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## ΠΡΟΓΡΑΜΜΑ ΔΙΑ ΒΙΟΥ ΜΑΘΗΣΗΣ ΑΕΙ ΓΙΑ ΤΗΝ ΕΠΙΚΑΙΡΟΠΟΙΗΣΗ ΓΝΩΣΕΩΝ ΑΠΟΦΟΙΤΩΝ ΑΕΙ (ΠΕΓΑ)

*«Οι σύγχρονες τεχνικές βιο-ανάλυσης στην υγεία, τη γεωργία, το περιβάλλον και τη διατροφή»*

1 A new set of 16S rRNA universal primers for identification of animal species

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3 Constantina Sarri\*, Costas Stamatis\*, Theologia Sarafidou, Ioanna Galara, Vassilis

4 Godosopoulos, Mathaios Kolovos, Constantina Liakou, Spyros Tastsoglou, Zissis Mamuris

5

6 Laboratory of Genetics, Evolutionary and Comparative Biology, Department of

7 Biochemistry and Biotechnology, University of Thessaly, 26 Ploutonos Street, 41221

8 Larissa, Greece

9

10 Constantina Sarri and Costas Stamatis equally contributed

11 Zissis Mamuris, corresponding author ([zmamur@bio.uth.gr](mailto:zmamur@bio.uth.gr))

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33 **Abstract**

34 In this study, bioinformatics were used to specifically design universal primers within 16S  
35 rRNA gene according to the following criteria: the priming sites needed to be sufficiently  
36 conserved to permit a reliable amplification (pooled samples) and the genetic marker  
37 needed to (a) be sufficiently variable to discriminate among most species and sufficiently  
38 conserved within than between species, (b) be short enough to allow also accurate  
39 amplification from processed samples (food) and non invasive approaches (fur, feathers,  
40 faeces etc) (c) convey sufficient information to assign samples to species and (d) be  
41 amplified under variable lab conditions and protocols. Furthermore, short sequences  
42 allow the accurate massive inter- and intra-species identification of point mutations by the  
43 SSCP technique. The size of the amplified segment ranged from 222 to 252 bp.  
44 Amplification and identification success was 100% with all kinds of tissue tested in both  
45 raw and processed samples in a wide range of species, mammals (n=27), fishes (n=32)  
46 birds (n=19), coleoptera (n=23), reptiles (n=5), crustaceans (n=5) and cephalopods (n=2),  
47 including almost all European mammal and avian game species. In addition, no intra-  
48 specific polymorphism was detected. Finally, gene fragments, homologous to those  
49 amplified by the primers used herein and retrieved from the GenBank for three animal  
50 sets [mammals (n=248), birds (n=231) and fishes (n=644)] showed a particular precise  
51 percentage of correct identifications. Therefore, this short segment of the 16S rRNA  
52 mitochondrial gene could be a good candidate for a rapid, accurate, low-cost and easy-to-  
53 apply and interpret method to identify mammal and avian game species by PCR  
54 amplification and sequencing that can be easily incorporated in integrated conservation  
55 and forensic programmes.

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65 **1. Introduction**

66 The ongoing need for accurate and secure animal identification for taxonomic,  
67 phylogenetic, forensic and conservation purposes together with the advances in  
68 technology and the low costs of DNA sequencing have placed great value on the use of  
69 short DNA sequences. The whole procedure is also well known under the term of DNA  
70 barcoding (Hebert, Cywinska, Ball, & deWaard, 2003; Tautz et al., 2003).

71 An important issue for the identification of species remains the choice of which genes  
72 to use. Because of its rapid pace of sequence changes that regularly results to pronounced  
73 divergences, even between closely related species, mitochondrial DNA (mtDNA) have been  
74 widely used in molecular phylogenetic studies (Brown, George, & Wilson, 1979; Moore,  
75 1995; Johns, & Avise, 1998). However, the fact that different parts of the mtDNA genome  
76 evolve at different rates (Avise, 1986; Roques, Fox, Villasana, & Rico, 2006) makes the  
77 decision of the suitable gene to evaluate the delimitation of species very crucial.

78 Nowadays, the criteria for a marker to reach universal applicability of DNA barcoding,  
79 are well established (Hebert, Cywinska, Ball, & deWaard, 2003). Thus, a genetic marker  
80 needs to (a) be sufficiently variable to discriminate among species, (b) be less variable  
81 within than between species, (c) have priming sites sufficiently conserved to permit a  
82 reliable amplification through different taxa (d) bring in sufficient phylogenetic  
83 information to assign species to major taxa (e) yield repeatable results under variable lab  
84 conditions and protocols, (f) give sequence alignment among distantly related taxa.

85 According to an increasing number of studies during the last decade, the gene region  
86 proposed for the standard barcode in animals is a 658 base pair region in the gene  
87 encoding the mitochondrial cytochrome c oxidase subunit 1 (cox1 or COI) (1 Hebert,  
88 Cywinska, Ball, & deWaard, 2003). This marker served for animal species identification  
89 and for the discovery of new or cryptic species (Hebert et al., 2004). Several studies have  
90 established the resolution power of this approach in several large groups of animals, such  
91 as birds (Hebert, Stoeckle, Zemplak, & Francis, 2004), fishes (Ward et al., 2005), cowries  
92 (Meyer, & Paulay, 2005), spiders (Barrett, & Hebert, 2005), Lepidoptera (Hebert et al.,  
93 2004; Janzen et al., 2005; Hajibabaei et al., 2006a) and reptiles [Nagy, Sonet, Glaw, &  
94 Vences, 2012]. The coordination of the efforts resulted to a comprehensive library of DNA  
95 sequences of thousands of species continuously updated and publicly available  
96 (<http://www.barcodinglife.org>).

