RESEARCH ARTICLE

Use of DNA barcoding in the assignment of commercially valuable fish species from Romania

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Abstract – DNA barcoding is a molecular technique frequently used either for biodiversity monitoring and fish conservation or in the identification of the species of origin for raw and processed food from restaurants or fish markets. The most important aspect of this technique is that it works for all stages of life and can be used to distinguish between closely related taxa. Also, the technique has been used to unmask attempts of frauds in fish markets where more desirable and expensive fish are sometimes substituted with lower valued species. Our study aims to test the utility of the COI barcoding gene in the correct identification of several economically and ecologically valuable fish species, and interspecies hybrids, from Romanian wild fauna, aquaculture and fish markets. By combining the 122 COI sequences generated here with additional barcodes downloaded from GenBank and BOLD we screened several members of Acipenseriformes and Salmoniformes. While COI was able to correctly classify the majority of Salmoniformes, the identification process for Acipenseriformes proved more difficult, especially in the case of hybrids. Although the results show that DNA barcodes present a low degree of interspecies variation making classification possible only at the genus level, this technique is still relatively cheap, fast, and useful in the identification of incorrectly labelled market products.

Keywords: DNA barcoding / Salmoniformes / Acipenseriformes / Wild fauna / Aquaculture / Fish market

1 Introduction

DNA barcoding is a molecular technique that is commonly used for biodiversity monitoring and fish conservation (Hebert et al., 2003; Holmes et al., 2009), as well as in the species-level identification of raw and processed food from restaurants or fish markets (Filonzi et al., 2010; Cline, 2012). DNA barcoding is a powerful tool because it works for all stages of life and it can be used to distinguish between closely related taxa (Stoeckle et al., 2004). Subunit I of the cytochrome oxidase (COI) gene has been used in numerous studies with high degree of success (Galimberti et al., 2012). For instance, COI has been successfully used to identify freshwater fish species with over a 93% success rate (Ward et al., 2009). There is great interest in using COI barcoding for aquatic species as indicated by the numerous scientific articles on this topic (Taylor and Harris, 2012). There are also efforts to identify shorter DNA fragments for use as barcodes, called mini-barcodes, for situations where

obtaining a fragment longer than 400bp is not possible (Meusnier et al., 2008; Dubey et al., 2011; Little, 2014; Shokralla et al., 2015). The barcoding technique alone is not suitable for the detection of hybrids for which both nuclear and mitochondrial markers should be assessed. Instead, DNA barcoding can be used to infer the maternal origin of hybrids.

Our study aims to test the utility of the COI barcoding gene and the public databases to accurately identify several economically and ecologically valuable fish species and interspecies hybrids from Romanian wild fauna, aquaculture and fish markets, to determine if COI can be used to identify the maternal lineage of known hybrids and to determine if cultured fishes and those sold in fish markets are correctly identified and labelled.

The issue of mislabelling fish or fish-derived products is a serious one, as there are laws that mandate correct product identification, regardless of its provenience [Council Regulation (EC) No. 1379/2013, <http://eur-lex.europa.eu>]. However, studies show that up to 30% of fish products are mislabelled in fish markets and restaurants with the problem being particularly acute in the latter (Pardo et al., 2016). For instance, Ludwig et al. (2015) showed in an analysis of 27

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caviar samples from Romania and Bulgaria, that only ten of them were correctly labelled, while the remaining samples were from *Cyclopterus lumpus*, a less economically valuable fish species whose roe are often used as a substitute for caviar.

