the taq polymerase is tolerant to mismatches, these primers are commonly degenerated at variable nucleotide positions to improve PCR outputs (Carrera et al. 2000; Kwok et al. 1995).

A PCR primer sequence is called degenerated if one or more of its positions have several possible bases (Linhart and Shamir 2005). Primers with degenerated positions increase the possibility to amplify, with a single PCR reaction, the same DNA fragment from a wide range of taxa (Lang and Orgogozo 2011) and, for this reason, are of particular interest in case of DNA sequencing approaches (Casiraghi et al. 2010). Therefore, the use of degenerated primers has become of great appeal with the development of procedures based on sequencing, such as FINS (*Forensically Informative Nucleotide Sequencing*) (Bartlett and Davidson 1992) and DNA barcoding (Hebert et al. 2003). In fact, this approach allows generating multiple data sets for evolutionary and forensic analysis and it is nowadays routinely and successfully applied to the identification of different kind of animal species. Even though the molecular methods based on sequencing are primarily used for seafood identification, they are also a useful tool for the authentication of other animal food products, in consideration of the vast array of marketable species and the consequent high rate of fraudulent substitutions (Galimberti et al. 2013). In

particular, the Regulation (EU) No.1379/2013 on the common organization of the markets of fishery and aquaculture products states that "*the available technologies, including DNA-testing, should be used to protect the consumer and in order to deter operators from falsely labeling catches*". Moreover, a recent report of the European Parliament asked the EU Commission to take further measures against food frauds and also to consider the creation of an EU Reference Laboratory (EURL) for food authenticity (Report 2013/2091 INI).

A wide variety of universal primers is now available for the amplification of Cytochrome b (*cytb*), 16S ribosomal RNA gene (*16SrRNA*) and Cytochrome oxidase subunit I (*COI*), the three mitochondrial genes most targeted for species identification. Among these primers, those targeting the *COI* gene are often degenerated (Armani et al. 2012) while the high degree of conservation of 1*6SrRNA* gene does not require these modification (Cawthorn et al. 2012; Kochzius et al. 2010).

Regardless the DNA technique and the target gene chosen, the quality of the amplification and of the sequences is crucial for a successful identification. For the improvement of PCR outputs, besides primer relative concentrations, reagent concentration and combination, DNA polymerases and template concentration also amplification facilitators, such as BSA, Dimethyl sulfoxide and Glycerol (Al-Soud and Rådström 2000) and mutants taq (Kermekchiev et al. 2009) can be used. However, all these expedients only acts during the PCR reaction, increasing the concentration and the overall quality of the final products. The use of tailed primers (bipartite primers), which include non-degenerated non-homologous sequences at their 5' ends (tails), has been proposed to improve both amplification and sequencing output (Binladen et al. 2007; Regier and Shi 2005; Roy et al. 1996; Steffens and Roy 1998).

M13 universal tails are the most used to date (Boutin-Ganache et al. 2001; Messing 1983; Missiaggia and Grattapaglia 2006; Oetting et al. 1995; Neilan et al. 1997; Schuelke 2000; Steffens et al. 1993). While most of the genome of the "wildtype" M13 phage, a filamentous bacteriophage with a genome of single-stranded circular DNA (Model and Russel 1988), contains the genetic

information that is essential for viral replication, a small region, called "intergenic sequence", can be used as cloning site (Van den Holden et al. 1976). In fact, the chain termination sequencing procedure of Sanger et al. (1977) requires single-stranded DNA as template and M13 can be easily obtained in this form (Schreirer and Cortese 1979). Nowadays, most sequencing services provide standard M13 primers at no additional cost and it has become customary to include tails on the PCR primers to simplify sequencing set-up in large projects.

The possibility to enhance the performance of PCR amplification and sequencing of DNA extracted from food products by using tailed primers could be of great interest also in order to favor standardization across European laboratories, which is still lacking (Griffiths et al. 2014). To the best of our knowledge, only few protocols use M13 tails in this field (Table 1SM). Consequently, every effort aimed at standardizing and enhancing the performance of a sequencing-based procedure could replace the need for expensive laboratory set-up and increase the overall quality and comparability of the results.

In this study, tailed and non-tailed universal primers (degenerated and non-degenerated) were used to amplify fragments of the *COI* and of the *16SrRNA* mitochondrial genes from different animal species (birds, fish and mammals). The primers characteristics were initially assessed *in silico*. Then, they were used for the amplification and sequencing of the selected gene fragments. Their performances during PCR amplification (bands recovery and intensity) and sequencing, evaluated on the basis of the sequences' recovery and quality (length and Phred score), were assessed and compared. Overall, this work aimed to assess the benefits of using tailed primers in standard laboratory procedures.

2. Materials and methods

2.1 Reference samples

Twenty fresh muscle tissue samples from different species (6 birds, 6 fishes and 8 mammals) were used (Table 1). All the tissues belong to specimens that have been morphologically identified at slaughterhouses or at wholesale fish markets.

2.2 DNA extraction and evaluation of DNA fragmentation by gel electrophoresis

Total DNA was extracted following the salting-out protocol proposed by Armani et al. (2011). The amount of DNA was determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) 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. DNA integrity was assessed ad reported in Armani et al. (2015a).

2.3 Reference genes, primers and tails selection

CO1 and *16SrRNA* genes were selected as targets and amplified using the primer pairs designed by Baldwin et al. (2009) and amended by Handy et al. (2011) and those designed by Palumbi (1996), respectively (Table 2). The M13 tails utilized were: M13F (-29) and M13R proposed by Steffens et al. (1993) and M13F (-21) and M13R (-27) proposed by Messing (1983). These couples of oligonucleotide tails are here referred as ST (Steffens' Tails) and MT (Messing's Tails), respectively (Table 2). NT stands for not tailed primers.

2.3.1 In silico evaluation of primers amplification performances. A *in silico* evaluation of all the primers was performed on the basis of their melting temperature (mt), GC content (%) and tendency to form hairpins and self and hetero-dimers using the software IDT's *Oligo Analyzer Version 3.1* (http://eu.idtdna.com/calc/analyzer) at standard conditions. The Multifunctional Oligo Property Analysis Tool (MOPS) (available at https://ecom.mwgdna.com/services/webgist/mops.tcl), which assigns an Annealing Score (AS) to the primers on the basis of their overall characteristic, was also used. Finally, the selected primers were aligned with the gene sequences of the reference species (Table 2SM and 3SM) using Clustal W in BioEdit version 7.0.9. (Hall 1999) and the number of mismatches was calculated.

2.4 DNA amplification and sequencing

2.4.1 Amplification of mtCOI and 16SrRNA using a PCR standard protocol. The amplification was performed according to the protocol reported in Table 4SM using both tailed and not tailed primers (Table 2). The DNA amplification was performed in triple.