97        Apart from COI other mitochondrial markers also have been used either for their  
98 utility in phylogenetics or to complement COI in DNA barcoding. Cytochrome b (cytb) has  
99 been suggested as a marker to determine species boundaries (Helbig, & Seibold, 1999;  
100 Bradley, & Baker, 2001; Lemer et al., 2007). In amphibians and Mollusca 16S ribosomal  
101 RNA gene has been proposed as DNA barcoding marker to complement COI (Vences et al.,  
102 2005; Feng, Li, Kong, & Zheng, 2011).

103        Beside taxonomists, DNA barcoding can be potentially useful for scientists from other  
104 fields such as ecology, forensics, biotechnology, food industries, animal diet, food quality  
105 etc (Valentini, Pompanon, & Taberlet, 2009). Furthermore, the identification of animal  
106 species in food is becoming a very important issue for the assessment of food composition  
107 and the provision of proper consumer information. However, in many of these samples the  
108 quality of DNA could be seriously affected and DNA degradation very often prevents PCR  
109 amplification of fragments longer than 250 bp (Goldstein, & Desalle, 2003; Hajibabaei et  
110 al., 2006b). Thus, conventional DNA barcoding could be problematic. Therefore, a genetic  
111 marker should to be short enough to allow also accurate amplification from processed  
112 samples (food), non invasive approaches (fur, feathers, faeces, saliva etc) and DNA from  
113 archive specimens. Furthermore, short sequences could allow the accurate massive inter-  
114 and intra-specific identification of point mutations by the SSCP technique, avoiding  
115 repetitive DNA sequencing of the analysed specimens. To overcome these problems  
116 Meusnier et al. 2008 (Meusnier et al., 2008) developed a universal set of primers,  
117 amplifying a 130 bp fragment of the COI gene within the barcoding region.

118        In this study, bioinformatics were used to specifically design universal primers within  
119 16S rRNA gene according to the above mentioned criteria, in order to create a “mini-  
120 barcode” marker. The designed primers were then tested with a battery of experimental  
121 procedures to verify if they met the assigned criteria.

122

## 123 **2. Materials and Methods**

124

125        Bioinformatic methods were used, based on sequence analysis of complete  
126 mitochondrial sequences of 150 species from very distant taxa retrieved from the  
127 GenBank, to specifically design a set of universal primers within 16S rRNA gene. The  
128 purpose was to define, after PCR amplification, a short segment variable enough to  
129 discriminate among species but with sufficiently conserved priming sites to permit a

130 reliable amplification throughout very distant animal taxa. Experimental procedures  
131 indicated that the following set of primers was the appropriate one: Forward: 5' -  
132 AYAAGACGAGAAGACCC - 3' and Reverse: 5' - GATTGCGCTGTTATTCC - 3'.

133 To verify the power of the primers even among very distantly related species, as well  
134 as their amplification ability in samples collected with non invasive approaches, tissue  
135 samples (muscle, blood, hair, sperm, faeces, saliva, fur, feathers etc) from 110 well defined  
136 animal species from four phylum: Chordata, Mollusca, Arthropoda [mammals (n=27),  
137 avian (n=19), (including almost all European mammal and avian game species) fishes  
138 (n=30) coleoptera (n=22), reptiles (n=5), crustaceans (n=5) and cephalopods (n=2)]  
139 (Table 1) were collected and appropriately stored till further treatment. DNA isolation  
140 from all tissues was performed using PureLink Genomic DNA Mini Kit (Invitrogen,  
141 Carlsbad, CA 92008, USA) according to the manufacturer's instructions with slight  
142 modifications regarding tissue and animal origin. PCR reactions (50  $\mu$ L) contained 200 ng  
143 DNA, 5  $\mu$ l of 10 x Taq buffer, 2 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP, 50 pmoles of each primer  
144 and 1 U Taq of proofreading polymerase (Platinum® Taq DNA Polymerase High  
145 FidelityInvitrogen, Carlsbad, USA). The optimal annealing temperature using a gradient  
146 thermocycler was found to be 53°C. The cycling conditions consisted of an initial  
147 denaturation at 95°C for 4 min followed by 35 cycles of denaturation at 95°C for 40 sec,  
148 annealing at 53°C for 40sec and extension at 72°C for 40sec, with a final extension at 72°C  
149 for 10min. To eliminate possible PCR artefacts leading to erroneous nucleotide  
150 substitutions for each specimen, except of the use of a proofreading polymerase, three PCR  
151 replications were performed. Amplified DNA segments were sequenced directly and bi-  
152 directionally by Macrogen Inc. Nucleotide sequences were aligned using ClustalX (Larkin  
153 et al., 2007).

154 When available, up to 30 specimens of each species were screened for polymorphisms  
155 within this fragment of the 16S rRNA gene using the Single-Strand Conformation  
156 Polymorphism (SSCP) method. This method allows the detection of single base  
157 polymorphisms in short DNA stretches due to mobility differences of single-stranded DNA  
158 fragments during electrophoresis in polyacrylamide gels (Orita et al., 1989). Preliminary  
159 SSCP tests were performed with samples known to carry different sequences. More  
160 specifically, 5  $\mu$ l of the PCR products were mixed with 10  $\mu$ l of loading dye (95% v / v  
161 formamide, 10 mM NaOH, 0.05% w/ v bromophenol blue, 0.05% w/ v xylene cyanol),