Romania's fisheries production has been in decline, from a harvest of 270 000 tons in 1986 to 16 238 tons in 2002 (www.fao.org). The most common species used in aquaculture in Romania are the common carp (*Cyprinus carpio*), the silver crucian carp (*Carassius auratus gibelio*), the rainbow trout (*Oncorhynchus mykiss*), and the brown trout (*Salmo trutta fario*). In the last two decades, sturgeon rearing has become more widespread in Romania with the most popular species being the stellate sturgeon (*Acipenser stellatus*), Russian sturgeon (*Acipenser gueldenstaedtii*), beluga sturgeon (*Huso huso*), and the sterlet (*Acipenser ruthenus*) (Smederevac-Lalić *et al.*, 2011). Exotic species such as *Acipenser baerii* and sturgeon hybrids such as: bester (♀ *H. huso* × ♂ *A. ruthenus*), sterbe (♀ *A. ruthenus* × ♂ *H. huso*) and best beluga [♀ (*H. huso* × ♂ *A. ruthenus*) × ♂ *H. huso*] are also farmed (Costache *et al.*, 2012; Dudu *et al.*, 2015). A more recent report states that in 2013 cyprinids made up more than 80% of fish production globally, while trout was only 11%, and sturgeon, along with pike-perch and perch, made up only 2% (www.eurofish.dk).

Sturgeons are listed in the CITES Appendices (Convention on International Trade in Endangered Species of Wild Fauna and Flora), due to habitat destruction, river regulation and overexploitation (Raymakers, 2007). Sturgeons' protected status as endangered has increased the need for aquaculture farming in order to satisfy the demand for caviar and other sturgeon derived products. To meet the growing demand, fish farms have introduced sturgeon hybrids in their aquaculture programs in order to take advantage of the rapid growth rate and high-quality caviar. This is the case for the bester hybrid (♀ *H. huso* × ♂ *A. ruthenus*), which produces high-quality caviar at a younger age compared to beluga sturgeon (Dudu *et al.*, 2015). Historically, six sturgeon species were native to the Danube River. However, due to the Iron Gate Dams, only four species now populate the Lower section of the river (Paraschiv *et al.*, 2006). The sturgeons found in the Lower Danube are the beluga sturgeon, Russian sturgeon, stellate sturgeon and sterlet (Bacalbasa-Dobrovici, 1997; www.danube-sturgeons.org).

Members of the *Salmonidae* family that populate Romanian waters are of great ecological and economical value such as the brown trout (*S. trutta fario*), brook trout (*Salvelinus fontinalis*), grayling (*Thymallus thymallus*) and the huchen (*Hucho hucho*). Most native species are found in mountainous streams with the non-native rainbow trout (*O. mykiss*) being predominantly raised in fish farms (Dudu *et al.*, 2011a; Georgescu *et al.*, 2011).

The IUCN (International Union for Conservation of Nature) status of these species is as follows: critically endangered for *A. stellatus*, *A. gueldenstaedtii*, *H. huso*; endangered for *A. baerii* and *H. hucho*; vulnerable for *A. ruthenus*; least concern for *S. trutta* and *T. thymallus*, while the statuses of *O. mykiss* and *S. fontinalis* have yet to be assessed (Gessner *et al.*, 2010).

2 Materials and methods

2.1 Sampling and DNA extraction

Samples used in this study were either wild caught or collected from participating fish farms throughout Romania. The wild individuals came from several Romanian rivers: Porumbacu, Avrig, Topolog, Cerna and the Danube river, while the fish farms were located in the following counties: Tulcea, Vâlcea, Gorj, Caras-Severin, Cluj-Napoca, Bistrița, Olt and Călărași (Supplementary Material 1). The fish market samples came from three supermarkets from Bucharest and two fish farms from Tulcea County.

We processed 64 samples from the family *Salmonidae* and 58 from the family *Acipenseridae* (Fig. 1), morphologically identified or using the product's market label. Tissue samples taken were either fin clips or muscle tissue. DNA was extracted from each tissue using a Chelex-resin based protocol (Estoup *et al.*, 1996), while the quality and quantity of the DNA were assessed by using NanoDrop 8000 (Thermo Scientific).