2.4.2 Gel electrophoresis and evaluation of PCR output and of PCR products intensity. Five µL of PCR products were checked by gel electrophoresis on a 2% agarose gel. The amplification of fragments of the expected length (~700bp for the *COI* gene and ~607bp for the *16SrRNA* gene, respectively) was assessed by a comparison with the standard marker SharpMass™50-DNA ladder (Euroclone, Wetherby, UK) and the concentration of PCR products by making a comparison with the intensity of the bands of the DNA ladder (Gu and Rajewsky 2004). A concentration of approximately 50 ng/µL was used as threshold to discriminate an amplification of good quality.

2.4.3 Annealing temperature (AT) selection for MT primers. Due to not repeatable outcomes during the amplification of the *COI* gene with MT primers, the standard PCR program was modified. In particular, the protocol was changed after testing different Annealing Temperatures (AT) (from 47°C to 59°C) using the DNA samples which was not amplified with the standard protocol (Table 1). The selected AT was 47°C. PCR outputs were assessed as reported in section *2.4.2*. The presence of false negatives and the need for improvement was considered as an index of low primer performance. All the samples were amplified in triple.

2.4.4 Evaluation of the sequencing success rate and sequence quality. All the obtained amplicons were purified and sequenced by High-Throughput Genomics Center (Washington, USA). Sequencing success rate was calculated by dividing the number of recovered sequences (of at least 500 bp in length) by the total number of sequenced samples. Sequences shorter than 500 bp were considered failures and removed from the analysis, as proposed by Handy et al. (2011). Then, forward and reverse chromatograms were analyzed using the program Codoncode Aligner 5.1 (CodonCode Corp., Dedham, MA) in order to calculate the length and the Phred quality score

(Ewing et al. 1998). In particular, the sequences bases were considered reliable (high quality) when presenting a Phred score higher than 20 (CodonCode Alignment User Manual). Then, the forward and reverse sequence of each samples were aligned using Clustal W in BioEdit version 7.0.9 (Hall 1999) and analyzed following the procedure described by Handy et al. (2011). Finally, each assembled sequence was used to run a BLAST analysis on GenBank and analyzed using the Identification System (IDs) on BOLD (Species Level Barcode Records) (Ratnasingham and Hebert 2007). For the *COI* gene identity values ≥ 98% were considered as an index of good quality (Barbuto et al. 2010). In the case of the *16SrRNA* gene an identity score of 100% was used as threshold for species level identification (Armani et al. 2015b). All the samples were sequenced in double.

2.4.5 Statistical analysis. Pearson's chi squared test and Fisher's exact test were used to compare the average concentration of the PCR products (bands intensity) obtained through the amplification with NT, ST, and MT primers. The two-tailed t Student test was used to compare the sequencing success rate obtained from NT, ST and MT primers. The same test was also used to compare both the single (one strand) and contig sequences' average length and the average percentage of high quality bp score (Phred score) within the sequences obtained from the three primers pairs.

3. Results and discussion

3.1 DNA quality evaluation

The spectrophotometric values of A260/A280 and A260/A230 were always within the optimal range (1.8-2), indicating a good level of purity of the DNA extracted (De Maeseneire et al. 2007; Sambrook and Russell 2001). The gel electrophoresis analysis showed fragments of at least 1 kb, which indicate a good value of DNA integrity (Teletchea 2009), in all the total DNA samples.

3.2 Reference genes, primers and tails selection

At present, the *COI* gene is the most targeted mtDNA gene due to its high intraspecific diversity, to a well-established molecular identification system with a dedicated database and to a broad range

198 of very robust universal degenerated primers for different animal organisms (Folmer et al. 1994; Hajibabaei et al. 2006; Hebert et al. 2004; Ivanova et al. 2007; Lorenz et al. 2005; Mikkelsen et al. 2006; Wells et al. 2001; see also Table 1SM). Considering the good performances on fish identification (Armani et al. 2015a, 2015b) and preliminary trials performed in our laboratory, which show that these primers were able to amplify also DNA samples of mammals and birds, the primers proposed by Handy et al. (2011) were selected.

The other selected gene was the *16SrRNA*, which allows the amplification of the same DNA fragment from different taxa, such as vertebrates, insects, gastropods and urchins (Palumbi et al. 1991), using non degenerated primers, such as those of Palumbi (1996). Also in this case previous trials highlighted the ability of these primers to amplify DNA from different taxa.

M13 tails were selected among those most used in phylogenetic and food inspection fields. In fact, several studies have indeed used primers with oligonucleotides tails designed by Messing (1983) and more recently re-proposed by Ivanova et al. (2007). M13 tailed primers LCO1490 and HCO2198 by Folmer et al. (1994) have been widely used for the amplification of *COI* gene from metazoan (James et al. 2010; Park et al. 2010; Porco et al. 2012; Prosser et al. 2013; Rougerie et al. 2009; Stoev et al. 2010; Van Houdt et al. 2010). Even though these primers have also been recently used in some studies that apply DNA barcoding to food inspection, their utilization is still limited (Table 1SM). In addition, the Official DNA barcoding protocol proposed by the US Food and Drug Administration for the authentication of fish-based commercial products (Handy et al. 2011) uses the tails proposed by Steffens et al. (1993). Thus, considering its authority in the field of food control, it is plausible that this tails will be used also by other official agencies. Therefore, in the present paper both M13 of Messing (1983) and of Steffens et al. (1993) have been tested and compared.

3.3 In silico evaluation of primers

Primers assessment extends beyond string matching and involves several real-valued criteria (Kämpke et al. 2001). First, primers melting temperature is of obvious relevance for the temperature cycling protocols (Hilier and Green 1991), but specificity of the priming reaction should also be enhanced through the minimization of hybridization effects among primers (self and heterodimers) (Kämpke et al. 2001). In fact, single stranded nucleic acid sequences may have a secondary structure (hairpin loops and dimers) which reduce the efficiency of the reaction by limiting their binding to the target site (Singh et al. 2000). Primer G+C content is also important: if the content is too high (higher than 40-60% of the total bases), the primer may tend to adopt a secondary structure or to nonspecifically anneal to GC-rich regions of the template DNA. However, if the G+C content is too low, the primer may not anneal to its target sequence. All these criteria were used to calculate the AS, an overarching value that provides a measure of base-pairing propensity of the primer with the various non-target sequences present in the reaction (Hilier and Green 1991).