162 denatured at 95°C for 6 min, cooled on ice and loaded onto a 10% polyacrylamide gel. The  
163 samples were electrophoresed in 0.5X TBE buffer at 220 V for 18–20 h at 4°C. Routine  
164 SSCP separations always included previously typed samples that served as standards to  
165 ensure correct genotype scoring. The resulting bands were visualized by silver staining,  
166 according to Sambrook, Fritsch, & Maniatis, (1989). PCR products that showed the same  
167 SSCP pattern were grouped and representative samples from each profile were sequenced  
168 directly and bi-directionally by MacroGen Inc. In total 21 species were screened for intra-  
169 species polymorphism [*Homo sapiens*, *Lepus europaeus*, *Lepus timidus*, *Bos taurus*, *Ovis*  
170 *aries*, *Sus scrofa* (both domestic and wild boar), *Equus caballus*, *Anas platyrhynchos*, *Anser*  
171 *anser*, *Tadorna tadorna*, *Alectoris graeca*, *Alectoris chukar*, *Phasianus colchicus*, *Turdus*  
172 *merula*, *Coturnix coturnix*, *Dicentrarchus labrax*, *Trachurus trachurus*, *Sparus aurata*,  
173 *Pagellus erythrinus*, *Nephrops norvegicus*, *Helix aspersa*]

174 To check if the amplified segments convey sufficient phylogenetic information to  
175 assign samples to species, all sequences obtained from the 110 animal species were  
176 compared against available sequences in Genbank, using BLAST scores and constructing  
177 neighbour-joining trees.

178 To verify if the primers also allow the accurate amplification from processed samples  
179 we analyzed 45 food products (Table 2) of some of the above species either as milks and  
180 cheeses or after subjection to various cooking methods or technological processes  
181 inherent to the food sector such as roasted, roasted roll, fried, boiled, smoked, canned and  
182 industrially processed meat, poultry and fish. Each sample was prepared and analyzed in  
183 triplicate. All solid samples were chopped with sterile surgical blade and subsequent DNA  
184 extraction was performed following the protocol described in Stamoulis et al 2010  
185 (Stamoulis, Stamatis, Sarafidou, & Mamuris, 2010). PCR reactions and cycling conditions  
186 were the same as those used for the raw meat, fish and poultry (Stamoulis, Stamatis,  
187 Sarafidou, & Mamuris, 2010).

188 To test the capacity of primers to reliably amplify species' DNA in pooled samples  
189 without false negatives, 92 artificial samples were prepared and analysed, after grinding  
190 an admixture of an increasing number (up to five) of different species chicken (*Gallus*  
191 *gallus domesticus*), turkey (*Meleagris gallopavo*), sheep (*Ovis aries*), pig (*Sus scrofa*  
192 *domesticus*), beef (*Bos Taurus*). Each admixture contained a combination of different  
193 species in different quantities. The smallest quantity for a species was 1% and 99% for the

194 other species; 49.5% for each one of the two other species; 33% for each one of the three  
195 other species;  $\approx 25\%$  for each one of the four other species. Additionally, several other  
196 combinations were tested, e.g. 2% for the first species, 18% for the second one, 30% for  
197 the third one and 50% for the fourth one. After DNA extraction and PCR amplification the  
198 SSCP method was applied (see above).

199 To test for universality of primers and cycling conditions, a number of randomly  
200 chosen samples from each of the above sets were analysed in parallel experiments with  
201 three different thermocyclers [Applied Biosystems (Veriti 96 Well Thermal Cycler), Labnet  
202 (MULTI GENE II), Eppendorf (Mastercycler ep534X)] and different biochemical products,  
203 but with the application of the same amplification conditions.

204 Finally to verify if priming sites were sufficiently variable to discriminate among most  
205 species and sufficiently conserved within than between species, sequences limited to the  
206 DNA segment studied from taxa of three animal sets [mammals (n=248), birds (n=229)  
207 and fishes (n=644)] (supplementary material) were retrieved from the GenBank aligned  
208 with CLUSTALX (Larkin et al., 2007) and checked for similarities or dissimilarities  
209 between species and/or between specimens within species when available. The ability of  
210 *16S* in assigning taxa to major clades was tested based on gene fragments homologous to  
211 those amplified by the primers used herein. PAUP\* (Swofford, 1998) was used with the  
212 neighbor-joining algorithm for a fast identification of taxa.

213 Additionally, to complement our results we used an identification approach based on  
214 direct sequence comparison, using TaxonDNA/SpeciesIdentifier 1.7.7-dev3 (Meier,  
215 Shiyang, Vaidya, & Ng, 2006). The *16S* rRNA gene sequences were evaluated according to  
216 the following criteria: "Best Close Match" and "Cluster". These methods are based on leave-  
217 one-out procedures, which consist of removing each individual in turn from the data set.  
218 The assignment methods are then tested for these individuals, considering the rest of the  
219 data set as the reference sample. The performance of each method is evaluated as the rate  
220 at which queried individuals are successfully assigned to the species or subspecies. "Best  
221 Close Match" identifies the best barcode match of a sequence and assigns a species name  
222 to a query only if the barcode is sufficiently similar. The clustering method clusters  
223 sequences into profiles in which all sequences are less than a threshold value from at least  
224 one other sequence in the profile but can be more than the threshold value from other  
225 sequences in the profile (Meier, Shiyang, Vaidya, & Ng, 2006). For this study the threshold

226 for “Best Close Much” was computed from pairwise summary and for “Cluster” was set at  
227 1%. All the other algorithms and parameters are incorporated into the software.