2.2 DNA Amplification and sequencing

DNA amplification of a 5' fragment of the cytochrome oxidase subunit 1 (COI) gene was done using the standard fish DNA barcoding primers, FishF1: 5'TCAACCAACCACAAA GACATTGGCAC3'/Fish R1: 5'TAGACTTCTGGGTGGC CGAAGAATCA3' (Ward *et al.*, 2005). However, in samples that failed to amplify we used the following primers which were designed using the Primer3 software (Untergasser *et al.*, 2012): StrSfo F: 5'TCCACCGCTTAACTCTCAG3'/StrSfo R: 5'CCGGGTCAAAGAAAGTGTA3', ThyF: 5'TCCACC GCTTAAACACTCAG3'/ThyR: 5'CTGGGTCAAAGAAAG TGGTA3' and Stur F: 5'GAAGGGGACTTTAACCTCTG3'/ Stur R: 5'TAGGCCCGTGTGTCTACGTCC3'. The PCR amplifications were performed in a 25 µl final reaction volume containing: 50 ng of DNA template, 1× PCR buffer (Applied Biosystems), 1.5 mM MgCl₂ (Applied Biosystems), 0.4 mM of each dNTP (Applied Biosystems), 0.4 µM of F primer, 0.4 µM of R primer, 1 U of AmpliTaq Gold Polymerase (Applied Biosystems). The PCR reaction conditions were: 95 °C for 10 min, 35 cycles of 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 60 s, followed by a final extension of 72 °C for 10 min and the amplifications were performed on a Verity Thermal Cycler (Applied Biosystems). The resulting PCR products were electrophoretically assessed for quality, and purified using Wizard SV Gel and PCR Clean-Up System (Promega). Separate forward and reverse strand sequencing amplification was done using BigDye Terminator v3.1 kit (Applied Biosystems). The resulting products were purified using the BigDye XTerminator Purification Kit (Applied Biosystems), followed by sequencing on the ABI Prism 3130 DNA Genetic Analyzer (Applied Biosystems) automatic platform.

2.3 Genetic diversity and phylogenetic analysis

COI sequences were edited using BioEdit Sequence Alignment Editor (Hall, 1999). To identify the sequences, we used the following steps proposed by Jo *et al.* (2014):

Fig. 1. Map of sampling sites for the 122 specimens collected from Romania (rivers and fish farms) and Romanian fish market. Each group (G) represents the specimens presented in [Supplementary Material 1](http://www.freeworldmaps.net/europe/romania/romania-hd-map.jpg) (<http://www.freeworldmaps.net/europe/romania/romania-hd-map.jpg>).

(1) search the available online database for a $\geq 98\%$ match with a known species and (2) construct a phylogenetic tree in order to observe the clustering of our sequences and known species. These authors propose an identification score of $\geq 98\%$ to classify a species, while a score of $< 98\%$ indicates a higher classification level (genus, family, etc.). The topology of the phylogenetic tree is taken into consideration when interpreting the identification scores. We used Web BLAST (Basic Local Alignment Search Tool) on GenBank ([Benson et al., 2013](#)) with the default settings, in order to identify the nearest matching sequences, the Ident and E(xpect) values. Additionally, we used the Identification option on BOLD (Barcode of Life Data Systems; [Ratnasingham and Hebert, 2013](#)) to obtain a list of sequences with the highest similarity. Furthermore, we downloaded the nearest matching sequences from our searches on BOLD and GenBank ([Supplementary Material 2](#)) in order to conduct a phylogenetic analysis. For this purpose, all the sequences were aligned and trimmed to 424 bp. The final dataset was comprised of 234 sequences: 122 generated here and 112 retrieved from the online databases. The sequences highlighted in grey in [Supplementary Material 1](#) were from individuals for which taxonomic classification, based on morphology, was conducted in the

