

Food Analytical Methods

Development of a simple and cost-effective bead-milling method for DNA extraction from fish muscles --Manuscript Draft--

Manuscript Number:	FANM-D-13-00552R1
Full Title:	Development of a simple and cost-effective bead-milling method for DNA extraction from fish muscles
Article Type:	Original Research
Keywords:	DNA extraction, bead milling, fish muscle
Corresponding Author:	Andrea Armani, PhD ITALY
Corresponding Author Secondary Information:	
Corresponding Author's Institution:	
Corresponding Author's Secondary Institution:	
First Author:	Andrea Armani, PhD
First Author Secondary Information:	
Order of Authors:	Andrea Armani, PhD Lara Tinacci Xiong Xiong Evgeniya Titarenko Alessandra Guidi Lorenzo Castigliengo
Order of Authors Secondary Information:	
Abstract:	<p>In the fish food sector, due to a growing globalization of the market, where intentional and unintentional frauds reach alarming levels, the molecular analysis is increasingly used by both Official agencies, to enforce the law on traceability, and private companies, to verify the quality of goods. DNA extraction represents a necessary and critical step for all types of DNA analysis. Among the drawbacks associated with this procedure, there are handling of toxic materials, low DNA yield and low throughput, due to time-consuming manual procedures. In this work, to overcome some of these problems, we developed an alternative method based on a bead milling procedure without proteinase K digestion. The new method was then compared with both a salting out protocol, developed in a previous work, and a commercial kit. Yield, spectrophotometric purity, electrophoretic degradation pattern, and amplificability of the extracted DNA were assessed. In particular, DNA amplificability was evaluated by comparing the band intensity on the gel, after amplification of the 16S rRNA and COI genes with a conventional PCR, and the take-off cycles, after amplification of the 16S rRNA gene with a real-time PCR. The results showed that the bead-based method allowed to obtain acceptable amounts of DNA, with good purity and good characteristics of amplificability. Although the salting out method remains the most effective protocol in terms of pure performances, the bead-milling procedure can be considered a valid alternative, in the light of its lower demand in terms of labor and costs.</p>

Dear Editor,

we revised the manuscript as suggested and here below you can find our answers, comments and rebuttals.

Reviewer #1: The paper is concerned with the development of a rapid and safe method for the extraction of DNA, with the fundamental characteristic of maximized cost-effectiveness in use in fish. I believe that it is well prepared article and presented and could be accepted for publication after major corrections

Major corrections

In general terms, I believe that the article can be shorter. The main information could be presented without losing its quality in a shorter way.

Also I believe that the authors should check the English grammar.

The manuscript has been edited.

Results and discussion could be shorter. It includes several known facts that can be skipped and replaced by references.

Results and discussion has been shortened as suggested.

Lines 296-314 could be shorter and moved to the methods as it does not contain any results.

The section 3.1 has been removed. The content has been shortened and moved to section 2.1 (line 91-95 and 103-106), which has been renamed as “*2.1 Species choice, tissue collection, sampling and storage*”

Line 346-354 could be deleted as they contained already known information.

Line 346-350 has been completely deleted as suggested, while the information reported in line 351-354 has been shortened and maintained in the manuscript (line 309-311). In our opinion these lines bring essential information to understand the spectrophotometric results. In fact, some information regarding the 260/230 ratio are reported at line 311-315.

Figure 1 could be deleted as the information could be easily described in the text

Figure 1 has been deleted as suggested

Table 1 could be restructured to be shorter and all types to be in one line

Table 1 has been restructured as suggested

Table 2SM is not clear

The table legend has been modified to better explain the meaning of the reported statistical results. As also explained in lines 226 (section 2.7 Statistical analysis), two models were created, which take into consideration the “species” and the “category” factors separately.

Minor corrections

Line 114: It is not clear. Rephrase the sentence

Line 114 has been rewritten

Line140: have should replaced

Line 140 has been checked

Line 273, 274: add reference

As specified in the sentence this evidence came from personal experience. In fact, due to this limitation, we have never used commercial kits for the DNA extraction. For this reason we propose to support the sentence with a personal citation (line 267)

1 **Development of a simple and cost-effective bead-milling method for DNA extraction from**
2
3 **fish muscles**

4
5 3
6
7 4 Armani Andrea*, Tinacci Lara, Xiong Xiong, Titarenko Evgeniya, Guidi Alessandra,
8
9 5 Castigliego Lorenzo.

10
11
12 6
13
14 7 *FishLab, Department of Veterinary Sciences, University of Pisa, Via delle Piagge 2, 56124, Pisa*
15
16
17 8 (*Italy*).

18
19 9
20
21
22 10
23
24 11
25
26
27 12
28
29 13
30
31 14
32
33
34 15
35
36 16
37
38
39 17
40
41 18
42
43
44 19
45
46 20
47
48 21 ***corresponding author:**

49
50
51 22 Postal address: *FishLab, Department of Veterinary Sciences, University of Pisa, Via delle Piagge*
52
53 23 2, 56124, Pisa (Italy)

54
55
56 24 Tel: +390502210207

57
58 25 Fax: +390502210213

59
60
61 26 Email: aarmani@vet.unipi.it

62
63
64
65

27 **ABSTRACT**

1
28 In the fish food sector, due to a growing globalization of the market, where intentional and
3
4
29 unintentional frauds reach alarming levels, the molecular analysis is increasingly used by both
6
730 Official agencies, to enforce the law on traceability, and private companies, to verify the quality of
8
9
31 goods. DNA extraction represents a necessary and critical step for all types of DNA analysis.
10
11
132 Among the drawbacks associated with this procedure, there are handling of toxic materials, low
13
14
33 DNA yield and low throughput, due to time-consuming manual procedures. In this work, to
15
16
1734 overcome some of these problems, we developed an alternative method based on a bead milling
18
19
35 procedure without proteinase K digestion. The new method was then compared with both a salting
20
21
2236 out protocol, developed in a previous work, and a commercial kit. Yield, spectrophotometric purity,
23
2437 electrophoretic degradation pattern, and amplificability of the extracted DNA were assessed. In
25
26
2738 particular, DNA amplificability was evaluated by comparing the band intensity on the gel, after
28
2939 amplification of the *16S rRNA* and *COI* genes with a conventional PCR, and the take-off cycles,
30
31
40 after amplification of the *16S rRNA* gene with a real-time PCR. The results showed that the bead-
32
33
3441 based method allowed to obtain acceptable amounts of DNA, with good purity and good
35
36
42 characteristics of amplificability. Although the salting out method remains the most effective
37
38
3943 protocol in terms of pure performances, the bead-milling procedure can be considered a valid
40
4144 alternative, in the light of its lower demand in terms of labor and costs.
42
43
4445

45
4646 **Keywords:** DNA extraction, bead milling, fish muscle.
47
48
4947
50
5148
52
5349
54
55
5650
57
5851
59
60
6152
62
63
64
65

INTRODUCTION

The several cases of fraudulent substitution often reported by media have made the unique identification of food a key factor. Consequently, especially in the fishery sector, the DNA based-analytical methods have become increasingly important and are nowadays applied for routine controls, also at the Official level. They represent a valid support not only to improve the compliance and traceability of goods, encouraging the enforcement of the law, but also to raise the protection level of consumers against fish allergies and of endangered animal species (Armani *et al.*, 2012a).

In order to meet the demand for reliable and sustainable food traceability systems, any DNA based analytical approach should be both effective and low-cost (Galimberti *et al.*, 2013). Effectiveness is mainly based on the possibility to rely on a successful PCR amplification, which greatly depends on the achievement of a sufficient amount of high quality DNA. In fact, it is fundamental that every extraction method be able to maximize the removal of contaminants that may inhibit PCR. For this reason, DNA extraction is considered the most critical step in the processing of samples for PCR-based analysis. Especially in the fishery sector, which deals with thousands of different species, the choice of the most appropriate technique should be accurately assessed.

Fundamental step of the DNA extraction method is the tissue disruption and the cell lysis. Many chemical and physical disruption methods, followed by enzymatic digestion using proteinase K, have been proposed for tissue lysis. Among those available, the rapid shaking of the samples in the presence of glass or steel beads has been shown to be effective for different kind of matrices of microbial, plant, and animal origin (Dilworth and Frey, 2000; Robe *et al.*, 2003; Allender *et al.*, 2004).

Due to the fact that beads-based tissue-disruption could significantly reduce times and costs, and that, to our knowledge, targeted studies on fish tissues have never been performed, in this work we developed a simple and cost-effective DNA extraction method, based on bead-milling, without

79 proteolytic enzyme, followed by a salting out procedure. A study was then performed to point out
1 strengths and weaknesses of this method by comparing it with a classical enzymatic digestion
280 2 coupled with a salting out procedure and with a commercial DNA extraction kit, both from fresh
3 4
581 5 coupled with a salting out procedure and with a commercial DNA extraction kit, both from fresh
6
782 7 and ethanol preserved tissues, using 38 different species. The yield, the quality and the degradation
8
983 9 pattern of the extracted DNA were assessed and their effects on subsequent downstream sample
10
11 analysis were evaluated by performing conventional and real time PCR.
1284 12

13
1485 14 Considering the high number of fish products marketed worldwide and the increased number of
15
1686 16 frauds reported, the development of a rapid and safe method for DNA extraction, with minimized
17
1887 18 costs and good effectiveness, would be required to better support the growing need for molecular
19
2088 20 analysis.
21
2288 22

23 2489 24 **2. MATERIAL AND METHODS**

25 2690 26 *2.1 Species choice, tissue collection, sampling and storage*

27
28
2991 29 Considering that the natural heterogeneity of the tissue composition can affect the efficiency of
30
3192 31 mechanical disruption, we decided to test the effectiveness of the new DNA extraction procedure by
32
33
3493 34 selecting the species on the basis of the fat content, according to the categorization proposed by
35
3694 36 Ackmann (1989). In fact, fats could contribute to the DNA precipitation and inhibit PCR (Wilson,
37
38
3995 39 1997; Besbes *et al.*, 2011).
40

4196 41 Muscle samples were collected from 38 different fish species (Table 1), according to the weight
42
43
4497 44 of the specimens. In case of fish with weight greater than 150gr, the tissue was excised from the
45
4698 46 dorso-lateral muscles of three different specimens and then grossly chopped with scissors. In case
47
48
4999 49 of small fish, where a single sampling would not suffices, three mixtures of at least five samples
50
5100 51 were prepared after skin and bone removal. From each of the three different specimens or mixtures,
52
53
5401 54 eighteen samples (one duplicate for each of the extraction method tested, see section 2.3) were
55
5602 56 produced and extracted.
57

58
5903 59 Considering that collection of tissues belonging to reference specimens, which are often stored in
60
6104 61 ethanol, since provided by museums or research institutes, represents a prerequisite to develop a
62
63
64
65

105 DNA barcoding for the identification of unknown fish samples (Armani *et al.* 2013; Galimberti *et*
1
106 *al.*, 2013), we also tested our methods on samples maintained in ethanol at 4°C for at least 30 days.

