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Title: Fish species identification in canned pet food by BLAST and Forensically Informative Nucleotide Sequencing (FINS) analysis of short fragments of the mitochondrial 16s ribosomal RNA gene (16S rRNA).

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Keywords: Species identification, pet food, BLAST analysis, FINS analysis, 16S ribosomal RNA gene, Mislabeling

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Abstract: Nowadays, pet food is available on the market, claiming high-valued fish among ingredients. Unfortunately, the modifications induced by processing make difficult the species identification by visual inspection and hinder the enforcement of the legislation on traceability. In this work, after aligning 819 sequences of the Clupeidae, Engraulidae, Salangidae and Scombridae families, we developed new universal primers for the amplification and sequencing of 2 short fragments (±118 and ~213) of the mitochondrial 16s ribosomal RNA (16srRNA) gene. Once tested on 130 DNA reference samples, these primers were used in the analysis of highly degraded DNA extracted from 43 canned cat food containing whole minnows (whitebait) (M) and tuna, or bonito and mackerel fillets (F). Three M and 2 F samples were analyzed for each can. A BLAST and a FINS analysis, the latter performed only on the 118bp fragment, were performed separately on the sequences obtained from M and F samples. All the M samples were identified at the species or genus level by both BLAST and FINS analysis. This allowed to highlight an impressive rate of mislabeling (100%). F samples, for which FINS was less performing in species identification, resulted mislabeled in 40% of the products.

***Detailed Response to Reviewers**

Dear Editor,
we revised the manuscript as suggested by the Reviewer.

Best Regards

Andrea Armani

Reviewers' comments:
It's a very interesting study in which a quick method was developed to identify the fish species by PCR based on 16s ribosomal RNA. However, the number of references seems to be to high for a regular paper and should be shorten wherever possible

The number of references has been reduced from 56 to 50.

***Cover Letter**

Dear Editor,

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

"*Fish species identification in canned pet food by BLAST and Forensically Informative Nucleotide Sequencing (FINS) analysis of short fragments of the mitochondrial 16s ribosomal RNA gene (16S rRNA)*"

Elite pet food products containing different kinds of boneless fish are usually sold in single-portion sterilized cans with a cost two or three times higher than the popular ones. For this reason, fish species substitution, often reported for products intended for human consumption, could be also pursued in the pet food sector to obtain a greater economic gain.

The official control, such as the visual inspection requested from the (EC) Regulation 882/2204 , is often ineffective to verify the compliance of the product due to the loss of the morphological characteristics of the species that have been used for the production. Such limitations emphasize the need of "*physical checks*" that often rely on the utilization of DNA based methods.

In this work, after aligning 819 sequences of different fish family (Clupeidae, Engraulidae, Salangidae and Scombridae) we developed new universal primers for the amplification and sequencing of 2 short fragments (~118 and ~213) of the mitochondrial 16s ribosomal RNA (*16srRNA*) gene. Once tested on DNA reference samples the protocol was used to analyze degraded DNA extracted from the 43 products reporting valuable species, such as Whitebait (Minnow-M) and Tuna, Bonito and Mackerel (Fillets-F), among the ingredients. The obtained M and F sequences were then analyzed by running both a BLAST analysis on GenBank and by performing a FINS analysis, separately.

The results showed that, even though the selected DNA marker does not allow to clearly differentiate certain closely-related fish species of the Scombridae family, it was effective in discriminating the species belonging to the Clupeiformes order. Overall, the analytical approach highlighted a high rate of incorrect labelling of 100% in case of M and 40% in case of F. If considered in the light of the two main ingredients contained in the products (M and F) the results showed that the 60% of the products were 100% mislabeled, while in the remaining products (40%), the mislabeling affected only M.

Fish species identification in canned pet food by BLAST and Forensically Informative Nucleotide Sequencing (FINS) analysis of short fragments of the mitochondrial 16s ribosomal RNA gene (*16S rRNA*).

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Abstract:

Nowadays, pet food is available on the market, claiming high-valued fish among ingredients. Unfortunately, the modifications induced by processing make difficult the species identification by visual inspection and hinder the enforcement of the legislation on traceability. In this work, after aligning 819 sequences of the Clupeidae, Engraulidae, Salangidae and Scombridae families, we developed new universal primers for the amplification and sequencing of 2 short fragments (±118 and ~213) of the mitochondrial 16s ribosomal RNA (*16srRNA*) gene. Once tested on 130 DNA reference samples, these primers were used in the analysis of highly degraded DNA extracted from 43 canned cat food containing whole minnows (whitebait) (M) and tuna, or bonito and mackerel fillets (F). Three M and 2 F samples were analyzed for each can. A BLAST and a FINS analysis, the latter performed only on the 118bp fragment, were performed separately on the sequences obtained from M and F samples. All the M samples were identified at the species or genus level by both BLAST and FINS analysis. This allowed to highlight an impressive rate of mislabeling (100%). F samples, for which FINS was less performing in species identification, resulted mislabeled in 40% of the products.

Keywords: Species identification, pet food, BLAST analysis, FINS analysis, 16S ribosomal RNA gene, Mislabeling

1 Introduction

The awareness that pets can contribute to the maintenance well-being of humans has led to an increase of their number all around the world (Wells, 2009). In the US, from 1970 to 2010, the number of dogs and cats has been estimated to be increased from 67 to 164 millions (http://www.humanesociety.org/issues/pet_overpopulation/facts/pet_ownership_statistics.html#.Up8y0cTuImE). In the EU, in 2012, the total number of pets was 204.947.400 and 72 million of homes had companion animals (FEDIAF, 2012).

In this new social contest, the relationship among pets and humans has completely changed and the owner has assumed personal responsibility even for their proper dietary management. Pet anthropomorphization and the rising of many food related pathologies (i.e obesity and food intolerances) have pushed the feed sector to search for solutions to satisfy their nutritional needs (Lund, Armstrong, Kirk & Klausner, 2006). Specific food for breed, size, life stage and high quality feed, in relation to the nutrient content (antioxidants, fibers, polyunsaturated fatty acids, etc.), are increasingly assuming greater appeal to the buyer, who is prone to pay for a higher price (Swanson *et al.*, 2013). Even though the ingredients' selection is a key element for pet food, tastiness and palatability also represent an important characteristic for the owner. In particular, the initial perception of quality and nutritional need satisfaction has evolved according to socio-cultural, environmental and ethical factors. This has brought to further increasing the variety of the offer on the market, nowadays representing a significant share of the international food industry, with an estimated value of 13.8 billion of euros in the Europe alone (FEDIAF, 2012). The pet food available on the market are mainly dry, moist, semi-moist, frozen chilled, and treats. In general, they can be grouped in two categories: “Popular”, usually sold in grocery stores or large-format pet retailers and “Premium”, typically sold in veterinary practices, and pet stores (Lund *et al.*, 2006). The latter are elite products that often recall recipes and typical dishes of the culinary tradition, which are able to meet food trends and preferences of the owners at the same time (Swanson *et al.*, 2013). Among them, the super-premium fish-based cat food, containing different kinds of boneless fish soaked in

brine or jelly, are usually sold in single-portion sterilized cans. The cost for these products are on an average two or three times higher than the popular ones (author's note). Considering that the use of valuable fish species directly affects the cost of the products, it is plausible that the practice of misleading labeling, widely reported for products intended for human consumption (Pepe *et al*., 2007; Armani *et al.,* 2013; Di Pinto *et al*., 2013), could be also applied in the pet food sector to obtain a greater market appeal.

With regard to fish-based products, unlike the provisions for fish sold for human consumption (Regulation (EC) 1379/2013), the Regulation (EC) 767/2009 (Regulation (EC) 767/2009) does not compel the Business Operators (BOs) to indicate the scientific name of the fish used for the manufacturing. However, it specifies that labels must not mislead, confuse or deceive, directly or indirectly, the buyer "claiming" or remind fish species not included in the product.

Visual inspection is often ineffective to verify the compliance of the product to the label due to the loss of the morphological characteristics of the species that have been used. Such limitations emphasize the need of physical checks (Regulation (EC) 882/20024) that, in case of seafood, often rely on the utilization of analytical methods capable to provide species identification (Armani, Castigliego & Guidi, 2012). The DNA-based techniques are routinely applied for the identification of processed fish based products and feedstuffs, and shows greater efficiency than protein-based techniques in heat processed products (Pepe *et al*., 2007, Armani *et al*., 2012b; Armani *et al*., 2013; Ardura *et al*., 2012). Among the several PCR-based methods, the sequencing, namely Forensically Informative Nucleotide Sequencing (FINS) and DNA Barcoding, are the most frequently applied to fish and seafood species identification (Hellberg & Morrissey, 2011). At present, the *COI* gene is the most targeted mtDNA gene due to a well-established molecular identification system for fish and seafood (FISH-BOL, www.fishbol.org). However, both the *cytochrome b* (*cytb*) and *16S ribosomal RNA* (*16SrRNA*) genes also represent useful targets for fish identification (Armani *et al*., 2012). This study was aimed to identify the fish species contained in 43 cat food products the label of which reported the presence of valuable species, such as Whitebait, Tuna, Bonito and Mackerel,

in the ingredients. We first developed a PCR and a sequencing protocol designing new primers for the amplification of *16SrRNA* gene fragments with different lengths. Then, the obtained short sequences were analyzed running a BLAST analysis on GenBank and by performing a FINS analysis to verify the labeling information in the light of the European provisions.

2. Material and Methods

2.1 Samples collection, visual inspection and DNA extraction

2.1.1 Reference samples. 107 reference tissue samples (RS) belonging to 22 species (from one to ten specimens per species) from Scombridae family were directly collected at the wholesale market or kindly provided by Research Institutes (Table 1SM).

2.1.2 Market samples. 43 cans of fish-based cat food belonging to 13 brands were collected from the Italian market (Table 1-2). Each can was brought to the laboratory and labeled with an internal code. The information reported on the label were registered and a visual inspection of the product content was performed by morphological analysis (Fig. 1). When possible, 3 whole minnows (M) specimens and 2 pieces of the Fillets (F) from each can were sorted randomly and washed with distilled water. In case of products containing chicken together with minnows (2 cans), only M were sampled and analyzed (Table 1-2).