field by ichthyologists. We consider these sequences as reference barcodes for the respective species. Sequences were aligned using MAFFT (www.mafft.cbrc.jp/alignment/server; [Katoh and Standley, 2013](#)). The best fitting molecular substitution model was assessed using jModelTest2 ([Guindon and Gascuel, 2003](#); [Darriba et al., 2012](#)) on CIPRES Science Gateway (www.phylo.org; [Miller et al., 2010](#)) with the default settings, while the phylogenetic tree construction was conducted using MEGA6 ([Tamura et al., 2013](#)). The trees were visualized with FigTree v1.4 (www.tree.bio.ed.ac.uk/software/figtree). The outgroups used here were *Plecoglossus altivelis* for salmonids (an *Osmeridae* family representative closely related to Salmoniformes) and *Polyodon spathula* for acipenserids (a *Polyodontidae* family representative belonging to the order Accipenseriformes). The robustness of the phylogenetic relationships was assessed using 1000 replicate bootstraps and the two Maximum Likelihood (ML) trees were constructed using the Tamura3 parameter model ([Tamura, 1992](#)), chosen based on the Akaike criterion. Additionally, we used the adegenet R package (along with its dependencies) ([Jombart and Ahmed, 2011](#)) for determining and plotting the genetic distances under the K2P model ([Kimura, 1980](#)).

Table 1. List of BOLD and GenBank screening results. Highlighted are the erroneous sequences from GenBank or BOLD offered for maximum identity scores.

Sample name	ID based on morphology	ID based on COI barcode	BOLD similarity	BOLD species	GenBank ident.	GenBank E-value	GenBank species
Str_4.5, Str_3.3, Str_3.2, Str_3.1, I11, Str_3.18, Str_4.18, Str_5.16, Str_5.24, Str_3.4, Str_4.4, Str_4.2, Str_4.1, Str_3.5, Str_4.7, Str_5.28, Str_5.1, Str_5.12	<i>Salmo trutta fario</i> (except I11 which was initially identified as <i>Oncorhynchus mykiss</i>)	<i>Salmo trutta fario</i>	100%	<i>Salmo fario</i> ides	100%	0.0	<i>Salmo fario</i> ides
Lo5	<i>Hucho hucho</i>	<i>Salmo trutta fario</i>	100%	<i>Salmo trutta</i> , <i>Salmo obtusirostris</i> , <i>Salmo fibreni</i> , <i>Salmo letnica</i> , <i>Salmo fario</i> ides, <i>Salmo zmanjaensis</i> , <i>Salmo visovacensis</i>	100%	0.0	<i>Salmo trutta fario</i> , <i>Salmo labrax</i> , <i>Salmo trutta</i> <i>caspius</i> , <i>Salmo trutta trutta</i> , <i>Salmo zmanjaensis</i> , <i>Salmo</i> <i>fibreni</i> , <i>Salmo letnica</i> , <i>Salmo visovacensis</i> , <i>Salmo</i> <i>obtusirostris</i> , <i>Salmo trutta</i>
19	<i>Salmo trutta</i>	<i>Salmo trutta fario</i>	100%	<i>Salmo labrax</i>	100%	0.0	<i>Salmo labrax</i>
Str_3a, I6	<i>Salmo trutta</i>	<i>Salmo trutta fario</i>	100%	<i>Salmo trutta</i>	100%	0.0	<i>Salmo trutta</i> , <i>Salmo trutta</i> <i>fario</i> , <i>Salmo Pellegrini</i> , <i>Salmo cettii</i> , <i>Salmo trutta trutta</i>
L1_FM	<i>Salmo salar</i>	<i>Salmo salar</i>	100%	<i>Salmo salar</i>	100%	0.0	<i>Salmo salar</i>
Hh_Lo	<i>Hucho hucho</i>	<i>Hucho hucho</i>	100%	<i>Hucho hucho</i>	100%	0.0	<i>Hucho hucho</i> , <i>Leucaspis</i> <i>delineatus</i>
Sfo_4a, Sfo_I13, Sfo_I5, Sfo_I7, Sfo_I2, B3_FM, Sfo_I16	<i>Salvelinus fontinalis</i> (except the B3_FM sample that was labelled as <i>Oncorhynchus mykiss</i>)	<i>Salvelinus fontinalis</i>	100%	<i>Salvelinus fontinalis</i>	100%	0.0	<i>Salvelinus fontinalis</i>
Li10	<i>Thymallus thymallus</i>	<i>Salvelinus fontinalis</i>	99.76%	<i>Salvelinus fontinalis</i>	99%	0.0	<i>Salvelinus fontinalis</i>
Omy_9a, Omy_18a, Omy_17a, Omy_16a,	<i>Oncorhynchus mykiss</i> (except the I4 and I3)	<i>Oncorhynchus mykiss</i>	100%	<i>Oncorhynchus mykiss</i>	100%	0.0	<i>Oncorhynchus mykiss</i>