107 **2.2 Development of a bead-milling non enzymatic method for total DNA extraction**

108 **2.2.1. Protocol optimization.** 0.1 mm and 0.5 mm glass beads (SI-BG01, SI-BG05 disruptor
8
109 beads Scientific Industries, Inc., Bohemia, NY, USA) and 2.5 mm stainless steel beads (precelllys
10
110 24-Bulk bead, Bertin Technologies Villeurbanne, France) were used to assess their destructive force
11
12
13
14
111 on fish muscle tissues. The trials were performed using fresh muscle tissue from ten species (Table
15
16
112 1). For the setting up of the bead-milling method, a solution composed of 200 µl of Lysis buffer
17
18
19
113 (500 mM Tris-HCl, 100 mM EDTA, 100 mM NaCl, 2% w/v SDS, pH 8.0) was added to 200 mg of
20
21
114 tissue. The glass beads were used in a 3:2, 1:1, 1:2, 1:6 w/w ratio with respect to the tissue,
22
23
115 according to the manufacturer suggestions, whereas the steel beads were added in number of 3, 5,
24
25
116 10 or 20 per 200 mg tissue. The samples were grinded at room temperature (RT) on the Cell
26
27
117 disruptor (Disruptor Genie[®], Scientific Industries, Inc., Bohemia, NY, USA) at 1200 rpm shaking
28
29
118 speed and the extraction was performed according to the method reported in section 2.3.2. The type
30
31
119 and the amount of beads were chosen on the basis of the DNA yield.

120 Finally, two times of mechanical disruption (30 and 60 min) were tried at both room temperature
32
33
121 and 60°C on a thermo-mixer (EuroClone T-shaker, EuroClone S.p.A, Pero, MI, Italy) at 1200 rpm
34
35
122 shaking speed. Time and temperature of disruption were chosen by evaluating A260/A280 ratio,
36
37
123 A260/A230 ratio, and yield as parameters for selection.

124 **2.2.2. Final Protocols (B30; B60).** For each sample, 200 mg of muscle tissue, 10 steel beads and
38
39
125 200 µl of Lysis buffer were put in a 2 ml round bottom tube. The tube was then placed on the T-
40
41
126 Shaker preheated at 60°C for 30 and 60 min at 1500 rpm. At the end of the milling step, the samples
42
43
127 were centrifuged at 15000 X g for 2 minutes to separate the clear supernatant from the tissues/beads
44
45
128 mixture and collected in a clean 1.5 ml tube. The extraction was then completed according to the
46
47
129 protocol reported in section 2.3.2, with the exception of those samples that, after the first sodium
48
49
130 acetate precipitation, presented a “cloud no-transparent supernatant phase”. In this case, a step of
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

131 reprecipitation with 0.5 volumes of sodium acetate, followed by a double washing with 70%
1
132 ethanol, were added. The optimized protocol was then evaluated on samples of decreasing
3
4
133 weights (200; 100; 50; 25; 10 mg). It was performed on 36 specimens, of which three individuals
6
134 for each species and three species for each of the four fat categories (Table 1 species in bold)
8
135 were processed according to methods SO, B30, and B60. The number of beads was
10
11
136 proportionally reduced.
13

14
137 *2.2.3. Stainless beads decontamination and sterilization procedure.* After use, the beads were
15
16
138 washed carefully under tap water in a Petri plate until cleaned from all tissue debris, then soaked
18
19
139 for one hour in a 5% sodium hypochlorite solution. After the removal of the hypochlorite
20
21
140 solution, the beads were washed with ultrapure water and ethanol 70%, air dried and then
22
23
141 submitted to a cycle in autoclave at 120°C for 30 minutes. The effectiveness of the
25
26
142 decontamination procedure was verified by performing a PCR for the amplification of a
27
28
143 fragment of the mitochondrial 16S ribosomal RNA (*16srRNA*) (section 2.5.1), in which the
30
31
144 autoclaved beads were directly placed into the reaction tubes.
32

33 145 **2.3. Comparison of different extraction procedures**

34
146 The DNA extraction method developed in this study was compared with other two different
37
38
147 methods: a salting out procedure (Armani *et al.* 2011) and a commercial kit, using all the samples
40
41
148 (Table 1) before and after the ethanol storage. The ethanol preserved samples were preliminary
42
43
149 rehydrated with a 30 minutes washing in 50 mM Tris solution, pH 7.8, before extraction. All the
44
45
150 muscle tissues were extracted in duplicate. Considering the low DNA yield obtained with the kit
47
48
151 from fresh samples (see section 3.3.1), this procedure was not tested in case of ethanol preserved
49
50
152 samples.
52

53
153 *2.3.1 Extraction with the commercial kit.* 40 mg of tissues were extracted with the EuroGold
54
55
154 Tissue DNA Mini Kit (EuroClone S.p.A), following the manufacturer's instructions, with the
57
58
155 exception of the final elution, which was obtained with 50µL of deionized sterile water instead of
59
60
156 100-200 µL as suggested by the manufacturer.
62
63
64
65

157 2.3.2 *Enzymatic digestion and Salting Out extraction protocol (SO)*. Total DNA extraction was
1
158 performed starting from 200 mg of tissue, following the protocol of Armani *et al.*, (2011), modified
2
3
4
159 by Armani *et al.*, (2012b). Briefly, after adding 200 μ l of lysis buffer and 200 μ l of 200 mM
5
6
160 Na_2HPO_4 , pH 8.0, the tissue was mechanically homogenized with scissors and incubated for one
7
8
161 hour digestion with 20 μ l of proteinase K, continuously stirring at 1200 X g on a Thermo mixer
9
10
162 (EuroClone T-shaker, EuroClone S.p.A, Pero, MI, Italy), at 60°C. The samples were then
11
12
163 centrifuged at 15000 X g for 2 min and the upper aqueous phase was collected in a new sterile
13
14
15
164 microcentrifuge tube. The proteins were precipitated adding 0.5 vol. of 4M sodium acetate, pH 8.3.
16
17
165 Then the mixture was incubated at RT for 5 minutes and then centrifuged at 15000xg for 5 min.
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

2.4 *Qualitative and quantitative determination of total DNA*

The overall quality of the extracted DNA with the three different procedures was determined by taking into account purity, yield and integrity of the DNA.

2.4.1. *Spectrophotometric assessment and yield*. The spectrophotometer NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, U.S.) was used to measure the concentration and the purity of the total DNA on the basis of the UV absorption ratio at 260/280 nm and at 260/230 nm on two subsequent measurements. Subsequently, the yield ($\mu\text{g}/\text{mg}$) was calculated.

2.4.2. *Evaluation of DNA integrity by gel electrophoresis*. The DNA integrity was checked by gel electrophoresis: 1 μg of total DNA of each sample, with the exception of those extracted with the kit for which a lower concentration was used (depending on to the yield of the extraction), was run for 1 h at 240V on a 0.8% agarose gel (GellyPhorLE, Euroclone, UK) prestained with GelRed™ Nucleid Acid Gel Stain (Biotium, Hayward, CA, USA) in 0.5x TBE buffer (pH 8.3). The result was visualized on a UV transilluminator. The DNA sizes were estimated by comparison with two

183 standard DNA markers, the SharpMass™50-DNA ladder and the SHARPMASS 1 kb-DNA Ladder,
1
184 (EuroClone S.p.A, Pero , MI, ITALY).

185 **2.5 DNA amplificability**

186 To test the amplificability of the DNA extracted, a conventional PCR and a real-time PCR were
187 performed on 36 specimens (three individuals each species; three species for each of the four
188 categories) (Table 1 species in bold) processed according to all the four methods of extraction.

189 **2.5.1. Conventional PCR.**

190 The DNA samples were amplified by conventional PCR by using two couple of universal
191 primers: 16SaR/16sbr (Palumbi *et al.*, 1996) and FISHCOILBC_ts/FISHCOIHBC_ts (Handy *et al.*,
192 2011), for a ~650bp partial sequence of the mitochondrial 16S ribosomal RNA (*16srRNA*) gene and
193 for a ~700bp partial sequence of the mitochondrial cytochrome oxidase I (*COI*) gene, respectively.
194 The amplification reactions were performed in 10 µl containing 1.25 U of PerfectTaq DNA
195 Polymerase (5Prime, Gaithersburg, USA), 200 µM each dNTP (dnTPmix, Euroclone S.p.A-Life
196 Sciences Division, Pavia, Italy), 0.25 µM of primer forward and reverse, 1.5 mM of MgCl₂
197 (5Prime, Gaithersburg, USA), 25 ng/µl of BSA (Purified BSA 100X, New England BIOLABS®
198 Inc. Ipswich, MA, USA), and 25 ng of total DNA. After an initial Taq Polymerase activation step (3
199 minutes at 94°C) both the amplification protocols were set up on a 35 cycles program as follow: 35
200 cycles of 94° C for 25s, 57.5°C for 15s, 72 °C for 2s (*16s rRNA* gene), 94°C for 30s, 55°C for 30s,
201 72°C for 30s (*COI* gene). Both the protocols were completed by a final elongation step at 72°C for
202 5 minutes. The amplification products were resolved on a 2% agarose gel (GellyPhorLE, Euroclone,
203 UK), stained with GelRed™ Nucleid Acid Gel Staining 10000X water solution (Biotium, Hayward,
204 CA, USA) in 0.5X TBE buffer. The final result was visualized on UV transilluminator and DNA
205 fragment size was estimated by comparison with the standard marker SharpMass™50-DNA ladder
206 (Euroclone, S.p.A-Life Sciences Division, Pavia, Italy).

207 The image of the gels were acquired with a digital camera and then analyzed using the software
208 Image J 1.47t (NIH, Bethesda, USA). The absolute values obtained were normalized as percentage

209 of the resultant of the sum of every single band within each group, in order to produce a relative
1
210 estimation of the intensity of each band.

211 2.5.2. *Real time PCR.*

212 For the real-time PCR the primers FOR16Spc/REV16Spc designed by Armani *et al.* (2012c)
8
213 were used for the amplification of a partial ~330bp gene fragment of the *16S rRNA* gene. The DNA
10
214 was amplified in a RotorGene 6000 thermocycler (Corbett Research, Sidney, Australia) and the
11
1214 was amplified in a RotorGene 6000 thermocycler (Corbett Research, Sidney, Australia) and the
13
14
215 reaction was run in 10 µl containing 5 µl of the premixed solution (QuantiTect SYBR Green PCR
15
16
216 Kit, Qiagen, Hilden, Germany), 250 nM of primer forward and reverse, and 25 ng tot of DNA
18
19
217 template. Cycling conditions were set as follows: initial hold at 95 °C for 10 min; 45 cycles
20
21
218 including denaturation 94 °C for 15s, annealing 53 °C for 30s, extension 72 °C for 30s. All samples
22
23
24
219 were run in duplicate with negative and positive controls. The threshold cycle (Ct) value was
25
26
220 registered and considered for statistical analysis.