2.1.3. DNA extraction. All the fish samples were stored at -20℃ until total DNA extraction, which was performed according to the protocol proposed by Armani *et al.* (2014), starting from a whole specimen in case of M and from 100mg of tissue in case of F or RS. The DNA concentration and purity were assessed by evaluating the absorbance at 260 nm and the ratios A260/280 and A260/230 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, US). Moreover, 23 DNA samples obtained from reference specimens molecularly identified at the species level in a previous work (Armani *et al*., 2012b) were also used to test the primer amplification performances (Table 1SM).

2.2 Evaluation of DNA fragmentation by gel electrophoresis

One µg of total DNA extracted from M and F was electrophoresed on 1% agarose gel (GellyPhorLE®, Euroclone, Pero, MI), stained with GelRed™ Nucleid Acid Gel Stain (Biotium, Hayward, CA, USA) and visualized under UV light. The degradation degree was assessed by comparison with the standard marker SharpMass™50-DNA ladder and SharpMass™1-DNA ladder (Euroclone, Wetherby, UK). degree

2.3 Sequence collection, primer design and PCR optimization

2.3.1 Sequence collection and primer design. A total of 819 GenBank sequences of the *16SrRNA* gene from the species belonging to the Clupeidae, Engraulidae, Salangidae and Scombridae families were aligned using Clustal W in MEGA version 6 (Tamura *et al.*, 2013). In particular, taking into consideration the DNA degradation level, the analysis focused on a fragment of ~ 335bp belonging to a sequence amplified by two universal primers (FOR16Spc- REV16Spc2 -2) developed in a previous work (Armani *et al.*, 2012b) (Table 3). Within this fragment, the regions with the highest identity level were identified and used to design new primers for the amplification of fragments of different length (77, 118, 213bp, length w/o primers calculated on the sequences FR849595 of *Sardina pilchardus*). Primers characteristics are shown in Table 3 and Fig. 2. The discrimination power of each fragment per each species included in the alignment was assessed by running an "*in vitro*" BLAST analysis on GenBank. This analysis also allowed to verify the availability of reference sequences for the *16SrRNA* gene (Table 2SM).

2.3.2 PCR optimization. The new primers were tested for their amplification performances on all the RS DNA by coupling them in all the possible combinations and in different concentrations. The optimal annealing temperature (Ta) was then determined using the temperature gradient function on the PeqSTAR 96 Universal Gradient thermocycler (Euroclone, Milan, Italy).

Even though all the selected couples were able to produce a readable PCR product from each of the RS species, the best results in terms of amplification yield (evaluated by visualization in UV light), specificity (no aspecific amplification products), absence of inter-oligo reaction (no amplification in the blank) and percentage of successfully amplified RS were obtained using the

two forward primers For16s-1, FOR16s-2 together with the Rev16s-2. These primers allowed the amplification of a 118 bp (short fragment) and 213 bp (long fragment), respectively. Thus, the selected primers were added with universal tails M13for(-21) and M13rev(-29) (http://www.htseq.org/services/dna_sequencing/sanger) and tested for assess their amplification performances (Table 3).

2.4 PCR amplification and DNA sequencing

2.4.1. Amplification of M and F DNA samples. All the PCR were performed in a final volume of 20µl containing 1µl of a 10×PCR buffer (5 Prime, Gaithersburg, USA), 100ng of DNA, 100µM of each dNTP, 100nM of each primer, 1U PerfectTaq DNA Polymerase and DNase free water applying a 35 cycles protocol (94℃ for 30s, 53℃ for 20s, 72℃for 30s) preceded by an initial activation at 94 ° C for 3 minutes and followed by a final elongation step at 72℃ for 10min. All the PCR products (5µL) were checked on a 1.8% agarose gel (GellyPhorLE, Euroclone, UK) stained with GelRed™ Nucleid Acid Gel Stain (Biotium, Hayward, CA, USA) and the presence of fragments of the expected length was assessed by a comparison with the standard marker SharpMass™50-DNA ladder (Euroclone, Wetherby, UK). The samples that presented the expected amplicon were sent to sequencing by the High-Throughput Genomics Center (Washington, USA).

2.4.2. Amplification and sequencing of DNA RS.

Part of DNA extracted from RS collected in this study were amplified using the primers proposed by Palumbi (1996) according to the protocol proposed by Armani *et al*. 2012a. In particular, we amplified the DNA from 2 to 5 samples belonging to the species *Euthynnus affinis, E. alletteratus, E. lineatus, Sarda chiliensis, S. orientalis, S. australis, T. maccoyii, Auxis rochei, A. thazard, Allothunnus fallai,* for which either only one sequence was deposited or no sequences were available. The PCR products were visualized and sequenced as reported in section 2.4.1. Totally, 28 reference sequences were obtained and deposited on GenBank via EBI (Table 2SM).

2.5 ***BLAST and phylogenetic analysis of the sequences***

The obtained sequences were visualized, edited and aligned with Clustal W employing MEGA 6.0. Fine adjustments were manually made after visual inspection. A total of 213 sequences belonging to the commercial samples with variable length in the range of 117-123 (short fragment) or 213-230bp (long fragment) (Table 1-2) were produced and used to run a BLAST analysis on GenBank. For distance analyses, the pairwise sequence divergences were calculated using a Kimura 2-parameter (K-2P) (Kimura, 1980) distance model computed on MEGA 6.0 software. The analysis was performed separately for M and F commercial samples, using as reference the 28 sequences produced in this study (section 2.4.2) and 191 sequences retrieved from GenBank, using, when available, five sequences per species (Table 2SM). In order to visualize the clustering pattern of the sequences two NJ dendrogram with 1000 bootstrap re-samplings (Saitou & Nei, 1987) were produced using MEGA 6.0.

3. Results and Discussion

Most of the studies on pet food were aimed to investigate the presence of harmful ingredients (Heller & Nocchetto, 2008) or microbiological contaminations (Weese, Rousseau & Arroyo, 2005) as a consequence of events of serious pets intoxication associated with the consumption of commercial feed. At present, to our knowledge, no studies exist on species identification in this kind of products aimed at verifying the labeling compliance.

At the European level, the Regulations on traceability and official controls (Regulations (EC) 178/2002, 882/2004 and Reg. 183/2005) on food and feed have been implemented with the introduction of the Regulation (EC) 767/2009 (Regulation (EC) 767/2009). This Regulation, in order to harmonize European legislation on feed, extended all the principles of Regulations 178/02 (Regulations (EC) 178/2002) also to pet food. Currently, Pet Feed Business Operators (PFBOs) become the guarantee and the solely responsible for the accuracy of all information on the label and, in particular, of the "claims" adopted to draw the attention of the final user.

In case of processed products PCR sequencing followed by a comparison with a dataset of reference sequences deposited in free accessible databases is the first choice for the fish species

identification (Hellberg & Morrissey, 2011). On this regard, it is interesting to note that also US FDA is considering to use DNA barcoding to detect mislabeling in imported pet food (http://ibol.org/fda-using-barcoding-to-spot-fish-fraud/).

3.1 Selection of the molecular target

As reviewed in Armani *et al*. (2012) mitochondrial DNA (mtDNA) is generally chosen as target for species authentication due to the high mutation rate, multi-copy nature and maternal inheritance. The main points to consider in the choice of a molecular marker are the inter-and intraspecific variability, the presence of reference sequences on public databases and the presence of highly conserved region for the universal priming site selection (Teletchea, Maudet & Hänni, 2005; Vences *et al*. 2005).

As a result of the implementation of the Barcode of Life campaign, several primer pairs are currently available for the amplification of the mtDNA *COI* gene from fish (Armani *et al*., 2012). Unfortunately, all these primers target a fragment of ~700bp, and are not suitable for the analysis of processed fish products because of the marked DNA degradation (Armani *et al*., 2013; Armani *et al*., 2014). On the other hand, the high level of sequence variation of the *cytb* gene makes difficult to locate conserved areas on which to design universal primers for the amplification of short gene fragments (Zhang & Hanner, 2012).

The *16SrRNA* gene, although characterized by a lower mutation rate with respect to the two mitochondrial genes *cytb* and *COI*, has been successfully targeted for the identification of Groupers (Trotta *et al*., 2005), Clupeiformes (Jerome *et al*., 2008) and many others fish species belonging to different families (Cawthorn, Steinman & Witthuhn, 2012; Ardura *et al*., 2012). Moreover, thanks to its high conservation rate, the *16SrRNA* has been used for the designing of universal primers able to amplify different length DNA fragments from many different fish species (Palumbi, 1996; Ardura *et al*., 2012).

All these reasons considered, the *16SrRNA* has been selected as molecular target for species identification in petfoods.

3.2 Samples collection

3.2.1. Reference samples. Initially, the choice of reference species to be used for the PCR optimization (Table 1SM) was made on the basis of the commercial and/or the scientific denomination reported on the labels of the pet food cans (Table 1-2). Then, other species were included, among those most commercially exploited belonging to the same genus or family, with the aim to develop universal primers capable to amplify a wide range of fish species. In case of "tuna-like fish" the choice of the species was also made taking into consideration those mentioned in the Regulation 1536/92 (Council Regulation (EEC) n. 1536/1992) on common labeling rules for tuna and bonito canned products. Moreover, due to the fact that Mackerel occasionally appeared among the ingredients, even the species belonging to the *Scomber* genus were considered. As for "*Bianchetto*" we took into consideration not only the *Sardina pilchardus*, but also other species belonging to both the Clupeidae or Engraulidae family, currently used for sardine and sardine-like canned products (Jerome, Lemaire, Verrez-Bagnis & Etienne, 2003). Finally, based on our previous study (Armani *et al*. 2011), also *Neosalanx taihuensis*, *N. anderssoni*, and *Protosalanx chinensis*, belonging to the Salangidae family, were included in the study because imported from Asian countries and frequently substituted with the juvenile form of *S. pilchardus*.