228

### 229 **3. Results and Discussion**

#### 230 *3.1. PCR product description*

231 *16S rRNA* gene has a length of 1557 bp in *H. sapiens* (situated between 1672-3229 bp  
232 of human’s mitochondrial genome). The *16S rRNA* segment analyzed here had a length of  
233 202 bp (*Homo sapiens*) situated between 2730-2932 bp of mitochondrial genome, near the  
234 3’ end of the gene. The pair of primers designed successfully amplified the *16S rRNA*  
235 segment from all tissues (muscle, blood, hair, sperm, faeces, saliva, fur, feathers) of all  
236 species analysed during this study. All species showed different sequences (Accession  
237 number KC984203 - KC984280) (Fig. 1) and in some cases this pair of primers  
238 distinguished even between breeds (horse) and different geographic populations (brown  
239 hare) (Fig. 2). Comparison of the obtained sequences against available sequences in  
240 Genbank and the construction of neighbour-joining trees (figure not shown) showed that  
241 the amplified segments convey sufficient phylogenetic information to assign samples to  
242 species.

243 Applying the same amplification conditions, the use of three different thermocyclers  
244 [Applied Biosystems (Veriti 96 Well Thermal Cycler), Labnet (MULTI GENE II), Eppendorf  
245 (Mastercycler ep534X)] and of different biochemical products produced identical results  
246 for the randomly chosen samples from the different sets of species and products analysed  
247 here.

248 *16S rRNA* gene, compared with protein coding genes, for which its third-position  
249 nucleotides show a high incidence of base substitutions, shows a three times lower rate of  
250 molecular evolution (Knowlton, & Weigt, 1998). Although the mitochondrial *16S* gene is  
251 highly conserved, mutations are common in some variable regions, corresponding to loops  
252 in the ribosomal RNA structure. Our results indicates that *16S* is sufficiently variable to  
253 unambiguously identify most species. As previously reported (Hebert, Cywinska, Ball, &  
254 deWaard, 2003; Vences et al., 2005) also in our study, PCR products from evolutionary  
255 distant taxa, showed a considerable length polymorphism, especially between the three  
256 major groups, ranging from 201 to 211 bp in mammals, from 213 to 217 bp in avian and  
257 from 225 to 249 in fishes. As usual, this polymorphism in nonpeptide-coding DNA, such as

258 the 16S rRNA gene, is due to a high number of insertions and deletions (indels). This  
259 length polymorphism occurred in different spots and mainly within a region situated 40  
260 bp after the middle of the amplified segment and appeared in direct relation with the  
261 taxonomic status of each species. Closely related species showed none or very low length  
262 polymorphism. The presence of indels poses for alignment difficulties and suggests the  
263 possibility of missing positional homology between parts of the alignment between  
264 distantly related taxa. There is a recent debate on the utility of the indels in phylogeny and  
265 of keeping or removing these problematic regions from the alignment in order to avoid  
266 biasing the resulting trees (Lutzoni, Wagner, Reeb, & Zoller, 2000). Nonetheless, there are  
267 indications that a large proportion of genetic variation between closely related individuals  
268 has to be attributed to indels, (Britten, Rowen, Williams, & Cameron, 2003) and therefore  
269 they should deliver important information about taxon separation.

270

### 271 *3.2. Processed samples and meat admixtures*

272 The designed primers successfully identified all kind of animal ingredients contained  
273 in processed products and described as food components in the products' labels (Table 2).  
274 DNA by its nature is a quite heat-tolerant molecule. Therefore it has a clear advantage  
275 compared with proteins in the molecular identification of processed food. During this  
276 study we analysed food products of different species either as milks and cheeses or after  
277 subjected to various cooking methods or technological processes inherent to the meat  
278 sector such as roasted, roasted roll, fried, boiled, smoked, canned and industrially  
279 processed meat, poultry and fish. Several studies already have pointed out the need of  
280 targeting small DNA fragments for PCR amplification of processed products (Stamoulis,  
281 Stamatis, Sarafidou, & Mamuris, 2010; Arslan, Ilhak, & Calicioglu, 2006). Conventional  
282 cooking (boiling/frying/baking) and industrial methods affected the quality of extracted  
283 DNA but they did not affect the PCR amplification procedure since PCR products were  
284 identical to those from the corresponding fresh samples.

285 Analyses of the admixtures of the five meat species in different quantities showed that  
286 the designed set of primers together with the SSCP method were capable of fully  
287 discriminate up to four species within an admixture regardless of the quantity of the  
288 species' meat (fig. 3 a,b,c). That was true even in highly asymmetric mixtures where the  
289 participation of the species in the mixture was the minimum (1%). This proves the  
290 capacity of primers to reliably amplify species' DNA in pooled samples without false

291 negatives. However, the addition of a fifth species blurred the image resulting to lower  
292 resolution after SSCP analysis, even for cases where all species participated equally (fig  
293 3d).

294 The usage of 16S rDNA universal primers facilitates the accurate and/or simultaneous  
295 identification of animal species (a) in products in which the species origin is not always  
296 obvious (packaging of meat pieces from various mammal, avian, fish, shellfish, game  
297 species) (b) in meat mixtures of processed foods after either conventional cooking  
298 (boiling/frying/baking) or industrial methods (Table 2). This set of primers reduces the  
299 time and cost of the procedure in comparison to approaches where species-specific  
300 primers are applied and multiple PCR reactions are performed for the species recognition.  
301 Furthermore, this method is definitely much simpler and economical relatively to multiple  
302 digests or sequencing, without interfering with the resolution of the analysis.