221 2.6 *Estimation of costs and time required by each method*

222 The cost per sample for the three experimental protocols was estimated on the basis of the
31
32
33
223 commercial price of chemicals and disposable items used. The time was estimated on a batch of ten
34
35
36
224 samples from the mechanical disruption of the tissue (with scissor or beads) to the pellet DNA
37
38
39
225 solubilization step.

226 2.7 *Statistical analysis*

227 Normality of data distribution was tested by Shapiro-Wilk W test. Homogeneity of variances was
42
43
44
228 tested by Levene's test.

229 To test the effect of the factors method (M), species (S) or category (C) (lean, low fat, medium fat,
48
49
50
51
230 and high fat content) on DNA yield, ratios of absorbance at 260/280 nm (A260/A280), and at
52
53
231 260/230 nm (A260/A230), after extraction, two mixed models with REML estimation of variance
54
55
232 were used. In the first model, M, S, and their interaction (M*S) represented the factors with fixed
56
57
58
233 effects, while the random effect was attributed to the individual factor "subject". The second model
59
60
234 was built as the previous one, replacing the factor S with the factor C.

235 Due to the fact that non-homogeneous variances were found, the differences among methods for
1
236 yield, A260/A280, and A260/A230 were analyzed by Friedman's test for repeated measures
3
4
237 followed by Dunn's multiple tests for pairwise comparison of means, for both fresh and ethanol-
5
6
238 preserved tissues. The analysis was performed overall and within each category of fish.
8

9
239 To test the effect of the factors M, initial amount of tissue (W), and S on DNA relative yield after
10
11
240 extraction, A260/A280, and A260/A230, a mixed model with REML estimation of variance was
12
13
14
241 used: M, W, their interaction (M*W), and S represented the factors with fixed effects, while the
15
16
242 random effect was attributed to the individual factor "subject".
18

19
243 The differences among methods of measured values were analyzed by Friedman's test for repeated
20
21
244 measures followed by Dunn's multiple tests for pairwise comparison of means.
23

24
245 To test the effect of the factors M and S on the band intensity of the DNA amplified by
25
26
246 conventional PCR and on the take-off cycle of the DNA amplified by real-time PCR, a mixed
27
28
247 model with REML estimation of variance was used: M, S , and M*S represented the factors with
29
30
31
248 fixed effects, while the random effect was attributed to the individual factor "subject".
32

33
249 A Friedman's test for repeated measures followed by Dunn's multiple tests for pairwise comparison
34
35
36
250 of means was used to compare values related to the band intensity and take-off cycles of PCR
37
38
251 performed on the DNA extracted with the four methods.
40

252 3. RESULTS AND DISCUSSION 42

43
253 An ideal extraction technique should maximize DNA yield, minimize DNA degradation, and be
44
45
254 effective in terms of costs, time, labor, and supplies. Moreover, it should also guarantee the removal
46
47
48
255 of most of the substances that can act as PCR inhibitors (Radstrom *et al.*, 2004; Bessetti 2007).
49
50
256 Even though the DNA based methods represent the most used techniques for fish species
51
52
257 identification, DNA isolation still represents one of the most time consuming step, which requires
53
54
258 active work of operators all along the process.
55
56
57

58
259 Among the traditional extraction approaches, the original phenol/chloroform protocol proposed
59
60
260 by Sambrook *et al.* (1989) is still widely used. A survey, carried out as a preliminary investigation
61
62
63
64
65

261 of this work and made by analyzing 51 articles published in the last five years, dealing with fish
1
262 species identification, clearly showed that the phenol/chloroform protocol and the commercial kits
3
4
263 are the only extraction methods used (Table 1SM). The phenol/chloroform method, even though
6
264 applicable to many different species and capable to guarantee high DNA recovery, involves the
8
265 handling of toxic materials. For this reason, it implies risks for operators and environment. On the
10
11
1266 other hand, to our experience, the main weakness of the commercial kits is the low DNA yield, if
13
14
267 compared with the classical procedures, although DNA purity may be sometimes higher (Author's
15
16
1268 note). Moreover, commercial kits often present disadvantages such as non-repeatability of the DNA
18
19
269 yield and purity declared by the manufacturers (Di Bernardo *et al.*, 2007; Akkurt, 2012).
20

21
270 In this light, the development of an user-friendly non-organic-based DNA extraction method
23
24
271 could represent the right compromise between the two procedure. Therefore, an alternative salting
25
26
272 out method was previously developed for daily utilization in an open-air laboratory environment
28
29
273 (Armani *et al.*, 2011) and then effectively used for the isolation of a high-quality total DNA from
30
31
274 many species of seafood (Armani *et al.* 2012b; Armani *et al.* 2012c; Armani *et al.* 2013).
32

33
34
375 In case of non-affinity extraction methods, the initial disruption of the tissue, which relies both
35
36
376 on chemicals or physical procedures, is generally the most time-consuming step and can also
37
38
377 greatly influence the yield and the quality of the DNA. Although the physical procedures are the
40
41
478 most employed, they can be very laborious and require operator training (Burden, 2008). Moreover,
42
43
479 all these methods are not easily standardizable and can lead to cross-contamination (Verollet, 2008).
45
46
480 In this study, a simple method based on a bead-milling homogenization protocol without proteases
47
48
481 was set up, not only to speed up and simplify our previous salting out method, but also to reduce the
49
50
5282 cost of the extraction, in the light of an increasing request for routinely biomolecular analysis in the
52
53
583 fish sector.
54

55 584 ***3.1 Bead-milling procedure: optimization and final protocol*** 57

58
585 The cell rupture and the protein digestion are usually achieved by incubation with proteolytic
59
60
6286 enzymes (proteinase K) for a variable time (Table 1SM). Even though several protocols have been
62
63
64
65

287 used for the DNA extraction from fish muscle tissue, the possibility to replace the step of enzymatic
1
288 proteolysis with only bead mill homogenization has never been assessed (Table 1SM).
3

4
289 One of the most important factor to obtain a good tissue disruption is to properly match the
6
290 sample size (mass and volume) with a suitable tube size and grinding ball (Burden, 2012). In our
8
291 protocol, the stain-less steel beads were chosen, due to the fact that they showed better
10
11
1292 performances, with respect to glass beads, in breaking myo-fibrillar tissue and releasing DNA in
13
14
293 solution. In fact, the DNA yield was found to be about 5 times higher (data not shown). Moreover,
15
16
1294 while the glass beads must be discarded together with the organic precipitate at the end of the
18
19
295 disruption step, the steel beads can be re-used after a simple decontamination procedure.
20

21
296 The procedure was optimized at 60°C due to the fact that already at 56°C many cellular proteins
23
297 and enzymes (including DNAase) are denatured (Lahiri and Schnabel, 1993).
25

26
298 Finally, when samples presented a “cloudy supernatant phase” after the first centrifugation at the
28
299 end of the lysis step, a further precipitation step with ammonium acetate, followed by a double
30
31
300 washing with ethanol 70%, was added to clear the solution. In particular, this occurrence was
32
33
301 observed in *Merlangius merlangus*, *Trisopterus minutus capelanus*, *Mullus barbatus*, *Salmo salar*,
35
36
302 *Sardina pilchardus* and *Squalus acanthias*.
37

303 **3.2. Comparison of different extraction procedures.**

40

41
304 **3.2.1 Yield and spectrophotometric quality.** The final yield of an extraction procedure may have
42
43
305 some importance, in that it has to provide not only a sufficient amount of DNA, but also a workable
45
46
306 concentration proportionally to the number and the type of analyses to be performed. This is
47
48
307 particularly important in case of samples provided by museums or collections, which are usually
49
50
308 few milligrams.

52
53
309 In general, an A260/A280 ratio between 1.8 and 2.0 indicates uncontaminated DNA (Sambrook
54
55
310 and Russell, 2001; Alaei *et al.*, 2005). Lower values indicate contamination by proteins, whereas
57
58
311 higher values may be associated to the probable presence of RNA (Varma *et al.*, 2007). The
59
60
312 A260/A230 ratio is considered acceptable when ranging in between 1.8 and 2.4. Values lower than
62
63
64
65

313 1.8 indicate the residual presence of significant amounts of organic compounds, such as phenol,
1
314 carbohydrates, or residual guanidine (De Maeseneire *et al.*, 2007; Morin *et al.* 2010;
3
4
315 <http://cancer.ucsf.edu/research/cores/genome/services/genome-analysis-service-analyze>).

316 A260/A230 ratio is less a precise parameter than A260/A280 ratio, which is often reported alone
8
9
317 (Besbes, *et al.*, 2011; Cawthorn *et al.*, 2011). However, considering that abnormal A260/A230
10
11
318 values may negatively influence downstream analyses, we decided to also consider this parameter
13
14
319 to better characterize the DNA obtained with the new method.

320 The results related to DNA yield, A260/A280, and A260/A230 ratios highlighted a high
18
19
321 variability within species (data not shown). This confirms the importance of the role played by
20
21
322 operators and the individual factor associated to each specimen. For this reason a mixed model
23
24
323 capable to take into consideration such factor of variability (random factor) was chosen to test the
25
26
324 influence of the method and category (on the basis of the fat content), or of the method and species
27
28
325 on the outcome of the extraction procedures. The statistical analysis showed that method, category
30
31
326 and species had a highly significant effect on yield. If the significance associated to method and
32
33
327 species remained very high for the other two parameters investigated (A260/A280 and A260/A230),
35
36
328 the significance associated to category decreased (Table 2SM). Important to notice that the
37
38
329 interaction of the aforesaid factors (method with species or method with category) played an
40
41
330 important role as well, indicating that the method influenced yield or absorbance parameters at a
42
43
331 different extent in different categories or species.

332 For samples stored in ethanol, only yield and A260/A280 are highly influenced by method and
47
48
333 species, while the factor “category” is not as important as the two formers. If these results and the
49
50
334 results related to fresh samples are taken together, it seems likely that the fat content influenced the
52
53
335 process of DNA purification and that ethanol may have leveled down such influence. In fact, it has
54
55
336 been previously reported that the extraction of lower DNA yields can be associated with samples
57
58
337 with higher fat content (Saunders and Rossi, 2008).