3.2.2. Market samples. According to the labels' information, all the pet food analyzed in this study were produced and imported from two major provinces of Thailand (Bangkok and Songkhla) for 10 distinct Companies holding one or more lines of sale for a total of 13 trademarks. These samples were purchased from pet stores and large retail supermarkets at a price in between 1.50 and 2 euros per 100g.

3.3 Evaluation of DNA fragmentation by gel electrophoresis

The high temperatures and pressures applied on fish based feed processing are similar to that used for the standard canning procedures for fish based preserved products. On the basis of FAO standards (http://www.fao.org/docrep/003/t0007e/t0007e05.htm), canning procedures used for tuna and tuna like products consist of a multi- step protocol comprising a steam pre-cooking carried at

95°-105 °C for one to several hours, followed by a final sterilization process with a typical temperature of 115°C.

Exposure to heat and other physical stressors is known to cause random breaks in DNA strands, thus reducing the DNA fragments size and determining the typical fragmentation pattern for tuna-like or sardine type canned food (from 100 to 350bp) (Jerome *et al*., 2003). As expected, the total DNA extracted from the M and F showed a marked level of fragmentation, with an electrophoretic pattern hardly visible above 500bp and, in most DNA samples, concentrated between 50 and 250bp.

3.4 Primers selection, PCR amplification and DNA sequencing

Two internal forward and 2 reverse primers were designed and tested for their amplification performances, together with other primers developed in a previous study (Armani *et al*., 2012b) (Table 3 and Fig.2). All the primers were designed on conserved areas spanning among region characterized by many base pairs gaps. The two forward primers For16s-1, FOR16s-2 together with the Rev16s-2 for the amplification of a 118bp short fragment and 213bp long fragment, respectively, were finally selected.

3.4.1 Reference samples. Totally, 28 reference sequences of variable length have been produced and deposited on GenBank (Table 2SM). Then, they were immediately released and used for the identification of the sequences obtained from the market products by BLAST analysis (Table 1-2).

3.4.2 Market samples. Despite the high level of DNA fragmentation (section 3.3) all the market samples were successfully amplified using the selected primers (section 3.4.1) and 213 *16srRNA* sequences (129 from M and 84 from F) of variable length were obtained.

In the case of M we obtained 34 long sequences (26%) with a length of ~213bp and 95 short sequences (76%) with length of ~118bp. In the case of F we obtained 84 short sequences (100%) with a variable length (from 119 of *Euthynnus* sp. to 140bp of *Trachurus novaezelandiae* sp.), due to the presence of a different number of indels. Since these sequences were not obtained from voucher specimens or expertly-identified fish specimens, they were not submitted to the databases and were only used to assess the labeling information reported on the cans.

3.5 BLAST analysis

The Basic Local Alignment Search Tool (BLAST), which is one of the most used application for calculating sequence similarity, was applied in this study for the identification of the sequences obtained from the market samples. This tool is able to return the results in the form of a ranked list on the basis of a normalized percent identity score, followed by individual sequence alignments (Quintero, Santaclara & Reihbein, 2008; Nicolé *et al.*, 2012).

In order to identify an unknown sample by BLAST analysis, a threshold of maximum divergence between the query and the sequences used as standard has to be defined. For the *COI* gene a cutoff threshold of 2% has been established (Barbuto *et al.*, 2010). In case of *16srRNA*, even though Ardura *et al.* (2012) suggested a sequence identity >99%, an universally accepted threshold has never been proposed. Thus, given the high degree of preservation of the *16SRNA* gene (Kochzius *et al.*, 2010; Cawthorn *et al.*, 2012) and the fact that we worked on a short fragment, an identity score of 100% was used as cut-off for the species identification. Overall, this identity threshold has been successfully achieved for 207 sequences out of 213 (97%) undergone to the BLAST analysis.

3.5.1 M sequences. Overall, 84% of the sequences analyzed (108 out of 129) were identified at the species level. According to the BLAST results, 126 M samples (98%) were identified as belonging to the genus *Encrasicholina* with an identity values of 99-100%. The remaining 3 samples (1.5%) were identified as *Anguilla anguilla* and *Neosalanx* sp. with identity values of 100%. As regard the *Encrasicholina* genus when a top match of 100% was obtained for one species, the lower identity value was only 93-94%. In particular, 102 LF samples (81%) were identified as *E. heteroloba*, 4 (3%) as *E. punctifer*, while 14 (11%) as *Encrasicholina* sp. On the countrary, the remaining 6 sequences from M samples (5%) had a maximum identity value of 99% with sequences deposited as *Encrasicholina* sp. and of 98% with *E. heteroloba* (Table 1). Therefore, the selected *16SrRNA* gene fragment could not unequivocally discriminate the two aforementioned species probably due to shared inter-specific variations in some specimens.

Considering that, at present, species specific sequences are only available for 3 species of *Encrasicholina* (*E. heteroloba*, *E. punctifer*, and *E. devisi*) out of the 5 ascertained species of this genus (http://www.fishbase.org/Nomenclature/ValidNameList.php?syng=encrasicholina&syns=&vtitle=Scientific+Names+where+Genus+Equals+%3Ci%3EEncrasicholina%3C%2Fi%3E&crit2=CONTAINS&crit1=EQUAL) and that the only two available sequences of *E. devisi* relate to the initial portion of the *16SrRNA* gene not including the fragment selected in the present study, the samples identified as *Encrasicholina* sp. could belong to the species *E. oligobranchus*, *E. purpurea* or *E. devisi*.

E. heteroloba, *E. punctifer* and *E. devisi* have been recorded from the Indian Ocean to the Pacific Ocean (Red Sea, Thailand, Indonesia, Australia North Island of Taiwan) Philippines, Tonga, Fiji and Japan (http://www.fao.org/docrep/009/t0835e/t0835e00.htm). On the countrary, the species *E. purpurea* has its natural habitat almost exclusively in the Pacific Ocean around the Hawaiian Islands, while *E. oligobranchus* is distributed mostly on the west coast of the Philippines and particularly in the Manila Bay. For the aforesaid reasons, and considering that the feed producers are all concentrated along the coast of Thailand, is highly unlikely that the species *E. purpurea* and *E. oligobranchus* were used in the products analyzed in this study. Moreover, the presence of *E. devisi* in a sample commercialized in Italy as *S. pilchardus* has already been reported (Riina *et al*., 2012).

Unfortunately, we did not succeed in identifying the species *E. oligobranchus*, *E. purpurea*, and *E. devisi*, due to the impossibility to collect reference specimens as a consequence of the strict regulations on exchanges for research purposes of samples coming from Asian and Indian waters (Rao & Gupta, 2003). This issue represents a significant limitation in the identification of the multitude of new exotic species continuously released on the Western market, considering that most of the fisheries are centered in the Pacific Ocean.

335 *3.5.2 F sequences.* A maximum identity score of 100% with at least one GenBank reference
336 sequence have been obtained for all the 84 sequences produced. In the 76 F analyzed the most
337 common species was *Katsuwonus pelamis* (62,5%), *E. affinis* (27,5%) and *A. rochei* (7,5%). Even
338 though the analyzed fragment does not seem to possess a discriminatory power comparable to that
339 highlighted for M, all the aforesaid sequences were correctly identified at the species level
340 according to the selected threshold. The inter-specific variability was found to be lower than in the
341 case of Clupeiformes. In fact, the identity values towards the species, other than those that matched
342 at 100%, were 99 and 98%. However, all the F sequences belonging to one species gave the same
343 results confirming the absences of intra-specific variability in the fragment analyzed in this study
344 (Table 2). On the countrary, 22 F sequences got a 100% identity value with the reference sequences
345 of both *E. affinis* and *E. lineatus*, suggesting the existence of shared intra-specific variations.
346 However, this circumstance does not influence the calculation of the mislabeling rate (see section
347 3.7).

348 Two sequences (2,5%) were identified as belonging to the genus *Thunnus* sp. with a maximum
349 identity score of 100% with all the species of this genus. The inability of the *16SrRNA* gene in
350 clearly differentiate species within the genus *Thunnus* was already highlighted in the work of
351 Cawthorn *et al.*, (2012).

352 Finally, the sequences obtained from pet food labeled as mackerel were identified (100% identity
353 value) as *T. novaezelandiae* (Yellowtail horse mackerel) (2 sequences) and *K. pelamis* (4
354 sequences), while other 2 products labeled as sardine fillets (2 sequences), were identified as
355 *Sardinella fimbriata* (Fringescale sardinella).

356 ***3.6 Phylogenetic analysis***

357 The phylogenetic analysis was performed using the *16SRNA* gene sequence amplified by the
358 primer pair For 16s-2 and Rev16s-2 (short fragment), due to the fact that this was the only fragment
359 obtained from 179 out of 213 (84%) samples analyzed. Two to 5 sequences belonging to the species
360 selected as RS were used in the phylogenetic analysis. Considering that the BLAST analysis results

highlighted the presence of species not taken into consideration during the first selection of the reference sequences, from 2 to 5 sequences belonging to different species of the genus *Encrasicholina*, *Anguilla* (Anguillidae) and *Trachurus* (Carangidae) were also included (Table 2SM). The distance analysis was performed separately for M and F, in order to assess the discrimination power of the selected *16srRNA* gene fragment for different group of species. The target sequence showed a variable length (from 117 to 140bp) probably owed to gaps in relation to the presence of indels, which are a common finding in the ribosomal genes due to the fact that they have a minimal impact on the rRNA function (Steinke, Vences, Salzburger & Meyr, 2005). In accordance with Doyle & Gaut (2000), all the gaps and insertions highlighted by the preliminary alignment were included in the neighbor joining (NJ) analysis, in order to maximize the overall number of nucleotide matches.