Primers with mt in the range of 52-58°C generally produce the best results, while primers with mt above 65°C tend to form secondary annealing. Moreover, a critical point that influences the amplification output is the proximity between the mt of the primer couple: in general, a difference higher than 5°C should be avoided (Kämpke et al. 2001). The mt of the primers used in this work was quite good for all the NTs, while, obviously, it exceeded the optimal value for all the STs and MTs primers (Table 2). All the primers pairs had a similar mt (maximum ΔT of 5.1°C in the case of 16sar-L MT/16sbr-H MT), except the 16sar-L/16sbr-H (Palumbi et al. 1996) (ΔT=11°C). However, the AT used in the amplification protocol was calculated on the basis of the mt of the NT primers, as proposed by Ivanova et al. (2007) and Handy et al. (2011). In fact, tails should not directly influence the choice of AT, because they do not pair to DNA regions during the first cycles of the reaction that are known to be most important for primers annealing. For this reason, the choice of the AT is especially critical during the first few cycles of PCR amplification, as any non-specific

annealing in this step will result in the amplification and accumulation of large quantities of non-specific products at the end of the PCR (van Pelt-Verkuil et al. 2008).

However, during the set up of the PCR protocol the AT had to be reduced to obtain the desired amplicon (see Section 3.5.1), suggesting their involvement in the overall ability of primers annealing.

Secondary structures of primers, such as loops and dimers, were evaluated taking into consideration their theoretical ΔG value (quantity of energy needed to fully break a given oligonucleotide pairing). Generally, the ΔG values for both self and hetero-dimers has to be less negative than -9 kcal/mole for non problematic primers (Olygo analyzer FAQ). This value was then taken as threshold for considering the primers as good. The ΔG value for self-dimers was lower than -9 kcal/mole for most of the primers used (both *mtCOI* and *16SrRNA* primers), with the exception of 16sar-L and 16sar-L ST (ΔG = -7.18 kcal/mole). However, the most part of the other primers had a ΔG values very close to -9 (Table 3). Regarding the hetero-dimers the ΔG values were lower than -9 kcal/mole for most of the primers used (both *COI* and *16SrRNA* primers), with the exception of 16sar-L/16sbr-H pair (ΔG = -3.61) and 16sar-L MT/16sbr-H MT pair (ΔG =-8.78). The ΔG value was not influenced by the presence of tails, with the exception of the primers of Handy et al. (2011) tailed with MT (ΔG =-14.84) (Table 3). The tendency to form hairpins resulted higher in the case of both forward primers (FISH-BCL and 16sar-L) tailed with ST (Table 3) and it was probably due to a complementary sequence within the M13F (-29). The higher tendency to form hetero dimers of FISH-BCL MT and FISH-BCH MT could be responsible for the presence of non-specific bands (see section 3.5.3).

The GC content of the *mtCOI* primers (tailed and not tailed) was always lower than 50%, while the 16sbr-H and the 16sbr-H MT primers slightly exceeded this value.

Overall, the AS was lower for the *mtCOI* primers (mean=11.2) than for the *16SrRNA* primers (mean=17.8). Among the *mtCOI* primers only the FISH-BCL ST exceeded the threshold value of

15 (AS=22). Among the *16SrRNA* primers, only the 16sar-L and the 16sar-L MT primers have an AS value lower than 15 (11 and 12, respectively) (Table 3). Therefore, based on the AS, the *mtCOI* primers seem to be better than *16SrRNA* primers. However, subsequent evaluations after amplification did not confirm these results (see section 3.5).

In silico evaluation of the number of mismatches between the primers and their annealing region was performed. To the best of our knowledge, this is the first time that the primers of Handy et al. (2011) have been used on species different from fish. In fact, the primers most used to amplify mammals and birds *mtCOI* are those of Ivanova et al. 2007 and Hebert et al. 2004, respectively. The number of mismatches found in the case of the *mtCOI* primers was similar among birds, mammals and fishes. There were no substantial differences between the Classes, with an overall range of 1-4 mismatches on the forward and of 0-3 mismatches on the reverse primer (Table 2SM). Conversely, differences in mismatches (number and position) were observed within the Classes (Table 2SM). With regard to the position, the hare, the beef, the deer and the duck had a mismatch within the first three bases near the 3' end which affects PCR more dramatically than those single mismatches located internally or at 5' end (Lindeman et al. 1991; Palumbi et al. 1991) (see section 3.5.2).

The results of the amplifications (see section 3.5), together with observations from a previous study, in which the introduction of mismatches in a critical position was found capable to prevent primers' annealing (Armani et al. 2014), suggest that the assessment of the number and position of mismatches represents a pivotal criteria to predict the primers performance.

In the case of the *16SrRNA* the number of mismatches was almost the same between the three animal Classes considered, with an overall range of 1-3 mismatches for the 16sar-L. No mismatches were observed for the 16sbr-H (Table 3SM). As expected, all the DNA samples were amplified using this primer pair (see section 3.5.1).

Even though the samples received by laboratories involved in species identification are usually from "unknown samples species", the Class to which they belong (birds, fish or mammals) is

generally known. Thus, with the aim to select primers with a low number of mismatches, it could be useful to proceed with a preliminary alignment of the selected primers with the reference sequences available on the databases. In fact, in most of the cases the position of the mismatches is conserved among classes (Table 2SM and 3SM).

3.4 Performance during PCR and PCR output

3.4.1. Amplification rate. Initially, two standard PCR protocols were used. However, while using the *16SrRNA* primers (tailed and NT) we obtained an amplification rate of 100%, in the case of the *COI* gene we observed some differences, even though non-significant (Table 1). In particular, the MT primers did not amplify 4 DNA samples (Table 1), with an overall amplification rate of 93.3%. On the basis of the electrophoresis outputs (see Section 3.2) and the amplification results (with both *COI* and *16SrRNA* primers) (Figure 1), these amplification failures are not attributable to a degradation of the DNA, but rather to a destabilizing effect of the MT. In fact, by modifying the AT of the PCR protocol, all the samples were amplified with MT primers (see section 3.5.2).

The primers of Handy et al. (2011), designed for the amplification of the *COI* gene in fish, were able to amplify the DNA of all the species of mammals and birds tested in this study, confirming what already hypothesized through primers *in silico* evaluation (see Section 3.4).

3.4.2 Implementation of the PCR protocol. Due to the low amplification rate with MT primers we decided to test different AT. Using an AT of 47°C all the samples gave the expected bands. Therefore, contrarily to Regier and Shi (2005), we found that the tails destabilize the primer annealing. This effect was evident when primers had more than one mismatch or when mismatches were located near the 3' end. In fact, 75% of the samples negative at the first amplification were those considered as "*potentially*" problematic during *in silico* evaluation (see Section 3.4 and Table 2SM). Two mismatches (not close to the 3') existed on both forward and reverse primers on the other species (anchovies) that was negative at the first amplification. Considering that all the other DNA samples that presented two mismatches both on forward and reverse primer in similar

positions were well amplified, we can suppose that interaction of the primers with the complementary DNA may be destabilized, according to the particular combination of the tail bases beside the 5' end of the primers and the corresponding bases on the DNA sequence. In fact, the stacking of the DNA bases is a strong contributor to the overall stabilization of the double helix and different combinations of unpaired bases have different destabilization potential. Furthermore, neighboring base can have a very significant influence on stacking energetics for a given unpaired base (Kool 2001). This could also explain because the DNA of beef, which presents the same number and position of mismatches existing on the DNA sequence of the deer, was amplified without problems.

