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»

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

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Abstract

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

1. Introduction

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

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

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

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

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

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

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

2. Materials and Methods

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

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

To verify the power of the primers even among very distantly related species, as well as their amplification ability in samples collected with non invasive approaches, tissue samples (muscle, blood, hair, sperm, faeces, saliva, fur, feathers etc) from 110 well defined animal species from four phylum: Chordata, Mollusca, Arthropoda [mammals (n=27), avian (n=19), (including almost all European mammal and avian game species) fishes (n=30) coleoptera (n=22), reptiles (n=5), crustaceans (n=5) and cephalopods (n=2)] (Table 1) were collected and appropriately stored till further treatment. DNA isolation from all tissues was performed using PureLink Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA 92008, USA) according to the manufacturer's instructions with slight modifications regarding tissue and animal origin. PCR reactions (50 μL) contained 200 ng DNA, 5 μl of 10 x Taq buffer, 2 mM MgCl2, 0.2 mM of each dNTP, 50 pmoles of each primer and 1 U Taq of proofreading polymerase (Platinum® Taq DNA Polymerase High FidelityInvitrogen, Carlsbad, USA). The optimal annealing temperature using a gradient thermocycler was found to be 53°C. The cycling conditions consisted of an initial denaturation at 95°C for 4 min followed by 35 cycles of denaturation at 95°C for 40 sec, annealing at 53°C for 40sec and extension at 72°C for 40sec, with a final extension at 72°C for 10min. To eliminate possible PCR artefacts leading to erroneous nucleotide substitutions for each specimen, except of the use of a proofreading polymerase, three PCR replications were performed. Amplified DNA segments were sequenced directly and bi-directionally by Macrogen Inc. Nucleotide sequences were aligned using ClustalX (Larkin et al., 2007).

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

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

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

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

To test the capacity of primers to reliably amplify species' DNA in pooled samples without false negatives, 92 artificially samples were prepared and analysed, after grinding an admixture of an increasing number (up to five) of different species chicken (*Gallus gallus domesticus*), turkey (*Meleagris gallopavo*), sheep (*Ovis aries*), pig (*Sus scrofa domesticus*), beef (*Bos Taurus*). Each admixture contained a combination of different 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; ≈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

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

3. Results and Discussion

3.1. PCR product description

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

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

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

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

3.2. Processed samples and meat admixtures

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

Analyses of the admixtures of the five meat species in different quantities showed that the designed set of primers together with the SSCP method were capable of fully discriminate up to four species within an admixture regardless of the quantity of the species' meat (fig. 3 a,b,c). That was true even in highly asymmetric mixtures where the participation of the species in the mixture was the minimum (1%). This proves the 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

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

4. Conclusion

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

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Legends

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

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

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

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).