338 Comparison of performances related to the different methods revealed that, overall, no
1
339 differences existed between B30 and B60 for all the three parameters (yield, A260/A280, and
3
4
340 A260/A230).
5
6

341 As a general outcome, it was found that SO method produced the highest yield and was more
8
342 efficient in removing contaminants from the DNA solution, with values of A260/A280 and
10
11
12
13
14
15
344 samples. With regard to the methods using milling beads, the yield was, on an average, one-half of
16
17
18
19
20
21
22
23
24
25
346 Values of A260/A280 were about 2, while A260/A230 were slightly beneath 1.8. Lastly, the kit,
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

349 Even though the commercial kits are used more frequently and are reported to be less technically
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

355 Patterns of performance are similar in the 4 groups, even though differences between methods
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

364 lowering of their means in medium and high-fat content groups determined the loss of significance
1
365 between values of SO and the kit, within this two categories.

366 As for samples stored in ethanol, differences in yield between SO and both B30 and B60
2
367 remained highly significant in all the 4 groups, as well as differences in A260/A280 and in
3
368 A260/A230.
4
5
6
7
8
9
10

11
1369 *3.2.2. Decreasing weights.* Starting from different amounts of tissue, different DNA relative
12
1370 yields and different values of purity indicators were obtained across the three methods.
14
15

16
1371 Statistical analysis based on the mixed model showed that both initial amount of tissue and
17
18
19
1372 method of extraction significantly influenced the relative yield ($\mu\text{g DNA/mg}$ of tissue) of DNA
20
21
22
2373 obtained and that such a parameter varied with a significantly different pattern in different methods.
23
24
25
1374 Such response was confirmed for both A260/A280 and A260/A230, despite different level of
26
27
28
2975 significance associated to the different factors (Table 3SM). The factor species was always found to
28
29
30
2976 have a highly significant influence on the three parameters considered.

31
32
33
34
35
36
37
38
39
377 If marked differences were observed between SO method and bead-milling methods, no
32
33
34
35
36
37
38
39
40
378 significant differences were observed increasing time from 30 to 60 minutes of bead milling. As
35
36
37
38
39
40
379 shown in Figure 1, the pattern of variation are very similar for B30 and B60, once more confirming
37
38
39
40
380 that 30 minutes of milling is a sufficient time to reach a good level of tissue disruption.

41
42
43
44
45
46
47
48
49
381 In particular, the initial amount of tissue that gave the highest DNA relative yield for SO method
42
43
44
45
46
47
48
49
382 was 50 mg. From lower or higher tissue amounts a significantly lower yield was obtained ($p < 0.01$).
45
46
47
48
49
383 On the contrary, differences in DNA purity indicators were not significant.

50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
384 As for bead-milling methods, a net gain in DNA relative yield was observed with the increase of
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
385 the initial amount of tissue processed. If A260/A280 did not vary appreciably, with only a
52
53
54
55
56
57
58
59
60
61
62
63
64
65
386 significantly higher value for 200 mg of tissue with respect to all the other amounts ($p < 0.01$),
53
54
55
56
57
58
59
60
61
62
63
64
65
387 A260/A230 was shown to have a minimum for 100 mg, which significantly differed from the other
56
57
58
59
60
61
62
63
64
65
388 values ($p < 0.05$).

389 From these results, it is possible to state that the best outcomes in terms of both yield and purity
1
390 were obtained with the lowest amounts of tissue (25 and 10 mg), with both absorbance ratios
3
4
391 around the threshold of the optimal range (Fig. 1). However, the DNA yield being relative, the total
6
392 amount of DNA obtained would be lower than that obtained starting from higher amount of tissue.
8
393 This is strictly dependent on the needs and scope of the analysis.

1394 *3.2.3. Integrity.* The purpose of an extraction method is not only to obtain an acceptable amount
13
14
1395 of highly pure DNA, but also to limit at most the DNA degradation processes. Agarose gel
15
16
1396 electrophoresis enables visualization, to some extent, of the degradation level of the extracted DNA.
18
19
1397 The visual analysis of the DNA degradation pattern did not show any evident difference among the
20
21
1398 extraction methods tested (data not shown).

1399 **3.3. DNA amplificability**

1400 In order to test the amplificability of the DNA extracted with different methods, a conventional
27
28
1401 and a real-time PCR were performed on a subgroup of the fresh tissue samples processed (the same
30
31
1402 used to test extraction from decreasing weight of tissues). The genes *16srRNA* and *COI* were chosen
32
33
1403 as target because are among the most used for fish species identification and because require
35
36
1404 different kind of primers for the amplification (Armani *et al.*, 2012a).

1405 *3.4.1. Conventional PCR.* The DNA was successfully amplified by PCR regardless the
40
41
1406 extractions procedures, even though some differences were observed among the four methods (Fig
42
43
1407 2).

1408 Once again, the mixed model showed that the amplification of the two targeted genes was
47
48
1409 significantly influenced by both the factors method and species, as well as their interaction,
49
50
1410 confirming that unknown factors associated to the kind of tissues (likely compositional and
52
53
1411 depending on the species) may strongly influence the extraction procedure and thus the downstream
54
55
1412 analysis (Table 4SM). In particular, even though overall means were not very far, significant
57
58
1413 differences ($p < 0.05$) were found only between SO and both B30 and B60, when the *COI* gene was
59
60
1414 amplified, while significant differences ($p < 0.01$) were found between the kit and SO, B30, and B60,
61
62
63
64
65

415 when the gene *16SrRNA* was targeted. The different influence of the extraction methods on the
1
416 amplificability of different genes may be due to the characteristics of the primer used, whose
3
4 capability to steadily match the complementary sequence on the DNA is likely influenced, at a
417
6
418 different extent, by the presence of residual molecules in the PCR reaction mixtures.

9
419 **3.4.2. Real time PCR.** Starting from the same amount of DNA, the take-off cycle is a function of
10
11
420 the PCR efficiency, which in turn is somehow proportional, other than to the characteristics of the
13
14
421 primers and the PCR conditions (identical for all the samples), also to the DNA integrity and the
15
16
422 presence of potential inhibitors. In this trial, only a fragment of the *16srRNA* gene was amplified,
18
19
423 due to the absence of universal primers for the amplification of a short fragment belonging to the
20
21
424 *COI* gene.

23
425 Statistics showed that both the two factors included in the model (method and species)
25
26
426 significantly influenced the take-off cycle ($p < 0.001$). The high significance ($p < 0.001$) associated to
27
28
427 the interaction of the two factors revealed that the factor method had a different effect on different
30
31
428 species (Tab. 4).

33
429 Overall, direct comparison between methods revealed significant differences between SO and
35
36
430 the other three ($p < 0.001$), while not significant differences were found between B30, B60, and the
37
38
431 kit. On an average, SO was associated to slightly but significantly lower take-off cycles, with
40
41
432 respect to the other methods (Fig. 3). The better performance of the downstream amplification in
42
43
433 real-time PCR obtained with SO extraction may reflect a higher quality of the extracted DNA and
44
45
434 thus a better global performance with regard to the extraction process.

48 **3.4 Cost and time evaluation**

49
50
436 The kit was the most expensive procedure with a cost of $\sim \text{€ } 2.25$ per sample, which was almost
52
53
437 2.5-fold higher than SO ($\sim \text{€ } 0.8$) and 3.5-fold higher than the bead-based procedures (Bs) ($\sim \text{€}$
54
55
438 0.62). This downside related to the utilization of the kit has already been reported by Ivanova *et al.*,
57
58
439 (2006). The cost of the Bs was further reduced with respect to SO due to the absence of the enzyme,
59
60
440 which is one the most expensive reagents used in the classic extraction procedures. Finally, it must
61
62
63
64
65

441 be underlined that the bead-milling completely replaces the manual work of the operator. This can
1
442 add some more economical advantage, due to the “working time” saved, which may become
3
443 conspicuous when a high number of samples have to be processed.
4
5
6

7
444 Differences were found comparing the global time required for the entire protocol from the
8
9
445 homogenization up to the DNA elution. Overall, the SO required around 3 hrs and 20 min, the B30
10
11
446 2 hrs and 30 min, the B60 3 hrs, while in the case of the kit the total time was extremely variable
13
14
447 ranging from 1 hr and 30 min to 3 hrs and 30 min. In fact, as reported by the manufacturer, the lysis
15
16
448 step can be extended up to 3 hrs in order to obtain a satisfactory result. Moreover, the total time for
18
19
449 SO and the kit can be influenced by the homogenization of the samples by manual scissor shearing,
20
21
450 variable from 20 to 40 min, according to the resistance to cutting.
22
23

451 CONCLUSIONS 25

26
452 In this work, a simple, rapid and cost-effective method, based on a mechanical grinding with
27
28
453 steel beads followed by a salting out procedure, was developed to isolate DNA from the fish
30
31
454 muscle.
32

33
455 From this experimentation emerged that it is not possible to indicate *a priori* the best extraction
35
36
456 method for a specific fish species or for a specific gene to be amplified, but general conclusions
37
38
457 may be draft on the basis of analysis performed on a number of different species, representative of
40
41
458 large categories, such as the very important ones related to the fat content.
42

43
459 Even though the comparison performed with other methods showed that the salting out
45
460 procedure is, on an average, the best in terms of both yield and spectrophotometric quality of the
47
48
461 total DNA, the bead-beating protocol allowed to obtain DNA of good quality and in acceptable
49
50
462 amount, which suffices for thousands of PCR amplifications, and significantly overcomes that
52
53
463 provided by the kit. Moreover, by eliminating the need of a physical disruption performed by the
54
55
464 operator, the bead-milling procedure allows to avoid the most undertaking and longest step of all
57
58
465 the DNA extraction procedures. This permits to improve the throughput of the procedure by
59
60
466 markedly increasing the number of samples processable by a single operator. Lastly, the automatic
62
63
64
65

467 tissue-disruption process also makes less important the relationship between goodness of the
1
468 outcome and ability or expertise of the operator.
3

4
469 In conclusion, the bead-milling method represents a valid alternative to the classical methods of
6
470 DNA extraction, especially for routine analyses that involve a high number of samples and demand
8
471 the lowest costs possible.
10

472 473 **“Compliance with Ethics Requirements”**

474
475 The research was performed with funds granted from the University of Pisa.
16

476
477 Armani Andrea declares that he has no conflict of interest.
18

478
479 Tinacci Lara declares that he has no conflict of interest.
21

480
481 Xiong Xiong declares that he has no conflict of interest.
24

482
483 Titarenko Evgeniya declares that he has no conflict of interest.
26

484
485 Guidi Alessandra declares that he has no conflict of interest.
28

486
487 Castiglieo Lorenzo declares that he has no conflict of interest.
31

488
489 This article does not contain any studies with human or animal subjects.
34

490 491 **References**

- 492 1. Ackmann RG.(1989) Nutritional composition of fats in seafoods. *Prog Food Nutr*
493 *Sci.*13(3-4):161-289.
- 494 2. Akkurt M. (2012) Comparison between modified DNA extraction protocols and
495 commercial isolation kits in grapevine (*Vitis vinifera* L.) *Genet. Mol. Res.* 11(3):
496 2343-2351 (2012)
- 497 3. Alaey M, Naderi R, Vezvaei A, Khalighi A, Salami, A. (2005). Comparing study
498 between four different methods of genomic DNA extraction from *Cyclamen persicum*
499 *Mill. Int. J. Agric. Biol.* 7: 882-884
- 500 4. Allender CJ, Easterday WR, Van Ert MN, Wagner DM & Keim P. (2004). High-
501 throughput extraction of arthropod vector and pathogen DNA using bead milling.
502 *Biotechniques* 37(5): 730-733
- 503 5. Armani A, Castiglieo L & Guidi A.(2012a) Fish frauds: the DNA challenge. *CAB*
504 *Reviews* 10/2012 7 (71).
- 505 6. Armani A, Castiglieo L, Tinacci L, Gandini G, Gianfaldoni D, & Guidi A. (2012b).
506 A rapid PCR–RFLP method for the identification of *Lophius* species. *Eur. Food Res.*
507 *and Technol.* 235(2):253-263
- 508 7. Armani A, Castiglieo L, Tinacci L, Gianfaldoni D & Guidi, A. (2011). Molecular
509 characterization of icefish, (*Salangidae* family), using direct sequencing of
510 mitochondrial cytochrome b gene. *Food Control* 22: 888-895.