Table 1. (continued).

Sample name	ID based on morphology	ID based on COI barcode	BOLD similarity	BOLD species	GenBank ident.	GenBank E-value	GenBank species
Omy_13a, Omy_10a, Omy_14a, Omy_4a, Omy_3a, Omy_1a, Omy_20a, Omy_12a, Omy_19a, Omy_8a, Omy_7a, Omy_6a, Omy_5a, Omy_2a, Omy_112, Omy_02-Damb, Omy_05-Cand, Omy_09-1m, Omy_09-3m, Omy_09-5m, L2_FM, L3_FM, M1_FM, I4, I3	samples which were initially labelled as <i>Salmo trutta</i>)						
M2_FM	Unlabelled	<i>Oncorhynchus mykiss</i>	99.76%	<i>Oncorhynchus mykiss</i>	99%	0.0	<i>Oncorhynchus mykiss</i>
B2_FM	<i>Oncorhynchus mykiss</i>	<i>Oncorhynchus mykiss</i>	98.55%	<i>Oncorhynchus mykiss</i>	99%	0.0	<i>Oncorhynchus mykiss</i>
Tthy_Li	<i>Thymallus thymallus</i>	<i>Thymallus thymallus</i>	100%	<i>Thymallus aeliani</i>	100%	0.0	<i>Thymallus aeliani</i>
Ast_184, Ast_185, Ast_186, Ast_187, Ast_188	<i>Acipenser stellatus</i>	<i>Acipenser stellatus</i>	100%	<i>Acipenser stellatus</i>	100%	0.0	<i>Acipenser nudiventris</i> , <i>Acipenser stellatus</i>
Ast_1886, Ast_4140, Ast_8038, Ast_7188, Ast_9296, Ast_1818	<i>Acipenser stellatus</i>	<i>Acipenser stellatus</i>	100%	<i>Acipenser stellatus</i>	100%	0.0	<i>Acipenser stellatus</i>
Agu_8_17_18	<i>Acipenser gueldenstaedtii</i>	<i>Acipenser gueldenstaedtii</i>	100%	<i>Huso huso</i> , <i>Acipenser gueldenstaedtii</i>	100%	0.0	<i>Acipenser gueldenstaedtii</i> , <i>Acipenser naccarii</i> , <i>Acipenser gueldenstaedtii</i> × <i>Huso dauricus</i> , <i>Acipenser sinensis</i> , <i>Acipenser persicus</i>
Agu_61, Agu_62, Agu_65, Agu_70, Agu_72, Agu_285, Agu_284, Agu_271, Agu_273	<i>Acipenser gueldenstaedtii</i>	<i>Acipenser gueldenstaedtii</i>	100%	<i>Huso huso</i> , <i>Acipenser gueldenstaedtii</i>	100%	0.0	<i>Acipenser gueldenstaedtii</i> , <i>Acipenser naccarii</i> , <i>Acipenser gueldenstaedtii</i> × <i>Huso dauricus</i> , <i>Acipenser gueldenstaedtii</i> × <i>Acipenser baerii</i> ,

Table 1. (continued).