3.*6.1M samples.* The dendrogram showed well-defined clusters with bootstrap values higher than 70% at both genus level and species level. In particular, 4 clades were produced according to the family Engraulidae, Clupeidae, Anguillidae and Salangidae (Fig. 1SM). Inside the Engraulidae clade family, all the species were clearly distinguished with bootstrap values higher than 70%, with the only exception of *E. encrasicolus* and *E. japonica* (bootstrap values 64 and 66%, respectively). The sequences identified as *E. heteroloba, E. punctifer* and *Encrasicholina* sp. by the BLAST analysis were grouped into specie-specifics sub-clades. Also in the case of the family Clupeidae, all the species were clustered in a well-defined family cluster with bootstrap values higher than 70%. The sequences identified as belonging to *Neosalanx sp.*, were clustered within the family Salangidae in the sub-clade made of the species *N. taihuensis* and *N. brevirostris.* These were separated from the species *N. jordani* and *N. oligodontis* (bootstrap value 100%) and from the species *P. chinensis* and *N. anderssoni* . The 2 M sequences identified as *A. anguilla* by the BLAST analysis were placed in the clade containing the genus *Anguilla* spp., but were not distinguished at the species level.

Overall, the distance analysis confirmed the results obtained with the BLAST analysis, showing that the short fragment selected as target allowed a clear discrimination at the species level of most of M samples. In the case of Engraulidae family, we obtained comparable results to those reported by Jerome *et al.* (2008), despite a shorter target fragment (~118bp against ~259bp). This study confirms the high discrimination power of the *16sRNA* gene within the order Clupeiformes.

3.6.2 F samples

The dendrogram obtained for the F samples (Fig. 2SM) appeared very different from that obtained for M samples. In fact, even showing a clear separation of the Scombridae, Clupeidae and Carangidae family (bootstrap 100%), gender specific clusters were detectable only within the family Clupeidae, even though not supported by high bootstrap values (<70%). Within the family Scombridae, the NJ analysis produced 3 major sub-clades: the first containing all the species belonging to the *Scomber* genus, the second grouping the genera *Thunnus, Katsuwonus, Auxis, Sarda* and *Euthynnus* and the third exclusively comprising the species *A. fallai*. Inside the second sub-clade, all the subsequent branching at the genus level were not supported by bootstrap values >70%, highlighting the low discriminating power of the 16sRNA gene for the family Scombridae.

In particular, the sequences identified as belonging to *A. rochei* by BLAST analysis were grouped into the genus *Auxis* spp. sub-clade in which, however, a further species grouping was not possible. The samples previously identified as *K. pelamis* and *E. affinis* with a BLAST top match of 100%, while being grouped into two distinct genus clusters, were not supported by significant bootstrap values. The phylogenetic analysis was not even able to clearly distinguish among the *Thunnus* and *Sarda* spp. sequences. The results are consistent with what already reported by Cawthorn *et al*. (2012) for the genus *Thunnus* and by Miya *et al*. (2013) in an evolutionary study of the Scombridae family. In the light of these findings it seems that the sole FINS analysis cannot be considered reliable for the species discrimination within the Scombridae family and that a BLAST analysis allow a better classification. However, the phylogenetic analysis allowed to correctly match all the F sequence at the family level.

412 Similar issues in species discrimination within the genus *Thunnus* spp were obtained even when
413 other mitochondrial genes, such as *COI*, *cytb*, and nuclear First Internal Transcribed Spacer for
414 rDNA (*ITS -1)* Vinas & Tudela, 2009) were used. These studies agree in the fact that both the low
415 genetic distance between species, especially those belonging to the Neo Thunnus subgenus, such as
416 *T. albacares, T. atlanticus, T. tonggol* (Chow & Kishino 1995), and introgression, described within
417 several tuna species, (Vinas & Tudela, 2009) can lead to misidentification according to the genetic
418 marker chosen.

419 In order to overcome this limit and reach a precise species discrimination, two or three markers
420 should be targeted in the same analysis (Vinas & Tudela, 2009), separately or pooled as
421 concatenated sequences to maximize the discriminatory effect (Jerome *et al*. 2008). Even though
422 useful, this approach would lead to a drastic increase of costs and working time, not always
423 affordable for routinely analysis.

424 Alternatively, a proteomics approach have been proposed to solve this issue (Pepe *et al*., 2010)

425 ***3. 7 Labeling***

426 Overall, the results show that the analyzed M samples were mislabeled in 100% of cases: in
427 Italy, the name of whitebait (*Bianchetto*), reported on all the labels analyzed, can be used
428 exclusively for the juvenile form of sardine (*S. pilchardus*) (MIPAAF, Decree n. 31, January 2008).
429 At the national level the juvenile form of this species has a great market appeal and is used for the
430 preparation of typical high-price products. Since 2006, in compliance with the EU policy aimed at
431 the conservation of fish species in the Mediterranean sea, this species has been subjected to a strict
432 fishing control (Council Regulation (EC) 1967/2006). For this reason, with the exception of
433 derogations granted annually for time-limited special fishing and experimental purposes the fishing
434 of *Bianchetto* is forbidden in the Mediterranean Sea.

435 On the other hand, no ban is imposed on the importation of the juvenile form of *S. pilchardus* or
436 similar species (whitebaits) from Non-Mediterranean Countries, which are not subjected to fishing
437 restrictions. Therefore, the commercial name "*Bianchetto*" reported on Asian imported products

438 does not constitute a formal breach of the regulations in force. Nevertheless, the declaration of
439 juvenile forms of *S. pilchardus* in products caught and processed along the coast of Thailand
440 constitutes a false, since the geographical distribution of this species is limited to the Mediterranean
441 Sea, the Black Sea and the Northeast Atlantic Ocean (http://www.fao.org/fishery/species/2910/en).
442 In addition to the commercial fraud, the use of undeclared juvenile anchovies of Asian origin for
443 the preparation of pet food poses a number of issues of sustainability for the fishing industry. In
444 fact, the complete replacement of species in the absence of effective traceability and labeling
445 systems could implies a progressive depletion of fish reserves. This occurrence is even more
446 evident in Asian countries, where there are no stringent policies aimed to fish stocks conservation
447 and where fishing belonging to Illegal Unreported and Unregulated (IUU) catches can be recycled
448 by unscrupulous FBOs (Morgan *et al.*, 2007, FAO document-Fishing capacity management and
449 IUU fishing in Asia).
450 At the international level, the genus *Thunnus*, *Euthynnus*, *Katsuwonus* and *Auxis* are referred as
451 tuna or true-tuna group, while bonitos (*Cybiosarda*, *Gymnosarda*, *Orcynopsis* and *Sarda*) are
452 referred as tuna-like groups (FAO 2007, Global fishery resources of tuna and tuna like species).
453 In EU, labeling rules for tuna and bonito canned product (Regulation (EEC) n.1536/1992)
454 attributes the trade description of preserved tuna only to those products prepared from species
455 belonging to genus *Thunnus* spp. and *K. pelamis.* On the contrary, the trade description of bonito
456 products must be applied to the species belonging to genus *Sarda, Euthynnus* and *Auxis*.
457 In this light, the comparison between the labels and the BLAST analysis results highlighted a
458 discordance rate of 37% (28 samples on 76 labeled as tuna or tuna like products) (Table 2). Even
459 though the BLAST analysis was not able to discriminate between 2 species belonging to the genus
460 *Euthynnus* (100% identity value with *E. affinis* and *lineatus*) this result does not affect the
461 mislabeling rate. In fact, the genus *Euthynnus* cannot be labeled as tuna.

As for misleading and deceiving aspects related to labeling and trade names, the use of invented names, such as "Pink Tuna" or "Pacific Tuna", not present in any official denomination list at both national and international level, was observed in 12% of products.

The 3 products labeled as Mackerel showed a 100% mislabeling, due to the fact that, according to the Italian regulation, this trade name can only be associated to the species belonging to the Scomber genus. This results were strongly supported by both BLAST and FINS analysis (Table 2 and Fig. 2SM). On the other hand, the samples labeled as “Sardine” were correctly labeled, since, according to the international standard (http://www.codexalimentarius.org/standards/list-of-standards/en/), canned sardines or sardine-like products can be prepared from fresh or frozen fish belonging to several genera of the Clupeidae family, including the genus *Sardinella*.

Altogether, according to the BLAST analysis, this results show an overall mislabeling rate of 40%.

If considered in the light of the two main ingredients contained in the products (minnows and fish fillets) on which we focused our analysis, the mislabeling results showed that the 60% of the products were fully mislabeled, while in the remaining 40%, the mislabeling affected only the minnows.

Food mislabeling and species substitution, especially for canned products, can accidentally occur because of the inadequate training of operators, who are not able to identify the species at the time of fishing, as well as the lack of effective traceability systems of raw materials during curing and filleting procedure that result in the loss of key morphological characters. In the case of F, the most plausible hypothesis is that of a misdescription caused by lack of accuracy in the identification and traceability system, considering that 10 out of the 28 mislabeled samples (36%) contained fish with higher commercial value than those reported on the labels. Finally, it has to be taken into consideration that pet food are frequently imported from non-EU Countries (mostly Asian), where the complexity of the market logistic and the lack of a traceability system make less effective the

487 efforts to control the fishery trade (Pramod, Nakamura, Pitcher & Delagran, 2014; D'Amico *et al*.,
488 2014).

489 **CONCLUSIONS**

490 In this work, short fragments of the *16srRNA* gene were used to verify the accurate labeling of
491 pet food products. The presence of highly conserved regions in the chosen gene allowed to obtain
492 readable DNA sequences from all the samples using few primers even in case of highly processed
493 products. The results of the BLAST and FINS analysis showed that, even though the selected
494 mitochondrial DNA marker does not allow to clearly differentiate certain closely-related fish
495 species of the Scombridae family, it was strongly effective in discriminating the species belonging
496 to the Clupeiformes order. Overall, the analytical approach was enough powerful to highlight a high
497 rate of incorrect labelling, which could determine misrepresentation at the moment of the
498 purchasing and encourage overfishing practices.