- 511 8. Armani A., Tinacci L., Giusti A., Castigliero L., Gianfaldoni D., Guidi A. (2013)
512 What is inside the jar? Forensically informative nucleotide sequencing (FINS) of a
513 short mitochondrial COI gene fragment reveals a high percentage of mislabeling in
514 jellyfish food products. *Food Res. Int.* 54 (2), 1383-1393.
- 515 9. Armani, A., Castigliero, L., Tinacci, L., Gianfaldoni, D., & Guidi, A. (2012c).
516 Multiplex conventional and real-time PCR for fish species identification of Bianchetto
517 (juvenile form of *Sardina pilchardus*), Rossetto (*Aphia minuta*), and Icefish in fresh,
518 marinated and cooked products. *Food Chem.* 133(1): 184-192
- 519 10. Besbes N, Fattouch S & Sadok S. (2011). Comparison of methods in the recovery and
520 amplificability of DNA from fresh and processed sardine and anchovy muscle tissues.
521 *Food Chem.* 129(2): 665-671
- 522 11. Bessetti J. (2007). An introduction to PCR inhibitors. *J. Microbiol. Meth.* 28: 159-167
- 523 12. Burden, D. W. (2008). Guide to the homogenization of biological samples. *Random*
524 *Primers* 7: 1-14
- 525 13. Cawthorn, DM, Steinman, HA, & Witthuhn, RC. (2011) Comparative study of
526 different methods for the extraction of DNA from fish species commercially available
527 in South Africa. *Food Control* 22(2): 231-244
- 528 14. De Maeseneire SL, Van Bogaert IN, Dauvrin T, Soetaert WK & Vandamme EJ.
529 (2007) Rapid isolation of fungal genomic DNA suitable for long distance PCR.
530 *Biotechnol. Lett.* 29(12): 1845-1855.
- 531 15. Di Bernardo G, Gaudio SD, Galderisi U, Cascino A & Cipollaro M. (2007).
532 Comparative evaluation of different DNA extraction procedures from food samples.
533 *Biotechnol. Progr.* 23(2), 297-301.
- 534 16. Dilworth E & Frey JE. (2000). A rapid method for high throughput DNA extraction
535 from plant material for PCR amplification. *Plant Mol. Biol. Rep.* 18(1): 61-64.
- 536 17. Galimberti A, De Mattia F, Losa A, Bruni I, Federici S, Casiraghi M, Martellos S &
537 Labra M. (2013) DNA barcoding as a new tool for food traceability. *Food Res. Int.*
538 50(1) : 55-63
- 539 18. Handy SM, Deeds JR, Ivanova NV, Hebert PDN, Hanner R, Ormos A, Weigt LA,
540 Moore, MM & Yancy, H.F. (2011). A single laboratory validated method for the
541 generation of DNA barcodes for the identification of fish for regulatory compliance. *J.*
542 *AOAC* 94(1): 201-210
- 543 19. Ivanova N.V, Dewaard J.R and P.D.N. HEBERT (2006). An inexpensive, automation-
544 friendly protocol for recovering high-quality DNA. *Mol. Ec. Notes.* 6(4), 998-1002.
- 545 20. Lahiri DK & Schnabel B. (1993). DNA Isolation by a Rapid Method from Human
546 Blood Samples: Effects of MgCl₂, EDTA, Storage, Time, and Temperature on DNA
547 Yield and Quantity. *Biochem. Gen.* 31: 321-328.
- 548 21. Löffler J, Hebart H, Schumacher U, Reitze H, & Einsele H. (1997). Comparison of
549 different methods for extraction of DNA of fungal pathogens from cultures and blood.
550 *J Clin. Microb.* 35(12): 3311-3312.
- 551 22. Morin N, Vallaeyts T, Hendrickx L, Natalie L & Wilmotte A. (2010). An efficient
552 DNA isolation protocol for filamentous cyanobacteria of the genus *Arthrospira*. *J*
553 *Microbiol. Methods* 80(2): 148-154.
- 554 23. Palumbi SR. (1996) Nucleic acids II: the polymerase chain reaction. In: *Molecular*
555 *Systematics* 2nd ed. Hillis DM, Moritz C, Mable BK. (eds), pp. 205–247. Sinauer &
556 Associates Inc., Sunderland, Massachusetts. USA
- 557 24. Rådström P, Knutsson R, Wolffs P, Lövenklev M, & Lofström C. (2004). Pre-PCR
558 processing. *Mol Biotechnol* 26(2): 133-146.
- 559 25. Robe P, Nalin R, Capellano C, Vogel TM & Simonet P. (2003). Extraction of DNA
560 from soil. *Eur J Soil Biol*, 39(4): 183-190

561 26. Sambrook J, Fritsch EF & Maniatis T. (1989). Molecular cloning: A laboratory
562 manual 2nd eds (Vol. 2). New York: Cold Spring Harbor laboratory press, New York,
563 USA
564 27. Sambrook J & Russell DW (2001) Molecular cloning, a laboratory manual, 3rd ed.
565 Cold Spring Harbor Laboratory Press, New York, USA
566 28. Saunders GC, & Rossi JM. (2008). DNA extraction. In J. T. Keer, & L. Birch (Ed).,
567 Essentials of nucleic acid analysis: A robust approach. Royal Society of Chemistry,
568 Cambridge, UK
569 29. Varma A, Padh H & Shrivastava N (2007). Plant genomic DNA isolation: An art or a
570 science. Biotechnol. J. 2: 386-392
571 30. Verollet, R. (2008). A major step towards efficient sample preparation with bead-
572 beating. Biotechniques 44(6): 832-833.
573 31. Wilson (1997). "Inhibition and facilitation of nucleic acid amplification." Appl
574 Environ Microbiol 63(10): 3741–3751.
575

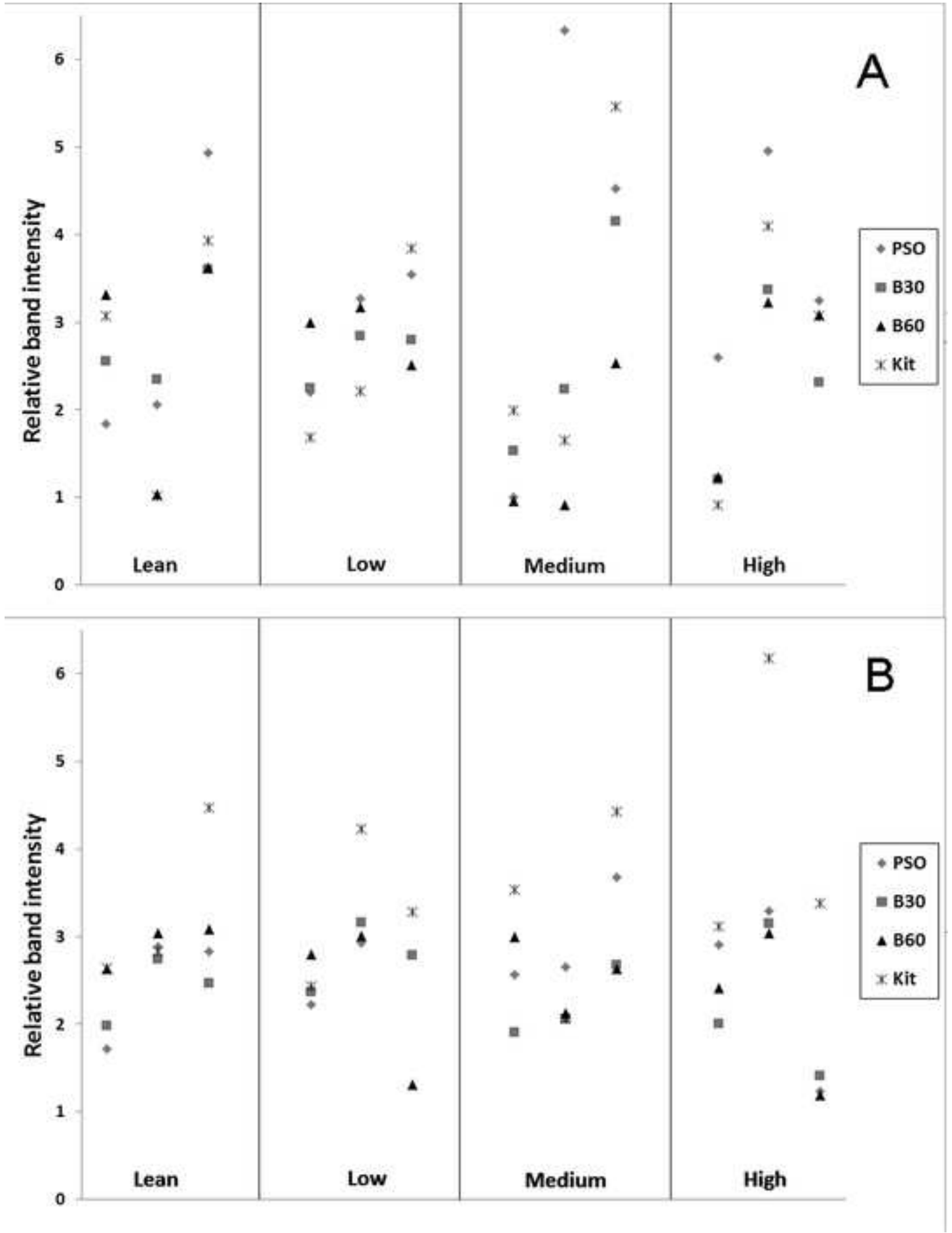
18
19
20
21
22 **Figure captions**
23

24 **Fig. 1** Performances related to scalar quantities of tissue processed.
25

26 **Fig. 2** Amplification performance of the two genes (A: *COI*; B: *I6S*) by conventional PCR,
27
28 starting from the DNA extracted with the four different methods, from a subgroup of twelve species
29
30 (lean, n=3; low fat, n=3; medium fat, n=3, high fat, n=3). Quantities are expressed as the relative
31
32 intensity of the single bands on the gel, corresponding to the percentage related to the sum of the
33
34 single intensity values calculated on a single gel, where all the samples belonging to a different
35
36 category were run. Overall values were not reported because of the impossibility of averaging
37
38 values obtained from different gels. Levels of significance were however calculated by comparing
39
40 values obtained by parallel comparison of samples run in the same gel.
41
42
43
44