303 Socio-religious reasons (e.g. vegetarianism, absence of pork for Jews and Muslims),  
304 health concerns (allergies) or economic reasons (replacement with low cost ingredients)  
305 have provoked a demand for transparency in the food industry and the need for  
306 appropriate detection methods that allow identification of different species in meat foods  
307 and of different ingredients in processed food. A considerable proportion of accidental  
308 exposures to allergenic foods, apart from failure to read labels and ignoring precautionary  
309 statements, are also attributed to inappropriate labelling (Sheth et al., 2010). Finally,  
310 recently, the unquestioned qualities of the game meat such as texture, flavour, low fat and  
311 cholesterol content as well as its lack of anabolic steroids or other drugs (Fajardo et al.,  
312 2006) gained the increasing preference of the consumers, inducing, however, fraud, such  
313 as mislabelling or selling less valuable meat as meat from more appreciated species (La  
314 Neve, Civera, Mucci, & Bottero, 2008). Therefore, clear and consistent labelling of food  
315 ingredients is necessary for the identification of potential mislabelling in specific sectors,  
316 whereas the improvement of existing laws with new amendments will also improve  
317 consumer confidence.

### 318 *3.3. Assignment methods*

319 Gene fragments, homologous to those amplified by the primers used herein, were  
320 retrieved from the GenBank for three animal sets [mammals (n=248), birds (n=231) and  
321 fishes (n=644)]. Mean sequence divergences within each group were 18.6% for mammals,  
322 13.4% for birds and 24.7% for fishes. Neighbor-joining algorithm and the trees produced

323 (not shown) showed a great ability of 16S in identifying different species. The percentage  
324 of correct identifications, using the assignment method of TaxonDNA/SpeciesIdentifier  
325 1.7.7-dev3, was particular precise: 97.5% for mammals, 97.1% for birds and 96.6% for  
326 fishes. Ambiguous identifications were detected in all three groups but at low frequencies  
327 (2.5% for mammals, 2.9% for birds and 3.4% for fishes). No incorrect identifications were  
328 detected for any group. When specimens were available the level of polymorphism within  
329 species was checked, using the neighbor-joining algorithm. Of the 34 species, with a  
330 number of specimens ranging from n=5 to n=36, analysed from the three groups, 27  
331 (79.4%) were monomorphic, four (11.8%) were polymorphic with two subgroups and  
332 three (8.8%) were polymorphic with three subgroups. However, as it is very difficult to  
333 correctly assess the geographic origin of the specimens within the species analysed it is  
334 probable that the observed monomorphism is due to geographically closely related  
335 specimens and conversely that the observed polymorphism is due to geographically  
336 distant groups.

337

#### 338 **4. Conclusion**

339 To conclude, this short segment of the 16S rRNA mitochondrial gene could be a very  
340 good candidate for a rapid, accurate, low-cost and easy-to-apply and interpret method to  
341 identify animal species by PCR amplification that can be easily incorporated in integrated  
342 conservation and forensic programmes. The ability of the designed pair of primers to  
343 identify animal species through non invasive approaches by examining fur, feathers,  
344 faeces, saliva etc, could also be very helpful in various ecological studies. The applicability  
345 of the primers to identify admixtures of different meats was shown during a routine  
346 survey of processed meat products from the local market. The ability to molecularly  
347 distinguish different species is of great commercial importance and prevents food  
348 mislabelling and wrong description, particularly if the food has been processed removing  
349 from all other methods the ability to distinguish one ingredient from another.

350

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359

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481 **Legends**

482 Fig. 1 UPGMA dendrograms that show different sequences for all the species in all groups  
483 after amplification of DNA with the set of primers of 16S rRNA (a) mammals, (b) fishes, (c)  
484 birds, (d) crustaceans, (e) reptiles, (f) coleoptera

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486 Fig. 2 SSCP profiles showing (a) three different electrophoretic profiles for horse breeds  
487 and (b) two different profiles between geographic populations of brown hare (*Lepus*  
488 *europaeus*)

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490 Fig. 3 SSCP profiles after PCR amplifications of the admixtures of the five meat species in  
491 different quantities (a) 1. Admixture of chicken-turkey, 2. Chicken, 3. Turkey. (b) 1. Pork, 2.  
492 Admixture of pork- chicken-turkey, 3. Chicken, 4. Turkey (c) 1. Beef, 2. Pork, 3. Admixture  
493 of beef-pork- chicken-turkey, 4. Chicken, 5. Turkey (d) 1. Chicken, 2. Turkey, 3. Admixture  
494 of chicken-turkey-sheep-beef-pork, 4. Sheep, 5. Beef, 6. Pork

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512 Table 1 List of the species analysed during this study for PCR DNA amplification. Numbers  
 513 of specimens for each group are indicated in parentheses (n).