499 In conclusion, it provides a valid tool to support the Official controls on pet foods, in the light of
500 the European provisions.

501

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Figures

Figure 1: A) Pet food can containing Minnow (M) and Fillets (F). **B)** Displaying of the content: M (on the right); F (on the left).

Figure 2: Position of the new primers designed for the amplification of the *16SrRNA* gene fragments.

Figures SM

Figure 1SM: Neighbor-joining (NJ) tree obtained using M sequences (from 117 to 123bp) of the *16srRNA* gene and reference sequences obtained in this study and retrieved from GenBank. Indels were included in the analysis. Boostrap values > 50% obtained from 1000 replications using Kimura two parameter genetic distances are reported in the tree.

Figure 2SM: Neighbor-joining (NJ) tree obtained using F sequences (from 118 to 140bp) of the *16srRNA* gene and reference sequences obtained in this study and retrieved from GenBank. Indels were included in the analysis. Boostrap values > 50% obtained from 1000 replications using Kimura two parameter genetic distances are reported in the tree.

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Highlights

Elite pet food with prized fish species is well regarded by pet-owners

Mislabeling may threaten fair trade and fish stock preservation

DNA-based analysis is often the only mean to verify species used as ingredients

We used new primers for PCR-sequencing analysis of the 16SrRNA gene in pet food

BLAST and FINS analysis highlighted a high rate of incorrect labelling

Figure

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Figure

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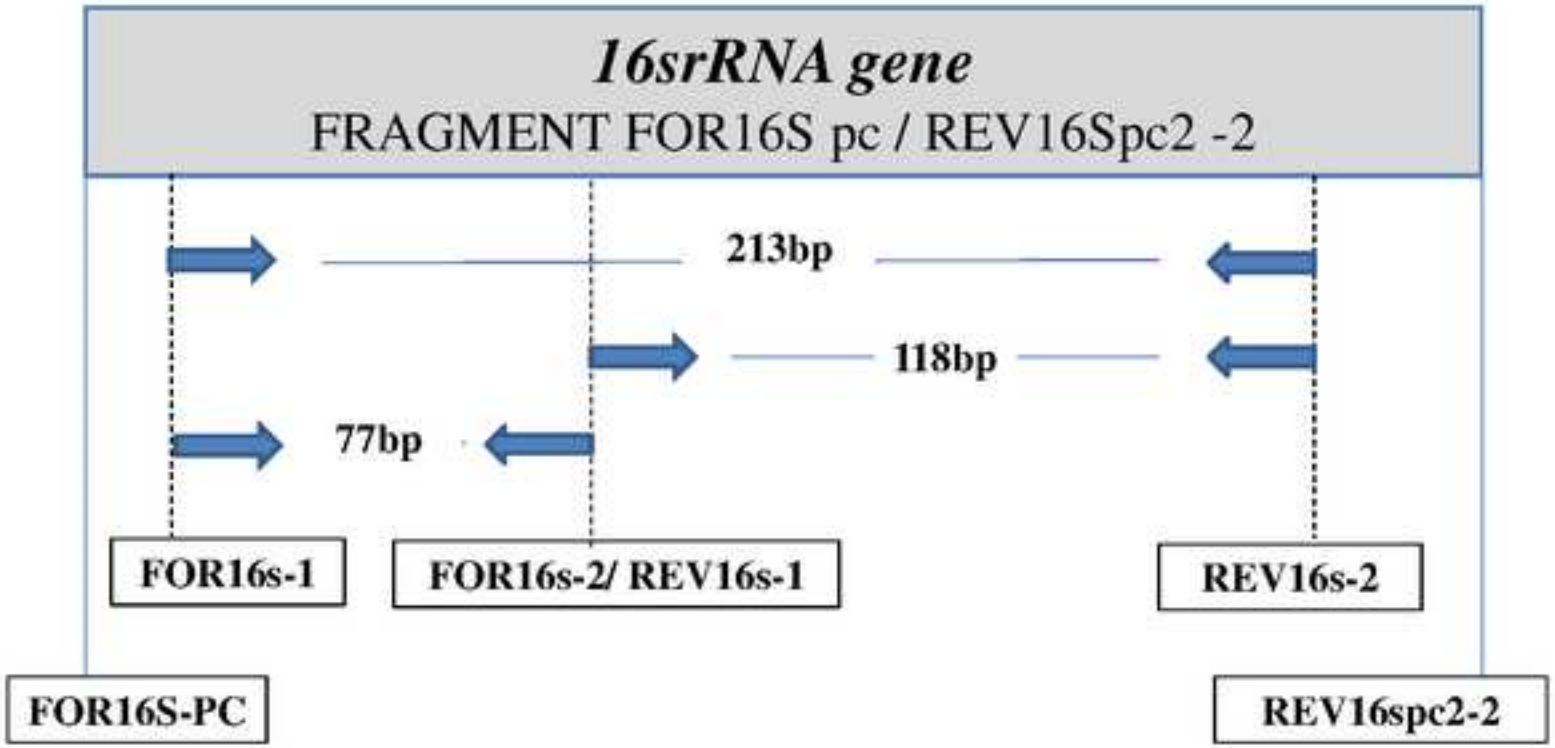

Table

BRAND	PROD.	LABEL	CODE	SEQ. L.	BLAST ANALYSIS	MISLAB.
N.1	CATF1	Whitebait	CATF1.1-CATF1.3	118	100% *E. heteroloba*	Y
	CATF15	Whitebait	CATF15.1-CATF15.3	213	100% *E. heteroloba*	Y
	CATF18	Whitebait	CATF18.1-CATF18.3	118	100% *E. heteroloba*	Y
	CATF24	Whitebait	CATF24.1-CATF24.3	118	100% *E. heteroloba*	Y
.N.2	CATF2	Whitebait	CATF2.1-CATF2.3	213	100% *E. heteroloba*	Y
	CATF6	Whitebait	CATF6.1-CATF6.5	118-213	100% *E. heteroloba*	Y
	CATF13	Whitebait	CATF13.1- CATF13.2	118	100% *E. heteroloba*	Y
		Whitebait	CATF13.4	214	100% *Encrasicholina* sp	Y
	CATF41	Whitebait	CATF41.1-CATF41.3	118	100% *E. heteroloba*	Y
N.3	CATF3	Whitebait	CATF3.1- CATF3.5	118	100% *Encrasicholina* sp	Y
		Whitebait	CATF3.2	119	100% *E. punctifer*	Y
	CATF4	Whitebait	CATF4.1 CATF4.2 CATF4.7	118	100% *E. heteroloba*	Y
	CATF12	Whitebait	CATF12.1	119	100% *E. punctifer*	Y
		Whitebait	CATF12.2	118	100% *Encrasicholina* sp	Y
		Whitebait	CATF12.4	213	100% *E. heteroloba*	Y
N.4	CATF5	Whitebait	CATF5.1-CATF5.2-CATF5.7	213	100% *E. heteroloba*	Y
	CATF20	Whitebait	CATF20.1-CATF20.2-CATF20.3	213	100% *E. heteroloba*	Y
	CATF31	Whitebait	CATF31.1-CATF31.2- CATF31.5	118-118-213	100% *E. heteroloba*	Y
N.5	CATF7	Whitebait	CATF7.1-CATF7.2	213	100% *E. heteroloba*	Y
		Whitebait	CATF7.5	214	99% *Encrasicholina* sp, 98%*E. heteroloba*	Y
	CATF39	Whitebait	CATF39.1-CATF39.2- CATF39.3	118	100% *E. heteroloba*	Y
	CATF40	Whitebait	CATF40.1-CATF40.2-CATF40.3	118	100% *E. heteroloba*	Y
N.6	CATF8	Whitebait	CATF8.1	213	100% *E. heteroloba*	Y
		Whitebait	CATF8.2	119	100% *E. punctifer*	Y
		Whitebait	CATF8.7	213	100%*Neosalanx* sp	Y
	CATF36	Whitebait	CATF36.1- CATF36.3	118	99% *Encrasicholina* sp, 98%*E. heteroloba*	Y
		Whitebait	CATF36.2	118	100% *E. heteroloba*	Y
	CATF37	Whitebait	CATF37.1-CATF37.2- CATF37.3	118	100% *E.heteroloba*	Y
N.7	CATF9	Whitebait	CATF9.1-CATF9.2	118	100% *E.heteroloba*	Y
		Whitebait	CATF9.3	214	99% *Encrasicholina* sp, 98%*E. heteroloba*	Y
	CATF29	Whitebait	CATF29.1- CATF29.3	118	100% *E.heteroloba*	Y
		Whitebait	CATF29.2	118	100% *Encrasicholina* sp	Y
	CATF45	Whitebait	CATF45.1- CATF45.2- CATF45.3	118	100% *E.heteroloba*	Y
N.8	CATF10	Whitebait	CATF10.1-CATF10.2-CATF10.7	118	100% E*ncrasicholina* sp	Y
	CATF16	Whitebait	CATF16.1- CAT16.2	118	100% *E.heteroloba*	Y
		Whitebait	CATF16.5	119	100% *E. punctifer*	Y
	CATF22	Whitebait	CATF22.1-CATF22.2-CATF22.3	213	100% *E.heteroloba*	Y
	CATF46	Whitebait	CATF46.1-CATF46.2-CATF46.3	118	100% *E.heteroloba*	Y
N.9	CATF11	Whitebait	CATF11.1- CATF11.2	118	100% E*ncrasicholina* sp	Y