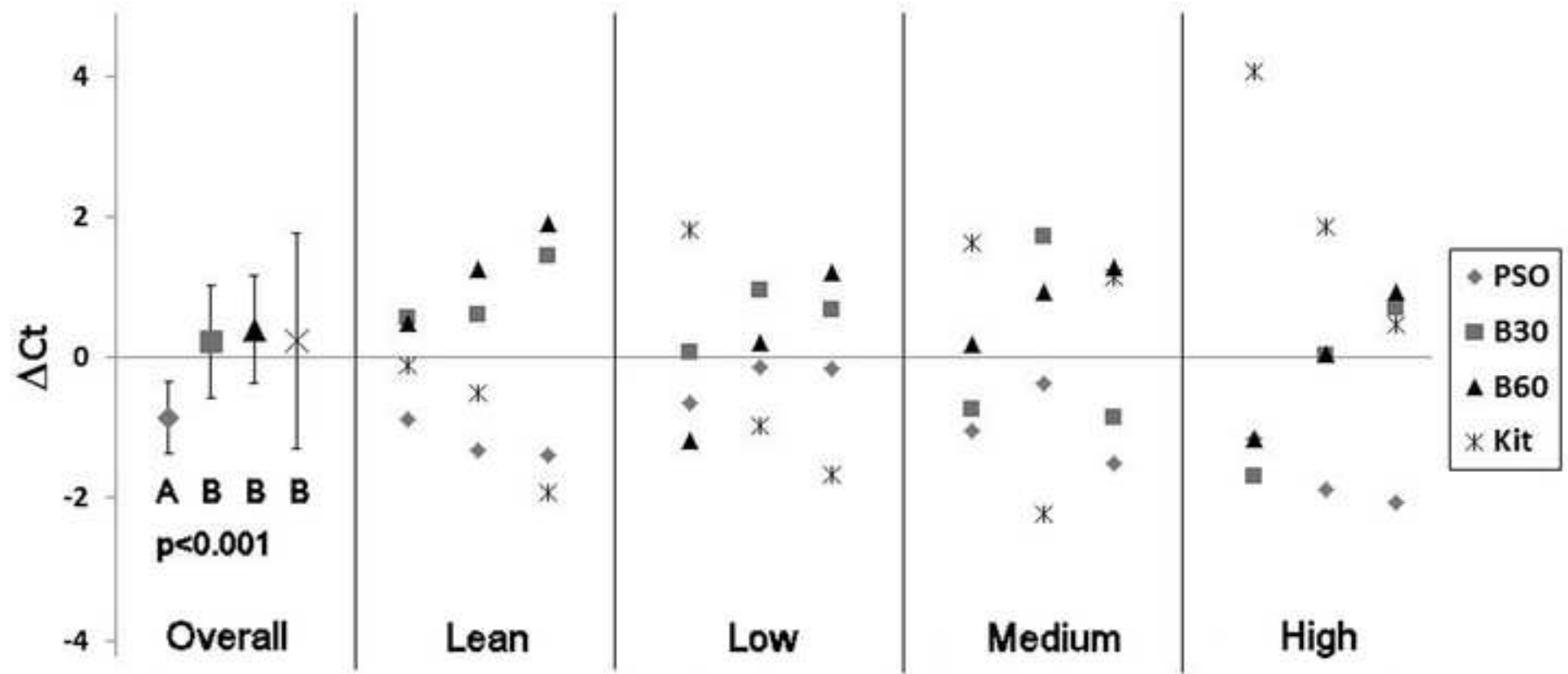
45 **Fig. 3** Amplification performance in real-time PCR of the DNA extracted with the four different
46
47 methods, from a subgroup of twelve species (lean, n=3; low fat, n=3; medium fat, n=3, high fat,
48
49 n=3), expressed as difference (ΔC_t) between the take-off cycle (C_t) for each subject and the overall
50
51 mean.
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Figure
[Click here to download high resolution image](#)



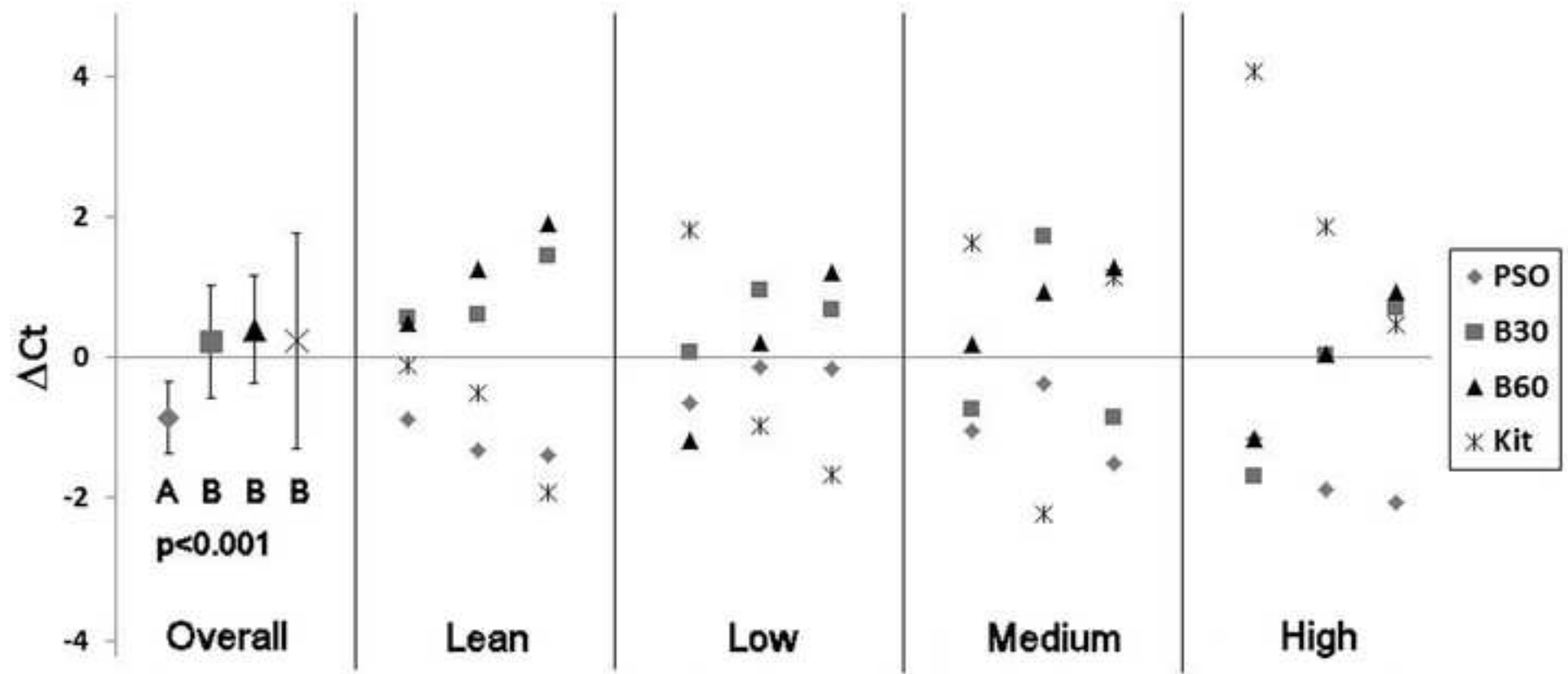
Figure

[Click here to download high resolution image](#)



Figure

[Click here to download high resolution image](#)



LEAN (<2%)	LOW FAT (2-4%)	MEDIUM FAT (UP TO 8%)	HIGH FAT (> 8%)
YELLOW GROUPER <i>Epinephelus awoara</i>	BROTULA <i>Brotula multibarбата</i>	TROUT <i>Onchorhynchus mykiss</i>	SALMON <i>Salmo salar</i>
NILE TILAPIA <i>Oreochromis niloticus</i>	POOR COD <i>Trisopterus minutus capelanus</i>	WHITE STURGEON <i>Acipenser transmontanus</i>	EUROPEAN EEL <i>Anguilla anguilla</i>
COD <i>Gadus morhua</i>	GRASS GOBY <i>Zosterisessor ohpicephalus</i>	RED MULLET <i>Mullus barbatus</i>	GREATER AMBERJACK <i>Seriola dumerili</i>
WHITING <i>Merlangius merlangus</i>	SEABASS <i>Dicentrarchus labrax</i>	GOLDEN GREY MULLET <i>Liza aurata</i>	ATLANTIC MACKEREL <i>Scomber scombrus</i>
HAKE <i>Merluccius capensis</i>	GILTHEAD SEABREAM <i>Sparus aurata</i>	SARDINE <i>Sardina pilchardus</i>	
GURNARD <i>Chelydonictis lucerna</i>	ANCHOVY <i>Engraulis encrasicolus</i>	SWORDFISH <i>Xiphia gladius</i>	
STARGAZER <i>Uranoscopus scaber</i>	ROUND SARDINELLA <i>Sardinella aurita</i>	SPINY DOGFISH <i>Squalus acanthias</i>	
JOHN DORY <i>Zeus faber</i>	YELLOWFIN TUNA <i>Thunnus albacares</i>		
SCORPION FISH <i>Scorpaena scrofa</i>	SMALL-SPOTTED CATSHARK <i>Scyliorhinus canicula</i>		
BLUESPOTTED SEABREAM <i>Pagrus caeruleostictus</i>			
YELLOW GOOSEFISH <i>Lophius litulon</i>			
COMMON SOLE <i>Solea solea</i>			
GUINEAN SOLE <i>Synaptura cadenati</i>			
SPOTTAIL SPINY TURBOT <i>Psettodes belcheri</i>			
BLUE SHARK <i>Prionace glauca</i>			
SMOOTH HOUND <i>Mustelus mustelus</i>			
PORBEAGLE <i>Lamna nasus</i>			
RAY <i>Raja sp.</i>			

Table 1 Species used in this study grouped on the basis of their fat content according to Ackmann, 1990. The fat content values of the species were retrieved from Prato & Biandolino, (2012); Ozogul & Ozogul (2007) and FAO values (<http://www.fao.org/wairdocs/tan/x5916e/x5916e01.htm>) In bold the species used for the optimization of the bead extraction method and the real time PCR.