Sample name	ID based on morphology	ID based on COI barcode	BOLD similarity	BOLD species	GenBank ident.	GenBank E-value	GenBank species
Abae_81, Abae_84, Abae_86, Abae_93, Abae_95, Abae_96	<i>Acipenser baerii</i>	<i>Acipenser baerii</i>	100%	<i>Acipenser baerii</i>	100%	0.0	<i>Acipenser sinensis</i> , <i>Acipenser persicus</i> <i>Acipenser gueldenstaedtii</i> , <i>Acipenser baerii</i> , <i>Acipenser baerii</i> × <i>Huso dauricus</i> , <i>Acipenser baerii</i> × <i>Acipenser schrenckii</i> , <i>Acipenser persicus</i>
Begu_99, Begu_100, Begu_112, Begu_113	Sturgeon hybrid, ♀ <i>A. baerii</i> × ♂ <i>A. gueldenstaedtii</i>	Placed in the clade with <i>A. baerii</i> , <i>A. persicus</i> and <i>A. gueldenstaedtii</i>	100%	<i>Acipenser baerii</i>	100%	0.0	<i>Acipenser gueldenstaedtii</i> , <i>Acipenser baerii</i> , <i>Acipenser baerii</i> × <i>Huso dauricus</i> , <i>Acipenser baerii</i> × <i>Acipenser schrenckii</i> , <i>Acipenser persicus</i>
Hh_742, Hh_743, Hh_280, Hh_281, Hh_282, Hh_1, Hh_12, Hh_18, Hh_20 F, Br_1 FM, Br_2 FM, Bester_5, Bester_9, Bb_RS, Bb_RL, Bb_4, Bb_9	<i>Huso huso</i> (except the Br_1 FM and Br_2 FM samples which were labelled as “sturgeon”; Bester_5 and Bester_9 samples were labelled as Sturgeon hybrid, ♀ <i>H. huso</i> × ♂ <i>A. ruthenus</i> ; Bb_RS, Bb_RL, Bb_4, Bb_9 were labelled as sturgeon hybrid, ♀ (<i>H. huso</i> × ♂ <i>A. ruthenus</i>) × ♂ <i>H. huso</i>)	<i>Huso huso</i>	100%	<i>Huso huso</i>	100%	0.0	<i>Acipenser ruthenus</i> × <i>Huso dauricus</i> , <i>Huso huso</i>
Ar_144_FM, Ar_148_FM, Ar_8112_FM, Aru_250, Aru_251, Aru_252	<i>Acipenser ruthenus</i> (except Ar_144_FM, Ar_148_FM, and Ar_8112_FM which were labelled as “sturgeon”)	<i>Acipenser ruthenus</i>	100%	<i>Huso dauricus</i> , <i>Acipenser ruthenus</i>	100%	0.0	<i>Acipenser ruthenus</i>
Sterbe_1, Sterbe_2, Sterbe_3, Sterbe_4	Sturgeon hybrid, ♀ <i>A. ruthenus</i> × ♂ <i>H. huso</i>	<i>Acipenser ruthenus</i>	100%	<i>Huso dauricus</i> , <i>Acipenser ruthenus</i>	100%	0.0	<i>Acipenser ruthenus</i>

3 Results

The majority of the investigated samples was successfully identified after querying against GenBank and BOLD, a result supported by the phylogenetic analysis. However, we observed several erroneous identifications from the online querying, suggesting that both BOLD and GenBank contain several miss-identified samples (Table 1).

3.1 Species identification through DNA barcoding in salmonids

All individuals from the Porumbacu, Avrig and Topolog rivers, that were initially morphologically identified as *S. trutta fario* were classified as *Salmo farioides* by both BOLD and GenBank databases. Interestingly, one individual, initially identified as *O. mykiss*, obtained from a fish farm (Caraş-Severin), was also classified as *S. farioides* based on COI.