Therefore, while in the study of Ivanova et al. (2007) the amplification was unaffected by tailed primers, we found that an implementation of the procedure may be necessary to avoid false negative during amplification. Therefore, in private and official laboratories, it is very important to set up any procedure considering that tails could affect the amplification process depending on the primers and the DNA samples used.

3.4.3 Band intensity. As for the amplification output, also in this case, our results show a discordance between the products obtained by amplifying the *16SrRNA* and the *COI* gene. In fact, PCR products of comparable intensity were generated with all the couples of the *16SrRNA* primers (tailed and NT) for all the DNA samples (data not shown). This could be explained considering that the tails did not influence the primer annealing, due to the high conservation of the gene (Table 3SM). The amplification performance of the *COI* gene varied according to the different primer couples used. In particular, MT primers reduced the amplification performance (overall lower bands intensity with mean= 35.4305 ng/µl and σ=6.071 ng/µl). Although in the study of Regier and Shi (2005) the intensity and purity of the PCR products were in most cases greater with tails than without, we observed the presence of evident non-specific bands in case of DNA samples amplified with MT primers (Figure 1). This result supports the hypothesis by Rudi et al. (2003), who

suggested that the tailed primers are more prone to generate unspecific products contributing to the overall reduction of the concentration of the target PCR product (Rudi et al. 2003).

A suboptimal quantity of template DNA can influence the success of the sequencing reaction. Considering that most of the DNA sequencing service providers recommend a concentration of PCR unpurified products between 10 and 50 ng/µl, and on the basis of our experience, we considered an estimated concentration >50 ng/µl as threshold of good quality. This optimal concentration was obtained for all the *16SrRNA* PCR products. In case of the *COI* gene, only 1.6% of the amplicons obtained with the MTs primers exceeded the selected threshold. The concentration of PCR products obtained with NT and ST primers exceeded this value in 36.6% and 28.3% of the samples, respectively. This result determined a significant difference between the NT/MT and ST/MT amplification outputs (P value = 0.0001), while no significant differences were observed between NT and ST amplification output (P value = 0.4358).

3.5 Sequencing success rates and evaluation of the sequence quality

3.5.1 Sequencing success rates. With regards to the *COI* primers, the overall sequencing success rate was 99.4%. In fact, only one reverse sequence from a beef sample amplified with ST primers was unreadable. Therefore, the sequencing success for PCR products amplified with NT and MT primers was 100% and for those amplified with ST primers 98.3%. However, in our opinion, this failure could be attributed to a random sequencing error not specifically linked to ST primers. The overall sequencing success rate was 100% for all the DNA samples amplified with both tailed and not-tailed *16SrRNA* primers.

3.5.2 Evaluation of the sequence quality. For the *COI* primers, the overall average length of the trimmed sequences was 597.0 (583.1, 614.6 and 593.4 for the sequences amplified with NT, ST and MT primers, respectively). The length of the expected sequences would have been ~655bp. In particular, the comparison between NT and ST primers highlighted a very high significant difference (P<0.0001). Similarly, the difference between ST and MT primers was significant

(P=0.0136), while no significant difference was found between NT and MT primers (P=0.2009). Although the *COI* sequences obtained from NT were slightly shorter, all of them exceeded 500bp.

On the contrary, no significant differences were observed between the lengths of the sequences obtained from *16SrRNA* primers.

Bases with quality values below 20 were considered not reliable (accuracy below 99%) (CodoCode Alignment User Manual). A Phred score >20 was obtained, on an average, in 573.12 bp, 602.84, and 581.63 bp for what concerns the *COI* trimmed sequence obtained from NT, ST, and MT primers, respectively. A comparison between the length of the sequences and their quality was performed, showing that in all the trimmed sequences an average of 98% of the bp had a Phred score >20, for tailed and NT primers (no significant difference). Moreover, the average length of the final contig was of 521.26, 581.06, and 542.46 bp for the sequences amplified with NT, ST and MT primers, respectively. In particular, 80%, 97%, and 90% of the sequence amplified with NT, ST and MT primers, respectively, was longer than 500 bp. All these sequences could be considered of high-quality according to Handy et al. (2011). However, it is interesting to point out that a significant difference in the length obtained was observed between NT and ST primers (P<0.0001), and ST and MT primers (P=0.0177). No significant difference could be observed between NT and MT primers (P value=0.1593). Therefore, in agreement with the results reported by Binladen et al. (2007), we showed that tailed primers, in this case the ST, improved the sequences output. Overall, non-significant differences were found in the case of the *16SrRNA* sequences.

3.5.3 BLAST analysis. All the sequences obtained from DNA samples amplified with both tailed and not tailed *mtCOI* and *16SrRNA* primers were unequivocally identified at the species level on GenBank and BOLD with values higher than 98% for *COI* (Barbuto et al. 2010) and 100% for *16SrRNA* gene (Armani et al. 2015c).

3.6 Overall comparison between tailed and NT primers

395 The main purpose of this study was to compare the overall efficiency of NT and tailed primers,
396 under the experimental conditions of our laboratory, in order to assess their strengths and
397 weaknesses in the field of biomolecular analysis applied to food inspection. To date, only a few
398 similar studies are available in literature and they refer only to a single step of the analytical process
399 (Binladen et al. 2007; Regier and Shi 2005). On the contrary, the comparison performed in this
400 study has taken into account the whole process starting from a preliminary *in silico* evaluation of
401 the primers until the sequencing output, in order to provide a complete and exhaustive overview on
402 the use of tails. Based on AS, both *16SrRNA* and *COI* tailed primers showed a higher tendency to
403 form non-specific structures compared to NT primers. This was probably due to the fact that the
404 longer the primers, the higher is the probability that unspecific combination of bases can occur. In
405 particular, the worst AS was obtained from both tailed (ST and MT) *16srRNA* primers, which in
406 turn showed a low number of critical mismatches. A higher number of mismatches, often localized
407 in critical positions were observed for *COI* primers. This preliminary assessment was then further
408 investigated by using primers couples in the PCR reaction: while a good and comparable
409 performance was observed for tailed and NT *16SrRNA* primers, contrasting results were observed in
410 the case of the *COI* primers. In fact, while an intense and specific band was obtained from all the
411 DNA samples amplified with NT and ST primers, MT primers performed worse and required an
412 implementation of the PCR. However, even after the PCR implementation, the band intensity (PCR
413 product concentration) after amplification with MT *COI* primers was significantly lower than that
414 obtained from NT and ST primers. Finally, the amplification with *COI* tailed primers revealed the
415 presence of non-specific bands, stronger with MT than with ST, which did not appear when NT
416 were used (Figure 1). Overall, these outcomes showed that critical primers mismatches had a
417 greater impact with respect to the other evaluated parameters on the amplification performance. In
418 fact, when amplifying the *16SrRNA* gene with tailed primers, the reaction was not affected by any
419 destabilizing effect of the tails, probably due to the high conservation of the annealing region

(absence or presence of few mismatches). The tails, in particular MT, could worsen the reaction further destabilizing an already unstable annealing. In fact, when the AT was reduced (less stringent binding conditions), the amplification was successful. Regarding the sequencing performances, both tailed and NT *16SrRNA* primers resulted equally and well performant. The *COI* primers performed differently, demonstrating that the ST *COI* primers allowed to enhance the outcomes of the whole analytical flow.