<b>Mammals (n=27)</b>	<b>Fishes (n=30)</b>	<b>Aves (n=19)</b>	<b>Reptiles (n=5)</b>	<b>Coleoptera (n=22)</b>
<i>Bos Taurus</i>	<i>Betta splendens</i>	<i>Alectoris chukar</i>	<i>Hemidactylus turcicus</i>	<i>Haplidia transversa</i>
<i>Bubalus bubalis</i>	<i>Boops boops</i>	<i>Alectoris graeca</i>	<i>Hierophis gemonensis</i>	<i>Leptura maculata</i>
<i>Canis lupus familiaris</i>	<i>Carassius auratus</i>	<i>Anas crecca</i>	<i>Lacerta viridis</i>	<i>Macraspis tristis</i>
<i>Capra hircus</i>	<i>Dicentrarchus labrax</i>	<i>Anas penelope</i>	<i>Platyceps najadum</i>	<i>Melolontha hippocastani</i>
<i>Capreolus capreolus</i>	<i>Engraulis encrasicolus</i>	<i>Anas platyrhynchos</i>	<i>Typhlops vermicularis</i>	<i>Melolontha melolontha</i>
<i>Equus caballus</i>	<i>Helicolenus dactylopterus</i>	<i>Anser anser</i>		<i>Monochamus sutor</i>
<i>Erinaceus europaeus</i>	<i>Katsuwonus pelamis</i>	<i>Columba livia</i>		<i>Morimus asper</i>
<i>Felis silvestris</i>	<i>Ladigesocypris ghigii</i>	<i>Columba palumbus</i>	<b>Crustaceans (n=5)</b>	<i>Morimus funereus</i>
<i>Homo sapiens</i>	<i>Limanda aspera</i>	<i>Coturnix coturnix</i>	<i>Callinectes sapidus</i>	<i>Neodorcadion sp.</i>
<i>Lepus brachyurus</i>	<i>Lophius budegassa</i>	<i>Coturnix japonica</i>	<i>Squilla mantis</i>	<i>Niphona grisea</i>
<i>Lepus capensis</i>	<i>Merluccius hubbsi</i>	<i>Gallinago gallinago</i>	<i>Astacus astacus</i>	<i>Oberea bipunctata</i>
<i>Lepus castroviejo</i>	<i>Merluccius merluccius</i>	<i>Gallus gallus</i>	<i>Nephrops norvegicus</i>	<i>Obezema pupillata</i>
<i>Lepus europaeus</i>	<i>Micromesistius poutassou</i>	<i>Meleagris gallopavo</i>	<i>Homarus gammarus</i>	<i>Oryctes nasicornis</i>
<i>Lepus granatensis</i>	<i>Mullus barbatus</i>	<i>Passer montanus</i>		<i>Parmena sp.</i>
<i>Lepus mediterraneus</i>	<i>Mullus surmuletus</i>	<i>Phasianus colchicus</i>	<b>Cephalopods (n=2)</b>	<i>Pedostrangalia verticalis</i>
<i>Lepus saxatilis</i>	<i>Oblada melanura</i>	<i>Scolopax rusticola</i>	<i>Sepia officinalis</i>	<i>Philleurus deshave</i>
<i>Lepus timidus</i>	<i>Pagellus erythrinus</i>	<i>Streptopelia turtur</i>	<i>Loligo vulgaris</i>	<i>Phytoecia nigricornis</i>
<i>Lepus victoriae</i>	<i>Phycis phycis</i>	<i>Tadorna tadorna</i>		<i>Plagionotus arcuatus</i>
<i>Martes martes</i>	<i>Prionace glauca</i>	<i>Turdus merula</i>		<i>Rhizotrogus sp.</i>
<i>Mus musculus</i>	<i>Raja miraletus</i>			<i>Saperda scalaris</i>
<i>Mustela nivalis</i>	<i>Salmo salar</i>			<i>Scarabaeus sp.</i>
<i>Oryctolagus cuniculus</i>	<i>Salmo trutta</i>			<i>Vadonia imitatrix</i>
<i>Ovis aries</i>	<i>Sardinella aurita</i>			
<i>Rupicapra rupicapra</i>	<i>Scomber scombrus</i>			
<i>Sus scrofa</i>	<i>Sebastes viviparous</i>			
<i>Ursus arctos</i>	<i>Sparus auratus</i>			
<i>Vulpes vulpes</i>	<i>Spicara smaris</i>			
	<i>Trachurus trachurus</i>			
	<i>Trigla lucerna</i>			
	<i>Zeus faber</i>			

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530 Table 2 List of processed samples of food products analysed in this study with the result of  
 531 the analysis. Sample constitution marked on the label of the food product is given in  
 532 parentheses.  
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<b>Food for animals</b>		
	<b>Product and composition stated</b>	<b>Results of Analysis</b>
<b>1</b>	Croquette (beef)	Beef
<b>2</b>	Croquette (poultry)	Chicken
<b>3</b>	Pâté (chicken)	Chicken
<b>4</b>	Beef	Beef
<b>5</b>	Chicken with vegetables	Chicken
<b>6</b>	Premium croquettes chunks (beef)	Beef
<b>7</b>	Meat	Beef
<b>Packaged yellow cheeses</b>		
<b>8</b>	Gouda (bovine)	bovine
<b>9</b>	Gouda (bovine)	bovine
<b>10</b>	Emmedal (bovine)	bovine
<b>11</b>	Kaser (bovine)	bovine
<b>12</b>	Edam (bovine)	bovine
<b>13</b>	Gruyere from Crete (sheep)	sheep
<b>14</b>	Gruyere from Mytilini (sheep)	sheep
<b>15</b>	Provolone Dolce (bovine)	bovine
<b>16</b>	Mozzarella from Italy (bovine)	bovine
<b>17</b>	Mozzarella from Denmark (bovine)	bovine
<b>18</b>	Kazer (sheep)	sheep
<b>Packaged white cheeses</b>		
<b>19</b>	Skim-milk cheese (sheep)	Sheep
<b>20</b>	White cheese (Bovine)	Bovine
<b>21</b>	White cheese (Bovine)	Bovine
<b>22</b>	White cheese (sheep, goat)	Sheep, goat
<b>23</b>	Cream cheese from Serifos (sheep, goat)	Sheep, goat
<b>24</b>	Feta (sheep, goat)	Sheep, goat
<b>25</b>	Feta (sheep, goat)	Sheep, goat
<b>26</b>	Feta (sheep, goat)	Sheep, goat
<b>Processed meats</b>		
<b>27</b>	Traditional sausage (pork)	Pork
<b>28</b>	Sausage (pork, beef, sheep)	Pork, beef, sheep
<b>29</b>	Traditional Italian prosciutto (pork)	Pork
<b>30</b>	Salami (pork, beef, sheep)	Pork, beef, sheep
<b>31</b>	Traditional sausage (pork)	Pork
<b>32</b>	Salami (pork, beef)	Pork, beef
<b>33</b>	Salami (pork)	Pork
<b>34</b>	Frankfurt sausage (chicken, turkey, pork)	Chicken, turkey, pork
<b>35</b>	Cocktail sausages (pork, turkey)	Pork, turkey
<b>36</b>	Liversausage (pork)	Pork
<b>37</b>	Bacon (pork)	Pork
<b>38</b>	Smoked bacon (pork)	Pork
<b>Frozen fish fillet</b>		
<b>39</b>	Breaded fillet (cod)	<i>Theragra chalcogramma</i>
<b>40</b>	Fish fillet ( <i>Limanda aspera</i> )	<i>Limanda aspera</i>
<b>41</b>	Fish fillet ( <i>Theragra chalcogramma</i> )	<i>Theragra chalcogramma</i>
<b>42</b>	Fish fillet ( <i>Theragra chalcogramma</i> )	<i>Theragra chalcogramma</i>
<b>43</b>	Fish fillet from Island (cod)	<i>Gadus morhua</i>
<b>44</b>	Breaded fillet ( <i>Theragra chalcogramma</i> )	<i>Theragra chalcogramma</i>
<b>45</b>	Taramas fillet (cod)	<i>Gadus morhua</i>