Table 1SM The table report the DNA extraction method (Commercial kit and Classical procedure) used in article dealing with fish species identification. The procedure market with * did not use enzymatic digestion with proteinase K.

Article	Samples	Method		Reference/ Company
		Commercial kit	Classical procedure	
Identifying Canadian Freshwater Fishes through DNA Barcodes Hubert, <i>et al.</i> (2008). Plos One 3(6): e2490.	Fresh	NucleoSpin96 kit		MACHEREY-NAGEL GmbH & Co. KG, D-52313 Düren, Germany
Authentication of Anglerfish Species (<i>Lophius</i> spp) by Means of Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) and Forensically Informative Nucleotide Sequencing (FINS) Methodologies. Espineira, et al. (2008). <i>Journal of Agricultural and Food Chemistry</i> 56 (22): 10594-10599.	Fresh/processed		Phenol-chloroform protocol *	Rogers, et al. (1988). Extraction of DNA from plant tissues. Plant Molecular Biology Manual. S. Gelvin, R. Schilperoort and D. Verma, Springer Netherlands: 73-83.
DNA barcoding detects market substitution in North American seafood. Wong & Hanner (2008). <i>Food Research International</i> 41(8): 828-837	Fresh/frying/ precooked		Glass Fiber protocol	Ivanova, et al. (2006). An inexpensive, utomation-friendly protocol for recovering high-quality DNA. <i>Molecular Ecology Notes</i> 6(4): 998-1002.
Detection of Mislabeling in Hake Seafood Employing mtSNPs-Based Methodology with Identification of Eleven Hake Species of the Genus <i>Merluccius</i> . Machado-Schiaffino, et al. (2008). <i>Journal of Agricultural and Food Chemistry</i> 56 (13): 5091-5095.	Fresh/frozen/ precooked		Chelex resin protocol	Estoup, et al. (1996). Rapid one-tube DNA extraction for reliable PCR detection of fish polymorphic markers and transgenes." <i>Molecular Marine Biology and Biotechnology</i> 5(4): 295-298.

DNA barcoding for the identification of smoked fish products Smith, et al. (2008). <i>Journal of Fish Biology</i> 72(2): 464-471.	Smoked		Phenol–chloroform– ethanol protocol	Taggart, et al. (1992). A simplified protocol for routine total DNA isolation from salmonid fishes. <i>Journal of Fish Biology</i> 40(6): 963-965.
Development of a method for the identification of scombroid and common substitute species in seafood products by FINS Espineira, et al. (2009). <i>Food Chemistry</i> 117(4): 698-704.	Fresh/frozen/ processed	NucleoSpin Tissue kit	Chloroform-isoamyl protocol*	MACHEREY-NAGEL GmbH & Co. KG., D-52313 Düren, Germany. Rogers, et al. (1985). Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. <i>Plant Molecular Biology</i> 5(2): 69-76.
PCR-based methodology for the authentication of grouper (<i>Epinephelus marginatus</i>) in commercial fish fillets. Asensio, et al. (2009). <i>Food Control</i> 20(7): 618-622.	Fresh/processed	Wizard [®] DNA Clean-Up System		Promega Corporation, 2800 Madison, WI 53711 USA
The Real maccoyii: Identifying Tuna Sushi with DNA Barcodes – Contrasting Characteristic Attributes and Genetic Distances Lowenstein, et al. (2009). <i>Plos One</i> 4(11): e7866	Sushi (fresh)	DNeasy Blood & Tissue Kit		QIAGEN GmbH, 40724 Hilden, Germany
A Validated Methodology for Genetic Identification of Tuna Species (Genus Thunnus) Vinas, et al. (2009). <i>Plos One</i> 4(10):e7606	Fresh		Phenol-chloroform-isoa myl protocol	Vinas, et al. (2004). Inter-oceanic genetic differentiation among albacore (<i>Thunnus alalunga</i>) populations. <i>Marine Biology</i> 145(2): 225-232.
Identification of shark and ray fins using DNA barcoding Holmes, et al. (2009). <i>Fisheries Research</i> 95(2-3): 280-288.	Fresh/dried		Chelex resin protocol Glass Fiber protocol	Walsh, et al. (1991). Chelex-100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. <i>Biotechniques</i> 10(4): 506-513. Ivanova, et al. (2006). An inexpensive, automation-friendly protocol for recovering

				high-quality DNA. <i>Molecular Ecology Notes</i> 6(4): 998-1002.
The application of PCR-RFLP and FINS for species identification used in sea cucumbers (Aspidochirotida: Stichopodidae) products from the market Wen, et al. (2010). <i>Food Control</i> 21(4): 403-407.	Fresh/dried	TIANamp Marine Animals DNA Kit		Tiangen Biotech Co. Ltd., Beijing, China
Identification of European Hake Species (Merluccius merluccius) Using Real-Time PCR Sanchez, et al. (2009). <i>Journal of Agricultural and Food Chemistry</i> 57(9): 3397-3403.	Fresh/ canned/ refrigerated/ precooked/battered	Wizard® DNA Clean-Up System		Promega Corporation 2800 Madison, WI 53711 USA
DNA barcoding for conservation and management of Amazonian commercial fish. Ardura, et al. (2010). <i>Biological Conservation</i> 143(6): 1438-1443.	Fresh/processed		Chelex resin protocol	Estoup, et al. (1996). "Rapid one-tube DNA extraction for reliable PCR detection of fish polymorphic markers and transgenes." <i>Molecular Marine Biology and Biotechnology</i> 5(4): 295-298.
A multiplex-PCR assay for the authentication of mackerels of the genus Scomber in processed fish products. Catanese, et al. (2010). <i>Food Chemistry</i> 122(1): 319-326.	Fresh/canned	FastDNA kit*		MP Biomedicals LLC; QBiogene Division 29525 Fountain Parkway, Solon, OH 44139
Species identification in anchovy pastes from the market by PCR-RFLP technique. Rea, et al. (2009). <i>Food Control</i> 20(5): 515-520.	Fresh/processed samples		Phenol-chloroform protocol	Sambrook, et al. (1987). <i>Molecular Cloning: A Laboratory Manual</i> , Cold Spring Harbor Laboratory Press. Woodbury, NY, USA
Molecular barcoding reveals mislabelling of commercial fish products in Italy Filonzi, et al. (2010). <i>Food Research International</i> 43(5): 1383-1388.	Fresh/processed	Aquapure Genomic DNA kit	Phenol-chloroform protocol	Bio-Rad Laboratories, Inc., 2000 A. Nobel Dr. Hercules, CA 94547 USA Moore, et al. (1999). "Manipulation of DNA." <i>Current protocols in molecular biology</i> , John Wiley & Sons Inc., New York (1999), 211-223
Application of a PCR-RFLP Method to Identify Salmon Species	Canned/smoked/	DNeasy Blood &		QIAGEN GmbH, 40724 Hilden, Germany

in U.S. Commercial Products Rasmussen, et al. (2010) . <i>Journal of Aquatic Food Product Technology</i> 19(1): 3-15.	jerky/fresh	Tissue Kit	
Misleading the masses: detection of mislabelled and substituted frozen fish products in South Africa Von der Heyden, et al. (2010) . <i>Ices Journal of Marine Science</i> 67(1): 176-185.	Fresh	DNeasy Blood & Tissue Kit	QIAGEN GmbH, 40724 Hilden, Germany
Authentication of swordfish (<i>Xiphias gladius</i>) by RT-PCR and FINS methodologies Herrero, et al. (2011) . <i>European Food Research and Technology</i> 233(2): 195-202.	Fresh/frozen/smoked		Phenol-chloroform protocol *
FINS methodology to identification of sardines and related species in canned products and detection of mixture by means of SNP analysis systems. Lago, et al. (2011) . <i>European Food Research and Technology</i> 232(6): 1077-1086.	Fresh/processed samples		Phenol-chloroform protocol *
Molecular identification of the black tiger shrimp (<i>Penaeus monodon</i>), the white leg shrimp (<i>Litopenaeus vannamei</i>) and the Indian white shrimp (<i>Fenneropenaeus indicus</i>) by PCR targeted to the 16S rRNA mtDNA Pascoal, et al. (2011) . <i>Food Chemistry</i> 125(4): 1457-1461.	Fresh samples	DNeasy Blood & Tissue Kit	QIAGEN GmbH, 40724 Hilden, Germany
Duplex real-time PCR for authentication of anglerfish species Herrero, et al. (2011) . <i>European Food Research and Technology</i> 233(5): 817-823.	Fresh/precooked		Proteinase K-phenol-chloroform protocol
Identification of fish species by 5S rRNA gene amplification Tognoli, et al. (2011) . <i>Food Chemistry</i> 129(4): 1860-1864.	Fresh		Phenol-chloroform-isoamyl- alcohol protocol
DNA barcoding unveils a high rate of mislabeling in a commercial freshwater catfish from Brazil.	Fresh		Phenol-chloroform protocol
			Rogers, et al. (1988) . Extraction of DNA from plant tissues. Plant Molecular Biology Manual. S. Gelvin, R. Schilperoort and D. Verma, Springer Netherlands: 73-83.
			Rogers, et al. (1988) . Extraction of DNA from plant tissues. Plant Molecular Biology Manual. S. Gelvin, R. Schilperoort and D. Verma, Springer Netherlands: 73-83.
			Aranishi. (2005) . "Rapid PCR-RFLP method for discrimination of imported and domestic mackerel." <i>Marine Biotechnology</i> 7(6): 571-575.
			Sambrook, et al. (1989) . Molecular Cloning: A Laboratory Manual, Cold Spring Harbor