In another case an individual that had been morphologically identified as *H. hucho*, was classified as *Salmo spp.* based on COI (Table 1). A different sample from the same fish farm was presented as *H. hucho* with both BOLD and GenBank agreeing on identification (Table 1).

Furthermore, for one sample morphologically identified as *S. trutta*, both databases showed it to be Black Sea salmon (*Salmo labrax*, Pallas, 1814) (Table 1). Two samples coming from two different fish farms (Cluj-Napoca and Gorj Counties) were both identified as *S. trutta*, by BOLD and GenBank with a 100% similarity and identity score, although GenBank has given various other species from the genus *Salmo* (Table 1). All samples coming from the fish farms that were morphologically identified as *S. fontinalis* were assigned by correctly. This was also true for one wild sample from Cerna River that was identified as a *T. thymallus* with a score of 99%. However the identification of one fish farm sample labelled as *T. thymallus* was identified by both GenBank and BOLD as *T. aeliani* a synonym of *T. thymallus* (EUNIS Species Database, <http://eunis.eea.europa.eu/species/12979>) (Table 1). Samples coming from two different fish farms were morphologically identified as *S. trutta*, but had a maxim score in BOLD and GenBank as *S. fontinalis* (Table 1). All the samples from a fish farm (Tulcea County) morphologically identified as *O. mykiss* had maxim scores in both databases (GenBank and BOLD) that agreed.

Concerning the fish market samples, the results were mixed with some products not listing species. A sample of smoked fish was identified as *S. salar*, as the product's label stated; however, eight other samples were classified as *O. mykiss* with scores ranging from 98.5 to 100%, but only two of these had labels indicating species (Table 1). Finally, one sample was identified as *S. fontinalis* and it was, along with another mislabelled sample, sold by the marketer as wild *S. trutta* (Table 1).

3.2 Species identification through DNA barcoding in acipenserids

The species names for the pure acipenserids based on morphology were in concordance with the results from the database query. For several samples, both BOLD and GenBank

gave more than one species name with maximum similarity or identity score. The *A. stellatus* samples coming from the fish farm (Tulcea County) and from the Danube River were identified as *A. stellatus*, based on both morphology and our database queries morphology except that GenBank gave a 100% Identity score for *A. nudiventris* as well. For the samples of *A. baerii*, *H. huso* and several hybrids, namely *H. huso* × *A. ruthenus*, *H. huso* × *bester* and *A. baerii* × *A. gueldenstaedtii*, BOLD queries resulted in 100% similarity per species with one result, while GenBank gave more than three matching results, all with 100% Identity scores. The BOLD classification for the samples above was in concordance with morphological assessments, while the hybrids were successfully barcoded as their maternal genitor species. Thus, begu samples were classified as *A. baerii*, and *bester* and *best beluga* as *H. huso*. For the best beluga and *bester* hybrids GenBank showed a 100% Identity with a hybrid sample of *A. ruthenus* × *Huso dauricus*, while for the begu hybrids, GenBank showed 100% Identity for two other sturgeon hybrids *A. baerii* × *H. dauricus* and *A. baerii* × *A. schrenckii*.

All the *A. gueldenstaedtii* samples were given a maximum similarity score for both *H. huso* and *A. gueldenstaedtii*, while the *A. ruthenus* samples had a maximum similarity score for both *H. dauricus* and *A. ruthenus*. Concerning the fish market samples, two samples were identified as either *H. huso* or *A. ruthenus* × *H. dauricus* (Table 1). This is also the case for the *H. huso*, *bester*, and *best beluga* from the fish farms. Several fish market samples were identified as either *H. dauricus* or *A. ruthenus*, which is also the case for the *A. ruthenus* samples we had from the fish farm (Table 1).