Figure 1

Figure 1

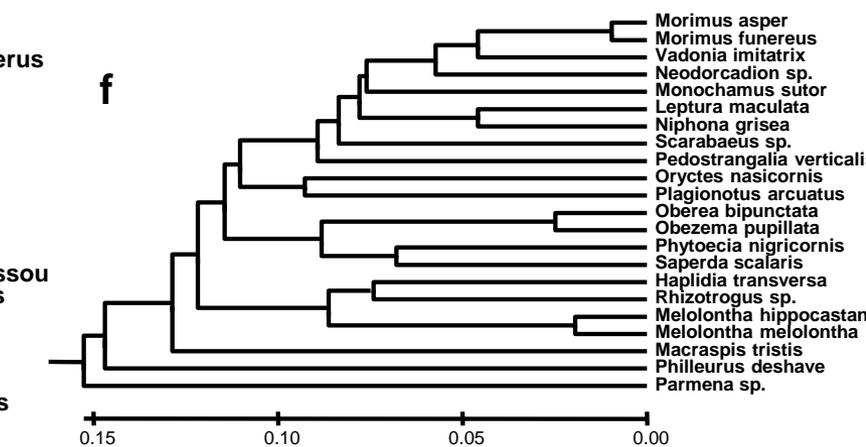
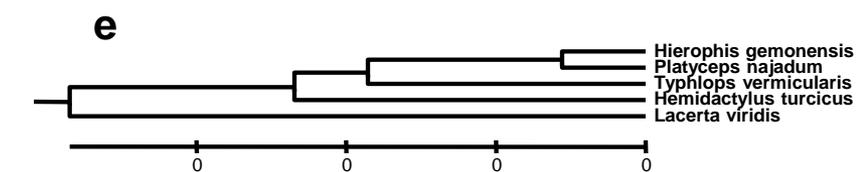
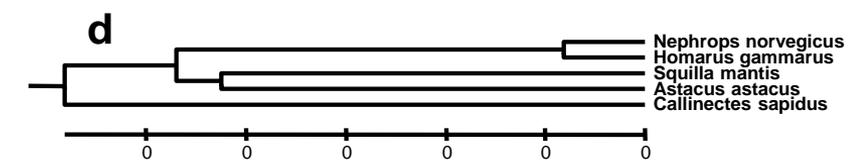
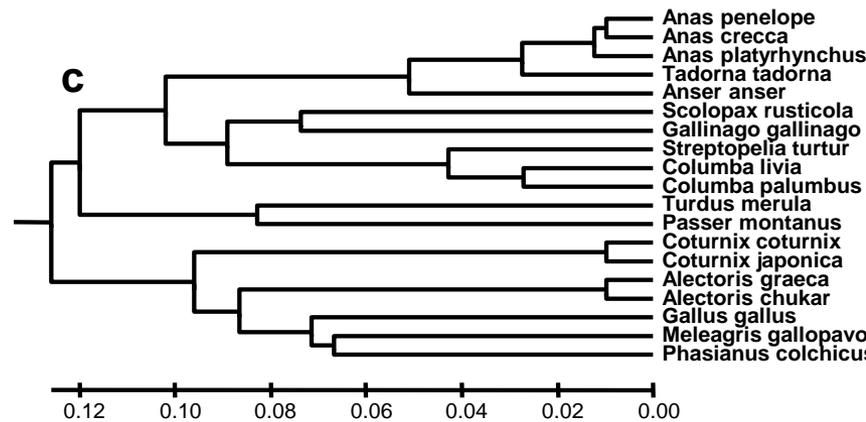
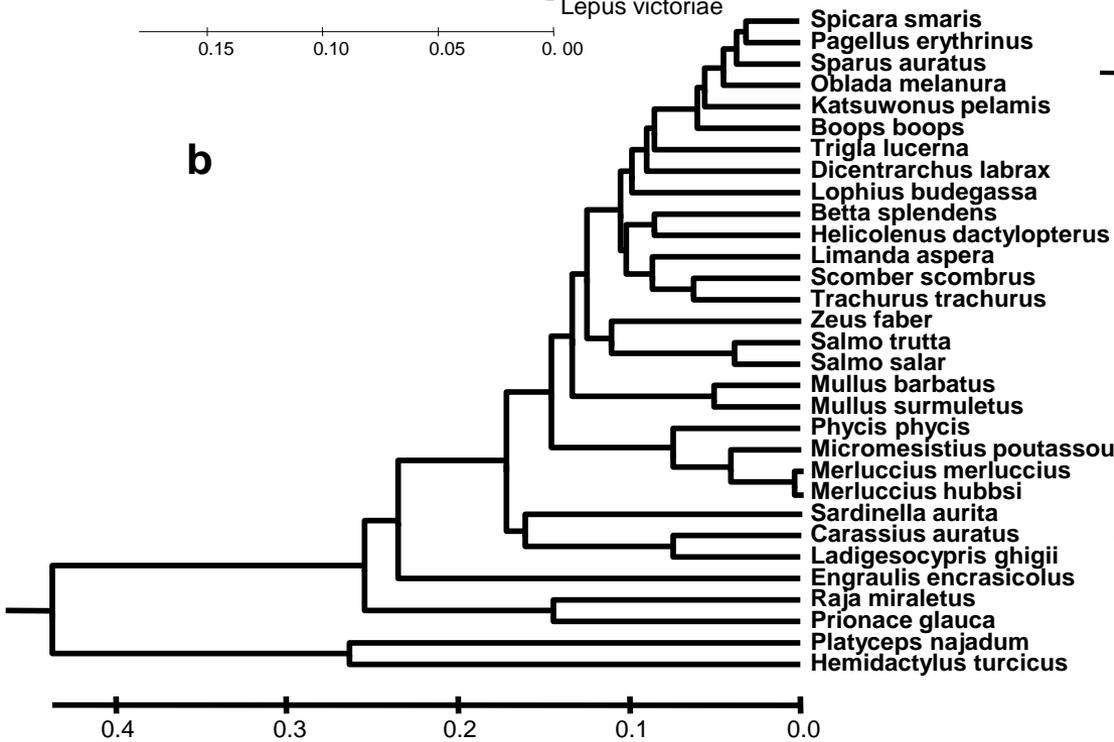
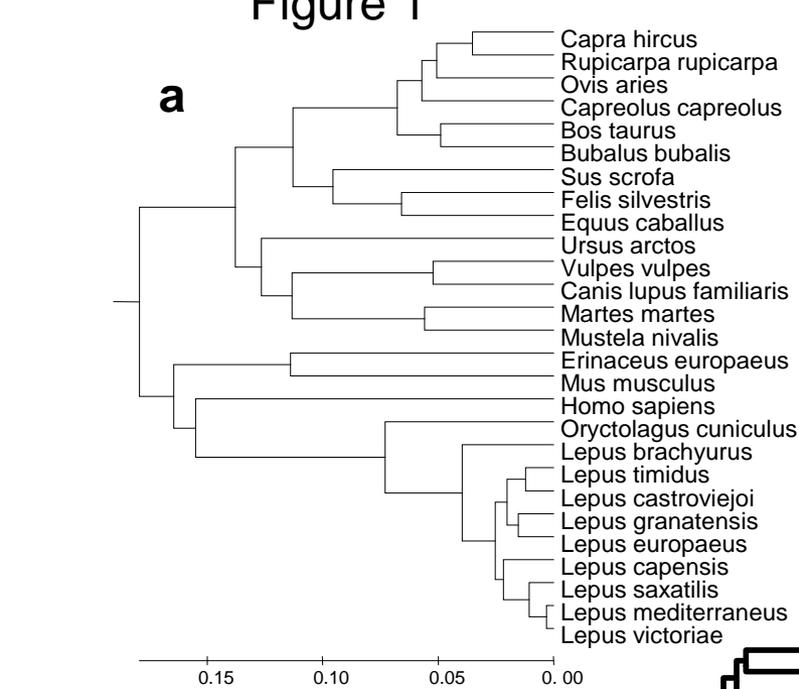