Overall, we found that under the experimental conditions adopted (reagents, instruments and DNA samples, which in the case of birds and mammals comprise the most part of the species used as food), the tails proposed by Steffens et al. (1993) performed better than those proposed by Messing (1983). Regarding fishes, even though only 6 species were analyzed in this study, we could confirm the results reported above also on the basis of our previous works (Armani et al. 2015a, 2015b), in which we examined a large number of different species.

Summarizing, no PCR implementation was required and no amplification failure were observed when ST primers were used. Moreover, we could observe a significant improvement of the length of the sequences obtained with ST primers.

Finally, we could actually assert that the utilization of tailed primers can reduce the time and the cost associated to the preparation of the samples to be sent for sequencing.

4. Conclusions

In this work, the amplification and the sequencing performance was assessed for tailed and NT primers among those most used for species identification. In our opinion, even though our outcomes cannot be generalized due to the inevitable existing differences among labs equipment and reagents, this study represents a useful guideline for the selection of the most appropriate tails to be used for the analysis of animal origin DNA. In fact, this work has demonstrated that proper tails can improve the overall throughput of the analysis by improving the quality of amplification and sequencing, speeding up the flow and reducing the costs. Even though the ST tails performed better under our

experimental conditions, the aim of this work was not to propose a standard protocol but rather point out the need to verify tails performances within each new project, since sometimes they could reduce amplification or sequencing quality.

Compliance with Ethics Requirements

The research was performed with founds granted from the University of Pisa.
Armani Andrea declares that he has no conflict of interest.
Giusti Alice declares that she has no conflict of interest.
Guardone Lisa declares that she has no conflict of interest.
Castigliego Lorenzo declares that he has no conflict of interest.
Gianfaldoni Daniela declares that she has no conflict of interest.
Guidi Alessandra declares that she has no conflict of interest.
This article does not contain any studies with human or animal subjects.

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Figure caption

Figure 1. Amplification of the COI gene of 20 different animal species with Handy NT primers (Line 1-21), Steffens' tails (Line 21-40) and Messing's tails (Line 41-60). Line 1;21;41: *Bos Taurus,* 2;22;42: *Ovis aries*, 3;23;43: *Cervus elaphus*, 4;24;44: *Equus caballus,* 5;25;45: *Sus domesticus*, 6;26;46; *Sus scrofa,* 7;27;47: *Lepus europaeus,* 8;28;48: *Oryctolagus cuniculus,* 9;29;49: *Gallus gallus,* 10;30;50: *Meleagris gallopavo,* 11;31;51: *Numida meleagris,* 12;32;52: *Anas platyrhynchos,* 13;33;53: *Coturnix coturnix,* 14;34;54*: Columba livia,* 15;35;55: *Solea solea,* 16;36;56: *Gadus morhua*, 17;37;57: *Engraulis encrasicolus* 18;38;58: *Mugil cephalus* 19;39;59: *Salmo salar* 20;40;60: *Scomber scombrus,* H1, H2 and H3: negative samples, L: ladder.

Figure

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Table
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Table 1. List of animal species analyzed in this study and amplification success rate.

Class	Species		1			2			3		
			NT	**ST**	**MT**	**NT**	**ST**	**MT**	**NT**	**ST**	**MT**
Birds	Chicken	*Gallus gallus domesticus*	+	+	+	+	+	+	+	+	+
	Duck	*Anas platyrhynchos*	+	+	+	+	+	+	+	+	+
	Guinea Fowl	*Numida meleagris*	+	+	+	+	+	+	+	+	+
	Pigeon	*Columba livia*	+	+	+	+	+	+	+	+	+
	Quail	*Coturnix coturnix*	+	+	+	+	+	+	+	+	+
	Turkey	*Meleagris gallopavo*	+	+	+	+	+	+	+	+	+
Fishes	Anchovy	*Engraulis encrasicolus*	+	+	-	+	+	-	+	+	-
	Cod	*Gadus morhua*	+	+	-	+	+	-	+	+	-
	Mackerel	*Scomber scombrus*	+	+	+	+	+	+	+	+	+
	Mullet	*Mugil cephalus*	+	+	+	+	+	+	+	+	+
	Salmon	*Salmo salar*	+	+	+	+	+	+	+	+	+
	Sole	*Solea solea*	+	+	+	+	+	+	+	+	+
Mammals	Beef	*Bos taurus*	+	+	+	+	+	+	+	+	+
	Boar	*Sus scrofa*	+	+	+	+	+	+	+	+	+
	Deer	*Cervus elaphus*	+	+	-	+	+	-	+	+	-
	Hare	*Lepus europaeus*	+	+	-	+	+	-	+	+	-
	Horse	*Equus caballus*	+	+	+	+	+	+	+	+	+
	Lamb	*Ovis aries*	+	+	+	+	+	+	+	+	+
	Pig	*Sus scrofa domesticus*	+	+	+	+	+	+	+	+	+
	Rabbit	*Oryctolagus cuniculus*	+	+	+	+	+	+	+	+	+

NT (not-tailed), **ST** (Steffens' tails) and **MT** (Messing's tails) *COI* primers. + : presence of the expected band; **-**: absence of the expected band. Samples that failed amplification have been highlighted in grey.

Table
Click here to download Table: Table 2.docx

Table 2. List of primers and tails used in this work.