		Whitebait	CATF11.4	214	99% *Encrasicholina* sp,	Y
	CATF14	Whitebait	CATF14.1-CATF14.2-CATF14.5	213	100% *E.heteroloba*	Y
	CATF42	Whitebait	CATF42.1-CATF42.2-CATF42.3	118	100% *E.heteroloba*	Y
N.10	CATF17	Whitebait	CATF17.1-CATF17.2-CATF17.3	118	100% E*ncrasicholina* sp	Y
	CATF32	Whitebait	CATF32.3-CATF32.9p CATF32.10p	213-118 118	100% *E.heteroloba*	Y
	CATF38	Whitebait	CATF38.1-CATF38.2-CATF38.3	118	100% *E.heteroloba*	Y
N.11	CATF19	Whitebait	CATF19.1-CATF19.2-CATF19.3	118	100% *E.heteroloba*	Y
	CATF25	Whitebait	CATF25.2-CATF25.3-CATF25.4	118	100% *E.heteroloba*	Y
	CATF35	Whitebait	CATF35.1-CATF35.5	118	100% *E.heteroloba*	Y
		Whitebait	CATF35.4	229	100% *A. anguilla*, 99%*A. rostrata*, 98% *A. reinhardtii*	Y
N.12	CATF23	Whitebait	CATF23.1-CATF23.2-CATF23.3	118	100% *E.heteroloba*	Y
	CATF33	Whitebait	CATF33.1-CATF33.9	118	100% *E.heteroloba*	Y
		Whitebait	CATF33.2	229	100% *A. Anguilla*, 99%*A rostrata*, 98% *A.reinhardtii*	Y
	CATF34	Whitebait	CATF34.1-CATF34.2-CATF34.3	118	100% *E.heteroloba*	Y
	CATF47	Whitebait	CATF47.1-CATF47.2- CATF47.3	118	100% *E.heteroloba*	Y
N.13	CATF30	Whitebait	CATF30.1-CATF30.2	118	100% *E.heteroloba*	Y
		Whitebait	CATF30.3	118	100% *Encrasicholina* sp	Y
	CATF43	Whitebait	CATF43.1-CATF43.2-CATF43.3	118	100% *E. heteroloba*	Y
	CATF44	Whitebait	CATF44.1-CATF44.2	118	100% *E. heteroloba*	Y
		Whitebait	CATF44.3	118	99% *Encrasicholina* sp, 98%*E. heteroloba*	Y

Table 1: List of Minnow specimens (M) analyzed in the study. Sampled cans are grouped by brand (from 1 to 13). The results of the BLAST analysis are reported up to an identity of 98%. PROD.= product; SEQ. L= Sequence Length; MISLAB: Mislabeled; Y=Yes; N=No.

Table

BRAND	PROD.	LABEL	CODE	SEQ. L.	BLAST ANALYSIS	MISLAB
N.1	CATF1	TUNA (E.affinis)	CATF1.4 CATF1.5	119	100% *E.lineatus E. affinis*, 98% *E. alletteratus, K. pelamis, P. triacanthus*	N
	CATF15	BONITO	CATF15.7 CATF15.8	119	100% *E.lineatus E. affinis*, 98% *E. alletteratus, K. pelamis, P. triacanthus*	N
	CATF18	TUNA	CATF18.6 CATF18.7	119	100% *Auxis rochei*, 99%*A.thazard*, 98%*K.pelamis*	Y
		SARDINE	CATF18.9s CATF18.10	120	100% *S. fimbriata*, 99% *S. albella*	N
	CATF24	BONITO (E.affinis)	CATF24.9 CATF24.10	119	100% *K. pelamis*, 99% *S.australis, S.orientalis, S. chiliensis*, 98% *A.rochei, E.alletteratus, E.lineatus, E.affinis*	Y
N.2	CATF2	TUNA	CATF2.4 CATF2.5	119	100% *K. pelamis*, 99% *S.australis, S.orientalis, S. chiliensis*, 98% *A.rochei, E.alletteratus, E.lineatus, E.affinis*	N
	CATF13	TUNA	CATF13.5 CATF13.6	119	100% *K. pelamis*, 99% *S.australis, S.orientalis, S. chiliensis*, 98% *A.rochei, E.alletteratus, E.lineatus, E.affinis*	N
	CATF41	TUNA	CATF41.9 CATF41.10	119	100% *K. pelamis*, 99% *S.australis, S.orientalis, S. chiliensis*, 98% *A.rochei, E.alletteratus, E.lineatus, E.affinis*	N
N.3	CATF3	TUNA	CATF3.3 CATF3.4	119	100% *K. pelamis*, 99% *S.australis, S.orientalis, S. chiliensis*, 98% *A.rochei, E.alletteratus, E.lineatus, E.affinis*	N
	CATF4	TUNA	CATF4.3 CATF4.4	119	100% *K. pelamis*, 99% *S.australis, S.orientalis, S. chiliensis*, 98% *A.rochei, E.alletteratus, E.lineatus, E.affinis*	N
	CATF12	TUNA	CATF12.5 CATF12.6	119	100% *A. rochei*, 99% *A.thazard*, 98% *K. pelamis*	Y
N.4	CATF5	TUNA	CATF5.3 CATF5.4	119	100% *K. pelamis*, 99% *S.australis, S.orientalis, S. chiliensis*, 98% *A.rochei, E.alletteratus, E.lineatus, E.affinis*	N
	CATF22	PINK TUNA	CATF22.6 CATF22.7	119	100% *K. pelamis*, 99% *S.australis, S.orientalis, S. chiliensis*, 98% *A.rochei, E.alletteratus, E.lineatus, E.affinis*	N
	CATF31	PACIFIC TUNA	CATF31.9 CATF31.10	119	100% *K. pelamis*, 99% *S.australis, S.orientalis, S. chiliensis*, 98% *A.rochei, E.alletteratus, E.lineatus, E.affinis*	N
N.5	CATF7	MACKEREL	CATF7.3 CATF7.4	140	100% *T. novaezelandiae*, 99% *D. Marusdsi*	Y
	CATF39	MACKEREL	CATF39.9 CATF39.10	119	100% *K. pelamis*, 99% *S.australis, S.orientalis, S. chiliensis*, 98% *A.rochei, E.alletteratus, E.lineatus, E.affinis*	Y
	CATF40	MACKEREL	CATF40.9 CATF40.10	119	100% *K. pelamis*, 99% *S.australis, S.orientalis, S. chiliensis*, 98% *A.rochei, E.alletteratus, E.lineatus, E.affinis*	Y
N.6	CATF8	EAST BONITO	CATF8.3 CATF8.4	119	100% *K. pelamis*, 99% *S.australis, S.orientalis, S. chiliensis*, 98% *A.rochei, E.alletteratus, E.lineatus, E.affinis*	Y
	CATF36	BONITO	CATF36.7 CATF36.8	119	100% *K. pelamis*, 99% *S.australis, S.orientalis, S. chiliensis*, 98% *A.rochei, E.alletteratus, E.lineatus, E.affinis*	Y
	CATF37	BONITO	CATF37.9 CATF37.10	119	100% *K. pelamis*, 99% *S.australis, S.orientalis, S. chiliensis*, 98% *A.rochei, E.alletteratus, E.lineatus, E.affinis*	Y

N.7	CATF9	TUNA	CATF9.5 CATF9.6	119	100% *E.lineatus E. affinis*, 98% *E. alletteratus*, *K. pelamis*, *P. triacanthus*	Y
	CATF29	TUNA	CATF29.6 CATF29.7	119	100% *E.lineatus E. affinis*, 98% *E. alletteratus*, *K. pelamis*, *P. triacanthus*	Y
	CATF45	TUNA	CATF45.6 CATF45.7	119	100% *E.lineatus E. affinis*, 98% *E. alletteratus*, *K. pelamis*, *P. triacanthus*	Y
N.8	CATF10	PINK TUNA	CATF10.9 CATF10.10	119	100% *K. pelamis*, 99% *S.australis, S.orientalis, S. chiliensis*, 98% *A.rochei*, *E.alletteratus*, *E.lineatus*, *E.affinis*	N
	CATF16	PACIFIC TUNA	CATF16.7 CATF16.8	119	100% *Thunnus* sp., 99% tuna like species	N
	CATF46	PINK TUNA	CATF46.4 CATF46.5	119	100% *K. pelamis*, 99% *S.australis, S.orientalis, S. chiliensis*, 98% *A.rochei*, *E.alletteratus*, *E.lineatus*, *E.affinis*	N
N.9	CATF11	BONITO	CATF11.6 CATF11.8	119	100% *E. affinis*, 98% *K. pelamis*, *P. triacanthus*	N
	CATF14	BONITO	CATF14.6 CATF14.7	119	100% *K. pelamis*, 99% *S.australis, S.orientalis, S. chiliensis*, 98% *A.rochei*, *E.alletteratus*, *E.lineatus*, *E.affinis*	Y
	CATF42	BONITO	CATF42.6 CATF42.7	119	100% *Auxis rochei*, 99%*A.thazard*, 98%*K.pelamis*, 97%*Thunnus* sp.	N
N.10	CATF17	TUNA	CATF17.9 CATF17.10	119	100% *K. pelamis*, 99% *S.australis, S.orientalis, S. chiliensis*, 98% *A.rochei*, *E.alletteratus*, *E.lineatus*, *E.affinis*	N
	CATF32	TUNA	CATF32.9 CATF32.10	119	100% *K. pelamis*, 99% *S.australis, S.orientalis, S. chiliensis*, 98% *A.rochei*, *E.alletteratus*, *E.lineatus*, *E.affinis*	N
	CATF38	TUNA	CATF38.9 CATF38.10	119	100% *K. pelamis*, 99% *S.australis, S.orientalis, S. chiliensis*, 98% *A.rochei*, *E.alletteratus*, *E.lineatus*, *E.affinis*	N
N.11	CATF19	BONITO (E.affinis)	CATF19.9 CATF19.10	119	100% *K. pelamis*, 99% *S.australis, S.orientalis, S. chiliensis*, 98% *A.rochei*, *E.alletteratus*, *E.lineatus*, *E.affinis*	Y
	CATF25	BONITO (E.affinis)	CATF25.6 CATF25.7	119	100% *E.lineatus E. affinis*, 98% *E. alletteratus*, *K. pelamis*, *P. triacanthus*	N
	CATF35	BONITO (E.affinis)	CATF35.6 CATF35.7	119	100% *E.lineatus E. affinis*, 98% *E. alletteratus*, *K. pelamis*, *P. triacanthus*	N
N.12	CATF23	TUNA	CATF23.6 CATF23.7	119	100% *E.lineatus E. affinis*, 98% *E. alletteratus*, *K. pelamis*, *P. triacanthus*	Y
	CATF33	TUNA	CATF33.9 CATF33.10	119	100% *K. pelamis*, 99% *S.australis, S.orientalis, S. chiliensis*, 98% *A.rochei*, *E.alletteratus*, *E.lineatus*, *E.affinis*	N
	CATF34	TUNA	CATF34.9 CATF34.10	119	100% *K. pelamis*, 99% *S.australis, S.orientalis, S. chiliensis*, 98% *A.rochei*, *E.alletteratus*, *E.lineatus*, *E.affinis*	N
	CATF47	TUNA	CATF47.4 CATF47.5	119	100% *K. pelamis*, 99% *S.australis, S.orientalis, S. chiliensis*, 98% *A.rochei*, *E.alletteratus*, *E.lineatus*, *E.affinis*	N
	CATF30	TUNA	CATF30.9 CATF30.10	119	100% *K. pelamis*, 99% *S.australis, S.orientalis, S. chiliensis*, 98% *A.rochei*, *E.alletteratus*, *E.lineatus*, *E.affinis*	N