Figure 2

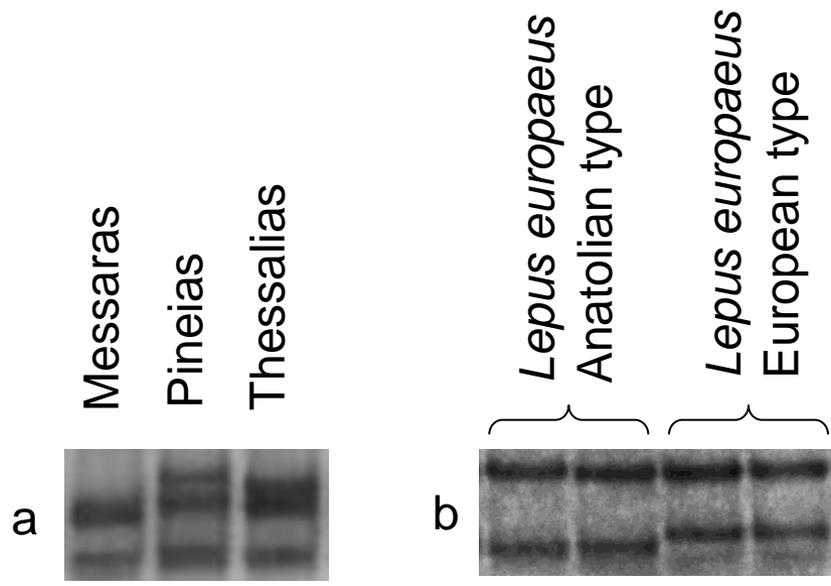


Figure 3

Figure 3