	Primer	Sequence (5' -> 3')	Lenght (bp)	Tm (°C)	ΔTm (°C)	References
COI	FISH-BCL	TCAACYAATCAYAAAGATATYGGCAC	26	59.3	1.3	Baldwin et al., 2009
	FISH-BCH	ACTTCYGGGTGRCCRAARAATCA	23	60.6		Handy et al., 2011
	FISH-BCL ST	CACGACGTTGTAAAACGACTCAACYAATCAYAAAGATATYGGCAC	45	71.7	0.6	Steffens et al., 1993; Baldwin et al., 2009
	FISH-BCH ST	GGATAACAATTTCACACAGGACTTCYGGGTGRCCRAARAATCA	43	72.3		Steffens et al., 1993; Handy et al.,2011
	FISH-BCL MT	TGTAAAACGACGGCCAGTTCAACYAATCAYAAAGATATYGGCAC	44	72.6	0.8	Messing, 1983; Baldwin et al., 2009
	FISH-BCH MT	CAGGAAACAGCTATGACCACTTCYGGGTGRCCRAARAATCA	41	73.4		Messing, 1983; Handy et al.,2011
16srRNA	16sar-L	CGCCTGTTTATCAAAAACAT	20	51.1	11	Palumbi 1996
	16sbr-H	CCGGTCTGAACTCAGATCACGT	22	62.1		
	16sar-L ST	CACGACGTTGTAAAACGACCGCCTGTTTATCAAAAACAT	39	69.5	3.8	Steffens et al., 1993; Palumbi 1996
	16sbr-H ST	GGATAACAATTTCACACAGGCCGGTCTGAACTCAGATCACGT	42	73.3		Steffens et al., 1993; Palumbi 1996
	16sar-L MT	TGTAAAACGACGGCCAGTCGCCTGTTTATCAAAAACAT	38	69.5	5.1	Messing, 1983; Palumbi 1996
	16sbr-H MT	CAGGAAACAGCTATGACCCCGGTCTGAACTCAGATCACGT	40	74.6		Messing, 1983; Palumbi 1996
Tails	M13F (-29) ST	CACGACGTTGTAAAACGAC	19	-	-	Steffens et al., 1993
	M13R ST	GGATAACAATTTCACACAGG	20	-		Steffens et al., 1993
	M13F (-21) MT	TGTAAAACGACGGCCAGT	18	-	-	Messing, 1983
	M13R (-27) MT	CAGGAAACAGCTATGACC	18	-		Messing, 1983

Table

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Table 3. Evaluation of primers used in this work with the Softwares Olygoanalyzer Version 3.1 and the Multifunctional Oligo Property Analysis Tool.

Primers	Self-dimers ΔG (kcal/mole)*	Hetero-dimers ΔG (kcal/mole)*	Hairpins ΔG (kcal/mole)	GC content (%)	Maximum Annealing Score (AS) (best <15)
FISH-BCL	-9.09	-10.75	-0.44	35,6	8
FISH-BCH	-12.37		0.67	47,8	7
FISH-BCL ST	-9.09	-10.75	-5.62	41,1	22
FISH-BCH ST	-12.37		-0.53	44,2	8
FISH-BCL MT	-9.28	-14.84	-1.05	42	12
FISH-BCH MT	-12.37		-1.8	48,8	10
16sar-L	-7.18	-3.61	-2.23	35	11
16sbr-H	-9.75		-2	54,5	16
16sar-L ST	-7.18	-11.1	-7.41	41	22
16sbr-H ST	-9.75		-2	47,6	30
16sar-L MT	-9.28	-8.78	-6.07	42,1	12
16sbr-H MT	-9.75		-2.52	52,5	16

*primers with **ΔG** values higher than -9 kcal/mole were considered as good. Those with a value lower than -9 kcal/mole were highlighted in grey.

Table
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Table 1SM. Main DNA barcoding studies for fishes identification with primers used.

Authors	Study title	Primers used and reference[1]	Oligonucleotide tails used	Field of application
Ward et al., 2005	DNA barcoding Australia's fish species	**Fish F1/ Fish R1** and **Fish F2/ Fish R2**	-	Food inspection
Ivanova et al., 2007	Universal primer cocktails for fish DNA barcoding	**FF2d/FR1d/FR1d_t1**; **VF1_t1/ VF1d_t1/ VF1i_t1/ VR1d_t1/ VR1i_t1** (Ivanova et al., 2006) **LepF1_t1** and **LepRI_t1** (Hebert et al., 2004) **VR1_t1/ VF2_t1/ FishF2_t1/ FishR2_t1** (Ward et al., 2005)	**M13F (-21) and M13R (-27)** (Messing, 1983)	Evolutionary studies
Wong and Hanner, 2008	DNA barcoding detects market substitution in North American seafood	**C_FishF1t1 and C_FishR1t1** (Ivanova et al., 2007)	**M13F (-21) and M13R (-27)** (Messing, 1983)	Food inspection
Smith et al., 2008	DNA barcoding for the identification of smoked fish products	**Fish F2 and FishR2** (Ward et al., 2005)	-	Food inspection
Baldwin et al., 2009	Genetic identification and color descriptions of early life-history stages of Belizean Phaeoptyx and Astrapogon (Teleostei: Apogonidae) with Comments on identification of adult Phaeoptyx	**FISH-BCL** and **FISH-BCH**	-	Evolutionary studies
Rasmussen et al., 2009	DNA Barcoding of Commercially Important Salmon and Trout Species(OncorhynchusandSalmo)from North America	**C_FishF1t1** and **C_FishR1t1** (Ivanova et al., 2007)	**M13F (-21) and M13R (-27)** (Messing, 1983)	Food inspection
Holmes et al., 2009	Identification of shark and ray fins using DNA barcoding	**C_FishF1t1** and **C_FishR1t1**	**M13F (-21) and M13R (-27)** (Messing, 1983)	Food inspection

		(Ivanova et al., 2007)		
Ardura et al., 2010	DNA barcoding for conservation and management of Amazonian commercial fish	**Fish F1/ Fish R1** and **Fish F2/ Fish R2** (Ward et al., 2005)	-	Food inspection
Barbuto et al., 2010	DNA barcoding reveals fraudulent substitutions in shark seafood products: The Italian case of ''palombo'' (Mustelus spp.)	**Fish R2** and **Shark-int** (Ward et al., 2005)	-	Food inspection
Filonzi et al., 2010	Molecular barcoding reveals mislabelling of commercial fish products in Italy	**COI-ff2d-fish and COI-fr1d-fish** (Ivanova et al., 2007)	-	Food inspection
Kochzius et al., 2010	Identifying Fishes through DNA Barcodes and Microarrays	**Fish F1** and **FishR1** (Ward et al., 2005)	-	Food inspection
Carvalho et al., 2011	DNA barcoding unveils a high rate of mislabeling in a commercial freshwater catfish from Brazil	**Fish F1** and **FishR1** (Ward et al., 2005)	-	Food inspection
Handy et al., 2011	A Single-Laboratory Validated Method for the Generation of DNA Barcodes for the Identification of Fish for Regulatory Compliance	**FISH-BCL and FISH-BCH** (Baldwin et al., 2009)	**M13F (-29) and M13R** (Steffens, 1993)	Food inspection
Zhang and Hanner, 2011	DNA barcoding is a useful tool for the identification of marine fishes from Japan	**FishF2_t1, VF2_t1, FishR2_t1, FR1d_t1** (Ivanova et al., 2007)	**M13F (-21) and M13R (-27)** (Messing, 1983)	Evolutionary studies
Cawthorn et al., 2012	DNA barcoding reveals a high incidence offish species misrepresentation and substitution on the South African market	**C_FishF1t1 and C_FishR1t1** (Ivanova et al., 2007)	**M13F (-21) and M13R (-27)** (Messing, 1983)	Food inspection
Haye et al., 2012	Authentication of commercialized crab-meat in Chile using DNA barcoding	**LCO1490** and **HCO2198** (Folmer et al., 1994)	**-**	Food inspection
Huxley-Jones et al., 2012	Use of DNA barcoding to reveal species composition of convenience	**VF1 and VR1d** (Ivanova et al., 2007)	**M13F (-21) and M13R (-27)** (Messing, 1983)	