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

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

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Figure 1

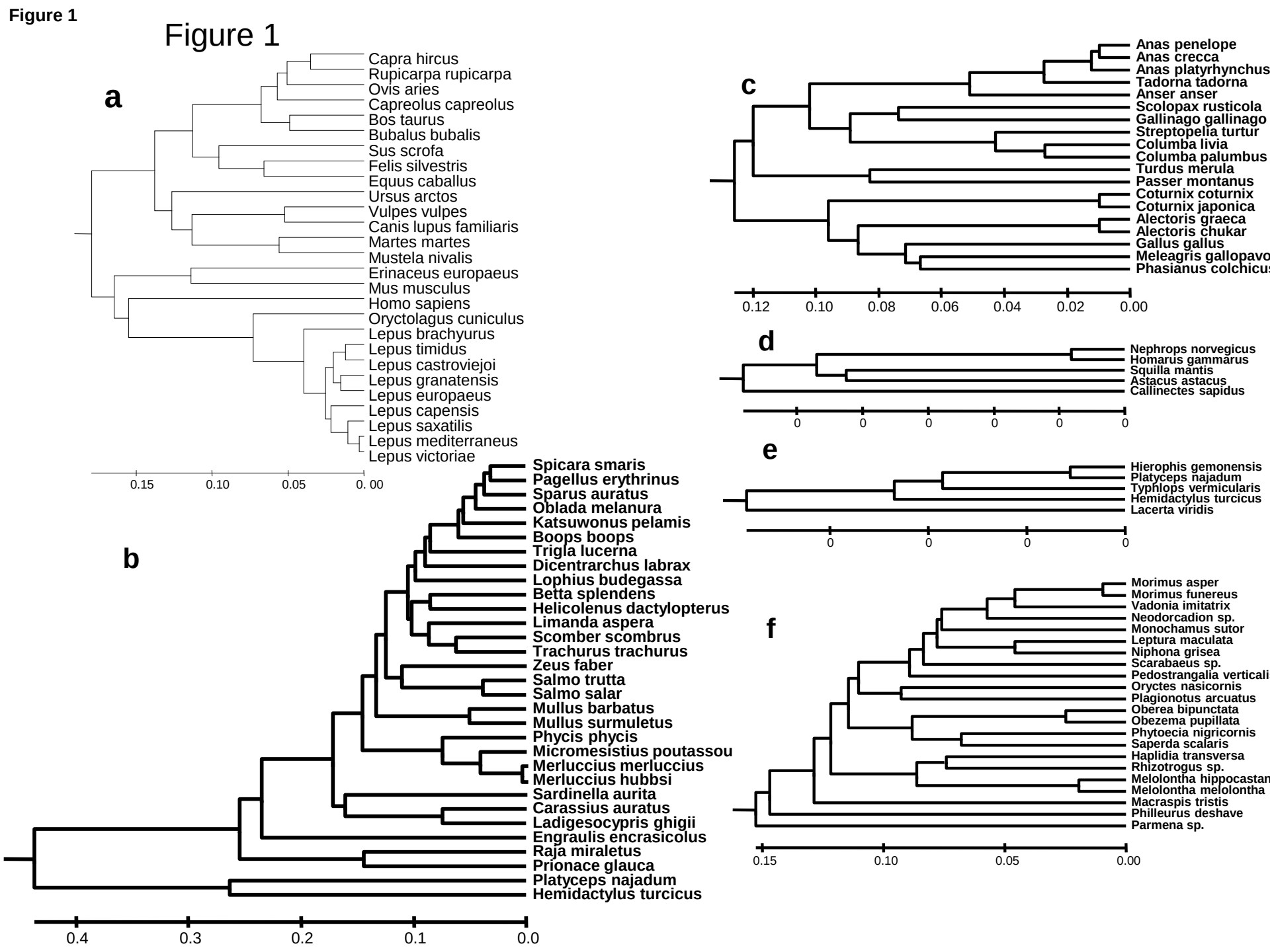
Figure 1
a
Capra hircus
Rupicarpa rupicarpa
Ovis aries
Capreolus capreolus
Bos taurus
Bubalus bubalis
Sus scrofa
Felis silvestris
Equus caballus
Ursus arctos
Vulpes vulpes
Canis lupus familiaris
Martes martes
Mustela nivalis
Erinaceus europaeus
Mus musculus
Homo sapiens
Oryctolagus cuniculus
Lepus brachyurus
Lepus timidus
Lepus castroviejoi
Lepus granatensis
Lepus europaeus
Lepus capensis
Lepus saxatilis
Lepus mediterraneus
Lepus victoriae
0.15
0.10
0.05
0. 00
b
Spicara smaris
Pagellus erythrinus
Sparus auratus
Oblada melanura
Katsuwonus pelamis
Boops boops
Trigla lucerna
Dicentrarchus labrax
Lophius budegassa
Betta splendens
Helicolenus dactylopterus
Limanda aspera
Scomber scombrus
Trachurus trachurus
Zeus faber
Salmo trutta
Salmo salar
Mullus barbatus
Mullus surmuletus
Phycis phycis
Micromesistius poutassou
Merluccius merluccius
Merluccius hubbsi
Sardinella aurita
Carassius auratus
Ladigesocypris ghigii
Engraulis encrasicolus
Raja miraletus
Prionace glauca
Platyceps najadum
Hemidactylus turcicus
0.4
0.3
0.2
0.1
0.0
c
Anas penelope
Anas crecca
Anas platyrhynchus
Tadorna tadorna
Anser anser
Scolopax rusticola
Gallinago gallinago
Streptopelia turtur
Columba livia
Columba palumbus
Turdus merula
Passer montanus
Coturnix coturnix
Coturnix japonica
Alectoris graeca
Alectoris chukar
Gallus gallus
Meleagris gallopavo
Phasianus colchicus
0.12
0.10
0.08
0.06
0.04
0.02
0.00
d
Nephrops norvegicus
Homarus gammarus
Squilla mantis
Astacus astacus
Callinectes sapidus
0
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0
e
Hierophis gemonensis
Platyceps najadum
Typhlops vermicularis
Hemidactylus turcicus
Lacerta viridis
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f
Morimus asper
Morimus funereus
Vadonia imitatrix
Neodorcadion sp.
Monochamus sutor
Leptura maculata
Niphona grisea
Scarabaeus sp.
Pedostrangalia verticali
Oryctes nasicornis
Plagionotus arcuatus
Oberea bipunctata
Obezema pupillata
Phytoecia nigricornis
Saperda scalaris
Haplidia transversa
Rhizotrogus sp.
Melolontha hippocastan
Melolontha melolontha
Macraspis tristis
Phileurus deshave
Parmena sp.
0.15
0.10
0.05
0.00

Figure 2

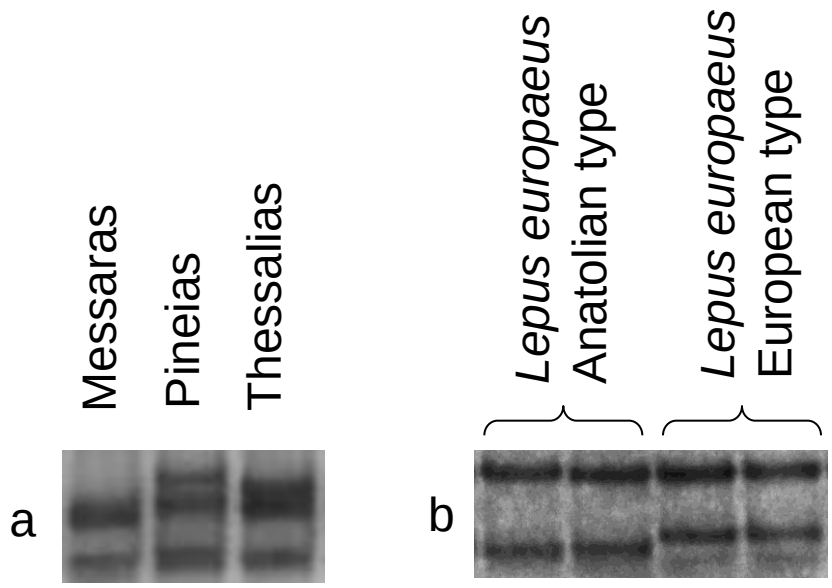
Messaras
Pineias
Thessalias
a
Lepus europaeus
Anatolian type
Lepus europaeus
European type
b

Figure 3