N.13	CATF43	TUNA	CATF43.6 CATF43.7	119	100% *E.lineatus E. affinis*, 98% *E. alletteratus*, *K. pelamis*, *P. triacanthus*	Y
	CATF44	TUNA	CATF44.6 CATF44.7	119	100% *E.lineatus E. affinis*, 98% *E. alletteratus*, *K. pelamis*, *P. triacanthus*	Y

Table 2: List of Fillets (F) analyzed in the study. The sampled cans are grouped by brand (from 1 to 13). The results of the BLAST analysis are reported up to 98% identity. The mislabeling are highlighted in grey. PROD.= product; SEQ. L= Sequence Length; MISLAB: Mislabeled; Y=Yes; N=No.

Table

Reverse primers: code and sequence	**TM(°C) PL bp**	**Forward primers: code and sequence**	**TM (°C) PL bp**	AL with and (w/o) primers
REV16S-1 GGTCGCCCCAACCKAAG	58.8 / 17	**FOR16S-1 5'-GACGAGAAGACCCTATGG-3'**	56.0 / 18	108 (73)
REV16S-2 CTGTTATCCCTAGGGTAACT	55.3 / 20			242 (207)
		FOR16S-2 5'-CTTMGGTTGGGGCGACC-3'	58.8 / 17	152 (117)
M13Rev(-29)- CAGGAAACAGCTATGACC	- / 18	M13For(-21)- TGTAAAACGACGGCCAGT	- / 18	

Table 3: Primers designed in this study and universal tails coupled to the selected primer for the amplification of *16SrRNA* gene fragments. TM: melting temperature, PL: primer length, AL: amplicon length calculated on the sequences FR849595 of *Sardina pilchardus*.

Table

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Family	Commercial name	Species	N. of specimens	Research Institute
SCOMBRIDAE	Atlantic bluefin tuna	*Thunnus thynnus*	5	Metabolic Physiology, Heinrich-Heine-Universitaet Duesseldorf, Germany
			5	AquaBioTech Group, Malta
	Yellowfin tuna	*Thunnus albacares*	2	Dept. Marine Biosciences, University of Marine Science and Technology, Tokyo, Japan
			1	Fish Lab, Pisa University, Italy
			2	Laboratory of Parasitology Joint Faculty of Veterinary Medicine Yamaguchi University, Japan
	Bigeye tuna	*Thunnus obesus*	3	Laboratory of Parasitology Joint Faculty of Veterinary Medicine Yamaguchi University, Japan
	Albacore	*Thunnus alalunga*	2	Laboratory of Parasitology Joint Faculty of Veterinary Medicine Yamaguchi University, Japan
			2	Fundação Universidade Federal do Rio Grande, Brazil
	Southern bluefin tuna	*Thunnus maccoyii*	1	Metabolic Physiology, Heinrich-Heine-Universitaet, Duesseldorf, Germany
	Longtail tuna	*Thunnus tonggol*	10	Dept. Marine Biosciences, University of Marine Science and Technology, Tokyo, Japan
	Pacific Bluefin tuna	*Thunnus orientalis*	2	Dept. Marine Biosciences, University of Marine Science and Technology, Tokyo, Japan
			8	Japan, Hiroshi Sato
	Frigate Tuna	*Auxis thazard*	2	Dept. Marine Biosciences University of Marine Science and Technology, Tokio, Japan
	Bullet tuna	*Auxis rochei*	2	Dept. Marine Biosciences, University of Marine Science and Technology, Tokyo, Japan
	Kawakawa (mackerel tuna)	***Euthynnus affinis***	1	Louisiana State University
			9	Dept. Marine Biosciences, University of Marine Science and Technology, Tokyo, Japan
	Little tunny	*Euthynnus alletteratus*	2	Fish Lab, Pisa University, Italy
	Black skipjack tuna	***Euthynnus lineatus***	1	Marine Vertebrate Collection Scripps, Institution of Oceanography University of California, USA
			8	NOAA National Marine Fisheries Service, USA
	Skipjack tuna	*Katsuwonus pelamis*	2	Dept. Marine Biosciences, University of Marine Science and Technology, Tokyo, Japan
	Slender tuna	***Allothunnus fallai***	2	Marine Vertebrate Collection Scripps, Institution of Oceanography University of California, USA
	Striped Bonito	***Sarda orientalis***	2	Dept. Marine Biosciences, University of Marine Science and Technology, Tokyo, Japan
			1	Marine Vertebrate Collection Scripps Institution of Oceanography University of California, USA
	Australian bonito	***Sarda australis***	5	NSW Department of Primary Industries, CFRC, Australia
	Pacific Bonito	***Sarda chiliensis***	10	NOAA Fisheries - Southwest Fisheries Science Center
	Mackerel	*Scomber scombrus*	1	Fish Lab, Pisa University, Italy
			1	NAFC Marine Centre, UK
	Chub mackerel	*Scomber japonicus*	1	Fish Lab, Pisa University, Italy
			2	Instituto de Investigaciones Marinas de Vigo (CSIC), Spain

			2	Fisheries Laboratory, Kinki University
	Blue mackerel	*Scomber australasicus*	4	Pepperell Research & Consulting Pty Ltd
	Atlantic Chub Mackerel	*Scomber colias*	4	Instituto de Investigaciones Marinas de Vigo (CSIC), Spain
			1	Direção de Serviços de Investigação e Desenvolvimento da Pesca - Direção Regional de Pescas, Madera
CLUPEIDAE	Sardine	*Sardina pilchardus*	2	Fish Lab, Pisa University, Italy
	Round sardinella	*Sardinella aurita*	2	Fish Lab, Pisa University, Italy
	European sprat	*Sprattus sprattus*	2	Fish Lab, Pisa University, Italy
	Atlantic herring	*Clupea harengus*	2	Fish Lab, Pisa University, Italy
ENGRAULIDAE	European anchovy	*Engraulis encrasicolus*	4	Fish Lab, Pisa University, Italy
	Japanese anchovy	*Engraulis japonicus*	2	Fish Lab, Pisa University, Italy
SALANGIDAE	Icefish	*Neosalanx taihuensis*	5	Fish Lab, Pisa University, Italy
	Icefish	*Neosalanx anderssoni*	2	Fish Lab, Pisa University, Italy
	Noodle fish	*Protosalanx chinensis*	2	Fish Lab, Pisa University, Italy

Table 1SM: Reference samples, tissue or DNA (highlighted in gray), used in the study. The DNA samples belong to reference specimens analyzed in a previous study (Armani *et al*., 2012). The species whose *16srRNA* sequences were produced and deposited in GenBank are in bold (see Table TBLE 2SM for Accession Number).

Table

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Family	Species	Genbank accs. number	References
SCOMBRIDAE	*Thunnus thynnus*	NC004901 NC014052 AB097669	Broughtoun Reneau 2006 Martinez Ibarra *et al*. Unpub (2009) Manchado *et al* 2004
	Thunnus albacares	GU946660-61 GU324164-65 HM071029	Cawthorn *et al* 2012 Nicole *et al.*, 2010 Little *et al.*, 2010
	Thunnus obesus	NC014059 HQ592266 to 68 HM071030	Martinez Ibarra *et al*. Unpub (2009) Cawthorn et al 2012 Little et al. 2010
	Thunnus alalunga	GU946662-63-64, NC005317 JN086151	Cawthorn et al 2012 Manchado et al unpub. Martinez Ibarra *et al*. Unpub (2009)
	Thunnus maccoyii	NC014101	Martinez Ibarra *et al*. Unpub (2009).
		LN558761	This study (Heinrich-Heine-Univ. Duesseldorf)
	Thunnus tonggol	GU325784 NC020673,JN086154	Hisieh et al unpub (submission 2009) Martinez Ibarra *et al*. Unpub (submission 2009).
	Thunnus orientalis	JN097816 KF906721 NC008455 GU256524	Ahn et al direct sub (2011) Araujo et al 2013 Takashima et al., 2006 Martinez Ibarra *et al*. Unpub (Submission2009)
	Auxis thazard	AB105447	Catanese et al 2008
		LN558762-63	This Study (Marine Biosciences, Tokyo University)
	Auxis rochei	AB103467-68, NC005313	Catanese et al 2008
	Euthynnus affinis	LN558764,66 to 68 LN558765	This study (Marine Sci. and Technol, Tokyo) This study(Louisiana State University)
	Euthynnus alletteratus	NC004530	Manchado et al Unpub. (submission 2003)
		LN558769, LN558770	This study (FishLab, Pisa University)
	Euthynnus lineatus	LN558771 LN558772 to 75	This study (Mar. Vertebrate Coll. Scripps University California) This study (NOAA National Marine Fisheries Service)
	Katsuwonus pelamis	HQ592230 to 32 NC005316 GU256527	Martinez Ibarra *et al*. Unpub (Submission2009)
	Allothunnus fallai	AY958653	Byrne *et al*. unpubl. (submission 2005)
		LN558788	This study (Mar. Vertebrate Coll. Scripps University California)
	Sarda orientalis	LN558781-82 LN558783	This study (Marine Biosciences, Tokyo University) This study (Mar. Vertebrate Coll. Scripps University California)
	Sarda australis	LN558784-87	This study (NSW Department of Primary Industries, CFRC)
	Sarda chiliensis	LN558776 to 80	This study (NOAA Fisheries - Southwest Fisheries Science Center)
	Scomber scombrus	FN688174 to 77 AB120717	Kochzius *et al*., 2010 Takashima *et al*., 2006
	Scomber japonicus	AB032521 HQ592254-56 FN688168	Sezaki *et al*., 2001 Cawthorn *et al*., 2012 Kochzius *et al*., 2010
	Scomber colias	NC013724; AB488406	Catanese *et al*., 2010
	Scomber australasicus	NC013725 AB032522 GU018106-07 DQ660418	Catanese *et al*., 2010 Sezaki *et al*., 2001 Ling et al (2009 unpublished) Casper *et al*. 2007
TRACHURIDAE	*Trachurus capensis*	GU946665 to 67	Cawthorn *et al*., 2012
	Trachurus japonicus	JQ178230 AP003091-92; NC002813	Kim *et al*., Unpub (2011) Mabuchi *et al* 2007
	Trachurus longimanus	AB642270 to 74	Yanagimoto & Hoshino un pub (2011)
	Trachurus mediterraneus	FN688250 to 52	kochzius *et al*., 2010
	T. novaezelandiae	DQ660424-25	Casper *et al*., 2007
	Trachurus picturatus	FN688253 to 57	Kochzius *et al*., 2010
	Trachurus symmetricus	JN387141 AY820735	Venegas *et al*., Unpub. (2011) Byrne *et al*., unpub (2004).