	seafood			
Di Pinto et al., 2013	DNA barcoding for detecting market substitution in salted cod fillets and battered cod chunks	**FISHCOILBC** and **FISHCOIHBC** (Handy et al. 2011)	**-**	Food inspection
Keskin and Atar, 2013	DNA barcoding commercially important fish species of Turkey	**Fish F2 and FishR2** (Ward et al., 2005)	**-**	Food inspection
Maralit et al., 2013	Detection of mislabeled commercialfishery by-products in the Philippines using DNA barcodes and its implications to food traceability and safety	**LCO1490** and **HCO2198** (Folmer et al., 1994)	**-**	Food inspection
Abdullah and Rehbein, 2014	Authentication of raw and processed tuna from Indonesian markets using DNA barcoding, nuclear gene and character‑ based approach	**Fish F1 and FishR1** (Ward et al., 2005)	**M13F (-21) and M13R (-27)** (Messing, 1983)	Food inspection
Chakraborty, 2014	An assessment of the DNA barcodes of Indian freshwater fishes	**Fish F1 and FishR1** (Ward et al., 2005)	**-**	Food inspection
Cutarelli et al., 2014[t]	Italian market fish species identification and commercial frauds revealing by DNA sequencing	**Fish F2 and FishR2** (Ward et al., 2005)	-	Food inspection
Galal-Khallaf et al., 2014	DNA barcoding reveals a high level of mislabeling in Egyptian fish fillets	**Fish F1** and **FishR1** **Fish F2** and **FishR2** (Ward et al., 2005)	-	Food inspection
Lamendin et al., 2014	Labelling accuracy in Tasmanian seafood: an investigation using DNA barcoding	**Fish F1 and FishR1** (Ward et al., 2005)	-	Food inspection
Carvalho et al., 2015	DNA Barcoding identification of commercialized seafood in SouthBrazil: A governmental regulatory forensic program	**Fish F1 and FishR1** (Ward et al., 2005)	-	Food inspection

[1]When not reported, the primers have been developed by the authors of the study. Rows in grey highlights studies which used oligonucleotides tails.

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Food Analytical Methods

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Table
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Table 2SM. Alignment between the *COI* primers of Handy et al. (2011) used in this study and the available *COI* gene sequences of the animal species used in this work.

Species	Available sequences	FISH BCL TCAACYAATCAYAAAGATATYGGCAC	NM	FISH BCH TGATTYTTYGGYCACCCRGAAGT	NM
Beef (*Bos taurus*)	**(5)** AF493541-42; AF490528; AB074962; AF492351	TCAACCAACCATAAAGATATTGGTAC	2	TGATTCTTTGGACACCCCGAAGT	2
Sheep (*Ovis aries*)	**(1)** AF010406	TCAACCAACCACAAAGATATCGGCAC	1	TGATTCTTTGGGCACCCTGAAGT	2
Deer (*Cervus elaphus*)	**(1)** AB245427	TCAACCAACCATAAAGATATCGGTAC	2	TGATTCTTTGGCCACCCTGAAGT	1
Horse (*Equus caballus*)	**(5)** X79547; AB859014; AP013080; AP013090; AP013096	TCAACTAACCACAAAGACATCGGCAC	2	TGATTCTTCGGACACCCCGAAGT	2
Pig (*Sus domesticus*)	**(2)** AP003428; KJ789952	TCAACAAACCACAAAGACATCGGCAC	3	TGATTTTTCGGACACCCAGAAGT	1
Boar (*Sus scrofa*)	**(1)** AJ002189	TCAACAAACCACAAAGACATCGGCAC	3	TGATTTTTCGGACACCCAGAAGT	1
Hare (*Lepus europaeus*)	**(1)** AJ421471	TCTACCAACCACAAAGACATTGGAAC	4	TGATTCTTCGGTCATCCTGAAGT	2
Rabbit (*Oryctolagus cuniculus*)	**(1)** AJ001588	TCTACCAACCACAAAGACATCGGCAC	3	TGATTTTTCGGGCACCCCGAAGT	2
Chicken (*Gallus gallus*)	**(5)** KM096864.1; AB086102.1; AP003580.1 AP003318.1; AP003317.1	TCAACCAACCACAAAGACATTGGCAC	2	TGATTCTTCGGTCACCCCGAAGT	0
Turkey (*Meleagris gallopavo*)	**(3)** EF153719.1; NC_010195.2; JF275060.1	TCAACCAACCATAAAGATATTGGCAC	1	TGATTTTTTGGCCACCCCGAAGT	1
Guinea fowl (*Numida meleagris*)	**(2)** AP005595.1; NC_006382.1	TCAACCAATCACAAAGACATTGGCAC	1	TGATTCTTCGGCCACCCTGAAGT	1
Duck (*Anas platyrhynchos*)	**(5)** EU755252.1; EU009397.1; EU755253.1 KJ739616.1; KJ833587.1	TCTACCAATCACAAAGACATCGGTAC	3	TGATTCTTCGGCCACCCAGAAGT	0

Pigeon (*Columba livia*)	**(5)** NC_013978.1; GU908131.1; GQ240309.1; KJ722068.1; KF926376.1	TCTACTAACCACAAAGACATCGGCAC	3	TGATTCTTTGGTCATCCTGAAGT	2
Cod (*Gadus morhua*)	**(3)** HG514359; AM489716; NC_002081	TCGACCAATCACAAAGACATTGGCAC	2	TGATTCTTCGGGCATCCCGAAGT	3
Anchovy (*Engraulis encrasicolus*)	**(2)** AP009137; NC_009581	TCAACAAATCACAAAGACATTGGCAC	2	TGATTCTTCGGACACCCCGAAGT	2
Mullet (*Mugil cephalus*)	**(3)** AP002930.1; KM368340; NC_003182.1	TCGACTAATCACAAAGACATCGGCAC	1	TGATTCTTTGGCCACCCAGAAGT	0
Salmon (*Salmo salar*)	**(5)** NC_001960.1; AF133701.1; KF792729.1; U12143.1; JQ390056.1	TCAACCAACCACAAAGACATTGGCAC	1	TGGTTCTTTGGCCATCCAGAAGT	2
Mackerel (*Scomber scombrus*)	**(2)** AB120717.1; NC_006398.1	TCAACAAACCATAAAGACATCGGCAC	3	TGATTCTTCGGACACCCAGAAGT	1

Mismatches have been highlighted in grey. NM= Number of mismatches.