Carvalho, et al. (2011). <i>Mitochondrial DNA</i> 22: 97-105.			Laboratory Press. Woodbury, NY, USA
High Level of Mislabeling in Spanish and Greek Hake Markets Suggests the Fraudulent Introduction of African Species Garcia-Vazquez, et al. (2011). <i>Journal of Agricultural and Food Chemistry</i> 59(2): 475-480.	Fresh	Chelex resin protocol	Moran, et al. (2006). "Identification of highly prized commercial fish using a PCR-based methodology." <i>Biochemistry and Molecular Biology Education</i> 34(2): 121-124.
Identification of barramundi (<i>Lates calcarifer</i>) and tilapia (<i>Oreochromis</i> spp.) fillets by DNA- and protein-analytical methods. Schiefenhoevel, et al. (2011). <i>Journal Fur Verbraucherschutz Und Lebensmittelsicherheit</i> 6(2): 203-214.	Fresh	Chloroform-isopropanol protocol	Rehbein. (2005). "Identification of the fish species of raw or cold-smoked salmon and salmon caviar by single-strand conformation polymorphism (SSCP) analysis." <i>European Food Research and Technology</i> 220(5-6): 625-632.
DNA Barcoding of Catfish: Species Authentication and Phylogenetic Assessment. Wong, et al. (2011). <i>Plos One</i> 6(3):e17812	Fin clip (fresh)	Genra Puregene Tissue Kit	QIAGEN GmbH, 40724 Hilden, Germany
DNA barcoding reveals a high incidence of fish species misrepresentation and substitution on the South African market Cawthorn, et al. (2012). <i>Food Research International</i> 46(1): 30-40.	Fresh/frozen/processed	SureFood® PREP Allergen Kit	R-Biopharm AG, 64297 Darmstadt, Germany
Chemical characterisation, biogenic amines contents, and identification of fish species in cod and escolar steaks, and salted escolar roe products. Hwang, et al. (2012). <i>Food Control</i> 25(1): 415-420.	Fresh/salted	Chemagic DNA Tissue 10 Kit *	PerkinElmer Chemagen Technologie GmbH 52499 Baesweiler, Germany
A unique specification method for processed unicorn filefish products using a DNA barcode marker. Yang, et al. (2012). <i>Food Control</i> 25(1): 292-302.	Canned/dried/fried	MasterPure™ DNA Purification Kit	Epicentre Biotechnologies, Madison, WI, 53719 USA
Authentication of commercialized crab-meat in Chile using DNA Barcoding. Haye, et al. (2012). <i>Food Control</i> 25(1): 239-244.	Fresh/canned	Phenol-Chloroform method	Sambrook, et al. (1989). <i>Molecular Cloning: A Laboratory Manual</i> , Cold Spring Harbor Laboratory Press. Woodbury, NY, USA

Inaccurate labelling detected at landings and markets: The case of European megrims. Crego-Prieto, et al. (2012) . <i>Fisheries Research</i> 129: 106-109.	Fresh samples		Chelex resin protocol	Estoup, et al. (1996) . "Rapid one-tube DNA extraction for reliable PCR detection of fish polymorphic markers and transgenes." <i>Molecular Marine Biology and Biotechnology</i> 5(4): 295-298.
Identification of 11 sea cucumber species by species-specific PCR method. Wen, et al. (2013) . <i>Food Control</i> 29(1): 287-287.	Fresh/fresh/dried	TIANamp Marine Animals DNA Kit		Tiagen Biotech Co. Ltd., Beijing, China
DNA Barcoding as a Reliable Method for the Authentication of Commercial Seafood Products Nicole, et al. (2012) . <i>Food Technology and Biotechnology</i> 50(4): 387-398.	Fresh/frozen/smoked/dried	GenElute Mammalian Genomic DNA Miniprep Kit		Sigma-Aldrich, LLC., St. Louis, Missouri 63103 United States
Forensic DNA analysis reveals use of high trophic level marine fish in commercial aquaculture fish meals Ardura, et al. (2012) . <i>Fisheries Research</i> 115: 115-120.	Canned/fresh/smoked/salty/dried	QIAamp® DNA Mini Kit		QIAGEN GmbH, 40724 Hilden, Germany
Authentication of the most important species of freshwater eels by means of FINS. Lago, et al. (2012) . <i>European Food Research and Technology</i> 234(4): 689-694.	Frozen		Phenol and chloroform protocol *	Rogers, et al. (1988) . Extraction of DNA from plant tissues. In: <i>Plant Molecular Biology Manual</i> . S. Gelvin, R. Schilperoort and D. Verma, Springer Netherlands: 73-83.
Development of real-time PCR assays for the detection of Atlantic cod (<i>Gadus morhua</i>), Atlantic salmon (<i>Salmo salar</i>) and European plaice (<i>Pleuronectes platessa</i>) in complex food samples. Hird, et al. (2012) . <i>Eu. Food Res.Tech</i> 234(1): 127-136.	Fresh/cooked/autoclave		Chloroform protocol	Meyer, et al. (1994) . Detection of pork in heated meat products by the polymerase chain reaction. <i>Journal of AOAC International</i> 77(3): 617-622.
Application of FINS and multiplex PCR for detecting genuine abalone products. Chan, et al. (2012) . <i>Food Control</i> 23(1): 137-142.	Dried/canned		Phenol-chloroform-isoamyl protocol*	Kang, et al. (1998) . A rapid DNA extraction method for RFLP and PCR analysis from a single dry seed. <i>Plant Molecular Biology Reporter</i> 16(1): 90-90.
Detection and quantification of tissue of origin in salmon and veal products using methylation sensitive AFLPs. Rodriguez	Fresh	NucleoSpin® Tissue Kit		MACHEREY-NAGEL GmbH & Co. KG, D-52313 Düren, Germany

Lopez, et al. (2012). <i>Food Chemistry</i> 131(4): 1493-1498.				
Identification of tuna species by a real-time polymerase chain reaction technique Chuang, et al. (2012). <i>Food Chemistry</i> 133(3): 1055-1061.	Fresh/canned samples	DNeasy Blood & Tissue Kit		QIAGEN GmbH, 40724 Hilden, Germany
Molecular identification of fish species from surimi-based products labeled as Alaskan Pollock. Keskin, et al. (2012). <i>Journal of Applied Ichthyology</i> 28(5): 811-814.	Surimi	DNeasy Blood & Tissue Kit		QIAGEN GmbH, 40724 Hilden, Germany
DNA barcoding commercially important fish species of Turkey Keskin, et al. (2013). <i>Molecular Ecology Resources</i> 13(5): 788-797.	Fresh muscle	DNeasy Blood & Tissue Kit		QIAGEN GmbH, 40724 Hilden, Germany
Detection of mislabeled commercial fishery by-products in the Philippines using DNA barcodes and its implications to food traceability and safety Maralit, et al. (2013). <i>Food Control</i> 33(1): 119-125.	Fresh/frozen		Chloroform protocol	Santos, et al. (2010). A pilot study on the genetic variation of Eastern little tuna (<i>Euthynnus affinis</i>) in Southeast Asia. <i>Philippine Journal of Science</i> , 139 (1): 43–50
Ensuring seafood identity: Grouper identification by real-time nucleic acid sequence-based amplification (RT-NASBA) Ulrich, et al. (2013). <i>Food Control</i> 31(2): 337-344.	Fresh/frozen/ ethanol preserved	DNeasy Blood & Tissue Kit		QIAGEN GmbH, 40724 Hilden, Germany
Rapid species identification of fresh and processed scallops by multiplex PCR Marin, et al. (2013). <i>Food Control</i> 32(2): 472-476.	Fresh/canned/ boiled		Phenol-chloroform protocol	Sokolov. (2000). "An improved method for DNA isolation from mucopolysaccharide-rich molluscan tissues." <i>Journal of Molluscan Studies</i> 66: 573-575.
A novel minisequencing test for species identification of salted and dried products derived from species belonging to Gadiformes Dalmasso, et al. (2013). <i>Food Control</i> 34(2): 296-299.	Fresh/salted/ dried samples	DNeasy Blood & Tissue Kit		QIAGEN GmbH, 40724 Hilden, Germany
Commercialization of a critically endangered species (largetooth sawfish, <i>Pristis perotteti</i>) in fish markets of northern Brazil: Authenticity by DNA analysis Melo Palmeira, et al. (2013).	Fresh/salted samples		Phenol-chlorophorm protocol	Sambrook, et al. (2001). <i>Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press. Woodbury, NY, USA</i>

<i>Food Control</i> 34(1): 249-252.				
DNA barcoding for detecting market substitution in salted cod fillets and battered cod chunks Di Pinto, et al. (2013) . <i>Food Chemistry</i> 141(3): 1757-1762.	Salted	DNeasy Blood & Tissue Kit		QIAGEN GmbH, 40724 Hilden, Germany
Development of a rapid genetic technique for the identification of clupeid larvae in the Western English Channel and investigation of mislabeling in processed fish products. Brechon, et al. (2013) . <i>Ices Journal of Marine Science</i> 70(2): 399-407.	Fresh/processed	Wizard kit for adult fish	Chelex resin protocol	Promega Corporation, 2800 Madison, WI 53711 USA ; Estoup, et al. (1996) . Rapid one-tube DNA extraction for reliable PCR detection of fish polymorphic markers and transgenes. <i>Molecular Marine Biology and Biotechnology</i> 5(4): 295-298.
Differentiation of Sparidae species by DNA sequence analysis, PCR-SSCP and IEF of sarcoplasmic proteins. Schiefenhoevel, et al. (2013) . <i>Food Chemistry</i> 138(1): 154-160.	Frozen/processed samples	Chloroform-isopropanol protocol		Rehbein. (2005) . Identification of the fish species of raw or cold-smoked salmon and salmon caviar by single-strand conformation polymorphism (SSCP) analysis. <i>European Food Research and Technology</i> 220(5-6): 625-632.

Table 2SM Level of significance, evaluated using two mixed models with REML estimation of variance, of the effects associated to the factors method (M), species (S), and interaction between method and species (M*S) (model 1), or method (M), category (C), and interaction between method and category (M*C) (model 2) on yield, A260/A280, and A260/A230 for fresh and ethanol preserved samples. *** p<0.001; ** p<0.01; * p<0.05; ns=non significant.

	Fresh			Ethanol		
	Yield	A260/A280	A260/A230	Yield	A260/A280	A260/A230
M	***	***	***	***	***	ns
S	***	***	***	***	***	ns
M*S	***	***	***	***	***	ns
M	***	***	***	***	***	ns
C	**	ns	*	ns	ns	ns
M*C	***	*	***	ns	ns	ns

Table 3SM Level of significance, evaluated using a mixed model with REML estimation of variance, of the effects associated to the factors method (M), initial amount of tissue (W), their interaction (M*W), and category (C) on DNA relative yield, A260/A280, and A260/A230. *** p<0.001; ** p<0.01; * p<0.05.

	Yield	A260/A280	A260/A230
M	***	***	***
W	***	***	*
M*W	***	***	**
S	***	***	***

Table 4SM Level of significance, evaluated using a mixed model with REML estimation of variance, of the effects associated to the factors method (M), species (S), and interaction between method and species (M*S) on the band intensity after conventional PCR of the COI and 16S genes and on the take-off cycle measured by real-time PCR on the gene 16S. *** p<0.001.

	Band intensity		Take-off
	<i>COI</i>	<i>16S</i>	<i>16S</i>
M	***	***	***
S	***	***	***
M*S	***	***	***