		EF458420-21, EF458453	Park *et al*., direct sub (2007)
	Trachurus trachurus	AB096007 FN688258 to 60 AB108498	Takashima *et a*l. 2006 Kochzius *et al*., 2010 Takashima *et al*. 2006
CLUPEIDAE	*Sardina pilchardus*	FR849595 to 98 NC009592	Armani *et al.*, 2012 Lavoue *et al.*, 2007
Family	Species	Genbank accs. number	References
CLUPEIDAE	*Sardinella aurita*	FR849559-60 DQ912067 EU552782 AM911207	Armani *et al.*, 2012 Li & Orti 2007 Wilson *et al.*, 2008 Jerome *et al.*, 2008
	Sardinella albella	NC016726	Lavoué *et al*., 2013
	Sardinella fimbriata	KC461222	De Battisti *et al*,. 2014
	Sprattus sprattus	FR849561-62 AM911201 NC009593	Armani *et al.*, 2012 Jerome *et al.*, 2008 Lavoué *et al*., 2007
	Clupea harengus	HQ592201 to 03 AM911204 NC009577	Cawthorn *et al.*, 2012 Jerome *et al.*, 2008 Lavoué *et al*., 2007
ENGRAULIDAE	*Engraulis encrasicolus*	FR849579-82 NC009581	Armani *et al.*, 2012 Lavoue *et al*., 2007
	Engraulis japonicus	FR851415-16 HQ592225-26 NC_003097	Armani *et al*.,2012 Cawthorn *et al* 2012 Inoue *et al*. 2001
	E. heteroloba	HM622117 AB246183	Yu, dir. Submission 2010 Akasaki *et al.*, 2006
	E.punctifer	AP011561	Lavoue *et al.*, 2010
	Encrasicholina sp.	HM622117	Yu, dir. Submission 2010
SALANGIDAE	*Neosalanx taihuensis*	FR849565-67 FR849571-72	Armani *et al.*, 2012
	Neosalanx anderssoni	FR849563-64 HM151509 to HM151511	Armani *et al.*, 2012 Guo *et al.* 2011
	Neosalanx brevirostris	HM151512-13	Guo *et al*. 2011
	Neosalanx jordani	HM151523-26	Guo *et al*. 2011
	Neosalanx oligodontis	HM151527-28	Guo *et al*. 2011
	Protosalanx chinensis	FR851413-14 HM151504 to 06	Armani *et a*l., 2012 Guo *et al.*, 2011
ANGUILLIDAE	*Anguilla anguilla*	EU315230-31 AJ244825-26 KJ564219	Frankowski *et al*., 2009 Bastrop *et al.*, 2000 *Jacobsen et al.*, 2014
	Anguilla australis	AJ244830 AB278721 to 24	Bastrop *et al*.2000 Minegishi *et al.*, 2014
	Anguilla bengalensis	AP007245	Minegishi *et al* .,2005
	Anguilla bicolor pacifica	AB278736 to 40	Minegishi *et al* .,2014
	Anguilla celebesensis	AB097748 -50 AB097723-24	Aoyama *et al*., 2003
	Anguilla dieffenbachii	AP007240 AB021754	Minegishi *et al* .,2005
	Anguilla interioris	AB021764 AB188422-24-25 AP007241	Aoyama, 1998 Kuroki 2007 Minegishi *et al.*, 2005
	Anguilla japonica	AB278885 to 89	Minegishi *et al.*, 2014
	Anguilla luzonensis	AB663553 to 57	Kuroki *et al.*, 2012
	Anguilla malgumora	AB021752, AB097711 AB188417-18-20	Aoyama, 1998 Kuroki dir. Submission 2007
	Anguilla megastoma	AP007243 AB021758	Minegishi *et al.*2005 Aoyama 1998

	Anguilla obscura	AB097702 AB021762	Aoyama *et al.*, 2003 Aoyama 1998
	Anguilla reinhardtii	DQ645686 AP007248 AB021761	Lopez *et al*.2007 Minegishi *et al*.2005 Aoyama *et al* 1999
	Anguilla rostrata	KJ564170 -71 AB021759 EU315233-34	Jacobsen *et al* 2014 Aoyama 1998 Frankowski *et al* 2009

Table 2SM: Reference sequences included into the FINS analysis. Those highlighted in gray were produced in this study.

Fifure

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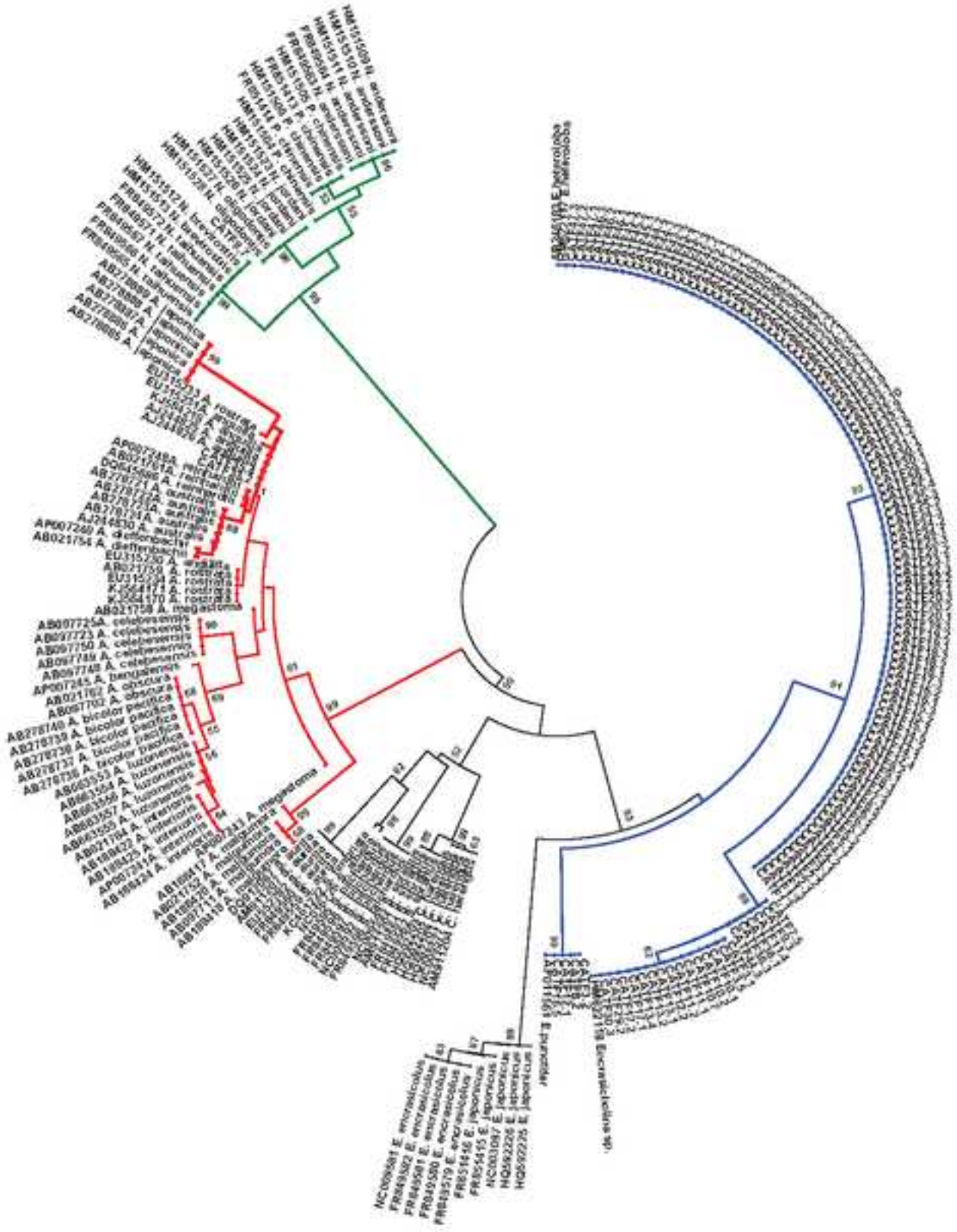

Figure

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