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Table
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Table 3SM. Alignment between the *16srRNA* primers of Palumbi (1996) used in this study and the available *16srRNA* gene sequences of the animal species used in this work.

Species	Available sequences	16sar-L CGCCTGTTTATCAAAAACAT	NM	16sbr-H ACGTGATCTGAGTTCAGACCGG	NM
Beef (*Bos taurus*)	**(5)** AF492351; KF926377.1; KF163094.1; HQ025805.1; JN817350.1	CGCCTGTTTACCAAAAACAT	1	ACGTGATCTGAGTTCAGACCGG	0
Sheep (*Ovis aries*)	**(5)** AF010406; NC_001941.1; KJ954145.1; KF938352.1; KF302459.1	CGCCTGTTTACCAAAAACAT	1	ACGTGATCTGAGTTCAGACCGG	0
Deer (*Cervus elaphus*)	**(5)** AB245427; NC_007704.2; GU457435.1;HQ191429.1; KJ025072.1	CGCCTGTTTACCAAAAACAT	1	ACGTGATCTGAGTTCAGACCGG	0
Horse (*Equus caballus*)	**(5)** X79547; AB859014; AP013080; AP013090; AP013096	CGCCTGTTTACCAAAAACAT	1	ACGTGATCTGAGTTCAGACCGG	0
Pig (*Sus domesticus*)	**(5)** AP003428; KJ789952; NC_012095.1; KC469587.1; KJ746666.1	CGCCTGTTTACCAAAAACAT	1	ACGTGATCTGAGTTCAGACCGG	0
Boar (*Sus scrofa*)	**(5)** AJ002189; AF034253.1; KM433673.1; KF888634.1; NC_000845.1	CGCCTGTTTACCAAAAACAT	1	ACGTGATCTGAGTTCAGACCGG	0
Hare (*Lepus europaeus*)	**(2)** AJ421471; NC_004028.1	CGCCTGTTTACCAAAAACAT	1	ACGTGATCTGAGTTCAGACCGG	0
Rabbit (*Oryctolagus cuniculus*)	**(2)** AJ001588; NC_001913.1	CGCCTGTTTACCAAAAACAT	1	ACGTGATCTGAGTTCAGACCGG	0
Chicken (*Gallus gallus*)	**(5)** KM096864.1; AB086102.1; AP003580.1; AP003318.1; AP003317.1	CGACTGTTTCCCAAAAACAT	3	ACGTGATCTGAGTTCAGACCGG	0
Turkey (*Meleagris gallopavo*)	**(3)** EF153719.1; NC_010195.2; JF275060.1	CGACTGTTTACCAAAAACAT	2	ACGTGATCTGAGTTCAGACCGG	0
Guinea fowl (*Numida meleagris*)	**(1)** AP005595.1	CGACTGTTTCCCAAAAACAT	3	ACGTGATCTGAGTTCAGACCGG	0

Duck (*Anas platyrhynchos)*	**(5)** EU755252.1; EU009397.1; EU755253.1; KJ739616.1; KJ833587.1	CGACTGTTTACCAAAAACAT	2	ACGTGATCTGAGTTCAGACCGG	0
Pigeon (*Columba livia)*	**(5)** NC_013978.1; GU908131.1; KJ722068.1;KF926376.1; GQ240309.1	CGACTGTTTACCAAAAACAT	2	ACGTGATCTGAGTTCAGACCGG	0
Cod (*Gadus morhua)*	**(3)** HG514359; AM489716; NC_002081	CGCCTGTTTACCAAAAACAT	1	ACGTGATCTGAGTTCAGACCGG	0
Anchovy (*Engraulis encrasicolus)*	**(2)** AP009137; NC_009581	CGCCTGTTTACCAAAAACAT	1	ACGTGATCTGAGTTCAGACCGG	0
Mullet (*Mugil cephalus)*	**(3)** AP002930.1; KM368340; NC_003182.1	CGCCTGTTTACCAAAAACAT	1	ACGTGATCTGAGTTCAGACCGG	0
Salmon (*Salmo salar)*	**(5)** NC_001960.1; AF133701.1; KF792729.1; U12143.1; JQ390056.1	CGCCTGTTTACCAAAAACAT	1	ACGTGATCTGAGTTCAGACCGG	0
Mackerel (*Scomber scombrus)*	**(1)** AB120717.1	CGACTGTTTACCAAAAACAT	2	ACGTGATCTGAGTTCAGACCGG	0

Mismatches have been highlighted in grey. NM= Number of mismatches.

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Table

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<table>
<tr><th></th><th></th><th>COI</th><th>16SrRNA</th></tr>
<tr><td rowspan="8">PCR program</td><td>Initial denaturation</td><td colspan="2">94°C for 3 minutes</td></tr>
<tr><td></td><td></td><td></td></tr>
<tr><td>Total 3 step cycling</td><td>40 cycles</td><td>45 cycles</td></tr>
<tr><td>Denaturation</td><td>94°C for 30 seconds</td><td>94°C for 25 seconds</td></tr>
<tr><td>Annealing</td><td>51°C for 30 seconds</td><td>57.5°C for 15 seconds</td></tr>
<tr><td>Extention</td><td>72°C for 35 seconds</td><td>72°C for 2 seconds</td></tr>
<tr><td>Final elongation</td><td>72°C for 5 minutes</td><td>72°C for 10 minutes</td></tr>
<tr><td rowspan="7">PCR reaction condition (final volume 20 µL)</td><td>Buffer
(5Prime, Gaithersburg, USA),</td><td colspan="2">2µL of 10× buffer</td></tr>
<tr><td>dNTPs
(dNTPmix, EurocloneS.p.A - Life Sciences Division, Pavia, Italy)</td><td colspan="2">200 µM each</td></tr>
<tr><td>BSA (Purified BSA 100×, New England BIOLABS® Inc. Ipswich, MA, USA),</td><td colspan="2">25 ng/µL</td></tr>
<tr><td>Primers</td><td colspan="2">300 nM</td></tr>
<tr><td>PerfectTaq DNA Polymerase (5Prime, Gaithersburg, USA),</td><td colspan="2">1.25 U</td></tr>
<tr><td>DNA template</td><td colspan="2">100 ng</td></tr>
<tr><td>DNase free water (Water Mol. Bio. Grade, DNase–RNase and Protease free, 5Prime GmbH, Hamburg, Germany).</td><td colspan="2">Up to final volume</td></tr>
</table>

Table 4SM. Standard PCR program and reaction condition used for the amplification of the *COI* and *16SrRNA* gene. The amplifications were carried on a LifePro™ Gradient Thermal Cycler (BIOER TECHONOLOGY CO., LTD).