3.3 Phylogenetic analysis in salmonids and acipenserids

The phylogenetic trees illustrating the clustering within Salmoniformes and Acipenseriformes orders are presented in Figure 2 and Supplementary Material 5.

Within the Salmoniformes two different clusters were highlighted: the one that groups all the *Thymallus* sequences, and the one that groups all the sequences from the genus *Salmo*, *Salvelinus*, *Oncorhynchus*, and *Hucho*. The *Salmo* cluster grouped together the sequences representing *S. trutta fario*, *S. trutta trutta*, *S. labrax*, *S. visovacensis*, *S. letnica*, *S. fibreni*, *S. farioides*, and *S. obtusirostris* along with our sequences obtained from individuals that were morphologically identified as *S. trutta fario*, *O. mykiss* and *H. hucho*. The phylogenetic analysis confirms the previous database query results and classified one sample, initially labelled as *H. hucho*, as a *Salmo spp.*, while one sample initially labelled as *O. mykiss* is, in fact, a *Salmo spp.* The analysis did not clarify the identity of the samples labelled as *S. trutta fario*, which had received maximum similarity and identity scores as *S. farioides*, since the tree topology shows that all *Salmo* sequences, except the different clade formed out of *S. salar*, are grouped without any clear boundary.

One fish market sample was grouped in the *S. salar* cluster, this being the species specified on the product label. The phylogenetic analysis grouped together seven sequences obtained in the laboratory into the *S. fontinalis* clade and 30 wild and fish market samples were grouped in the same

Fig. 2. The phylogenetic trees were inferred by using the Maximum Likelihood (ML) methods (a) for salmonids and (b) for acipenserids based on the Tamura 3-parameter model. For the ML analysis, the tree with the highest log likelihood is shown. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site. With light blue we depicted those samples that were considered reliable from taxonomic and technical points of view, while in orange we depicted the samples which were considered mislabelled after database query.

O. mykiss cluster, as the sequences retrieved from GenBank and BOLD. The *H. hucho* clade was formed out of other *H. hucho* sequences downloaded from GenBank and BOLD and one of our samples.

Regarding the acipenserids group, four samples of sturgeon hybrids along with six fish market samples were placed in the same *A. ruthenus* clade as the sequences from GenBank. All the samples that represented *A. stellatus* individuals were placed in the *A. stellatus* clade. None of our sequences were placed in the *A. nudiventris* clade, despite that the BLAST analysis gave 100% identity with *A. nudiventris* sequences from GenBank for five of our *A. stellatus* specimens. The *H. huso* clade includes, besides all of our samples labelled as *H. huso*, and the sequences retrieved from data bases designated as *H. huso*, all the best beluga sturgeon hybrid sequences, the two best sturgeon hybrids (from aquaculture), the two sturgeon fish market samples and one GenBank sequence coming from a hybrid of (♀ *A. ruthenus* × ♂ *H. dauricus*). One sturgeon hybrid is also placed in the *A. ruthenus* clade with two other sequences. All our *A. gueldenstaedtii* samples were placed in the *A. gueldenstaedtii* clade. While the *A. ruthenus*, the *A. nudiventris*, the *H. huso* and the *A. stellatus* clades formed well-defined clades, this is not the case for the rest of the acipenserids representatives, as *A. persicus*, *A. gueldenstaedtii*, *A. nacarii*, *A. baerii*, and *A. sinensis* which formed mixed clades. Our *A. baerii* sequences, along with the begu sturgeon hybrid ones were placed in the clade that contained *A. baerii*, *A. persicus* and *A. gueldenstaedtii*.

We computed the genetic distances (Supplementary Material 3) between our COI sequences using the *dist.dna*

function in *adegenet* and plotted them as histograms using all the sequences grouped based on their orders and then on their corresponding species (Fig. 3 and Supplementary Material 4). The genetic distance between the members of Salmoniformes and Acipenseriformes was 0.27, the same as the genetic distance value obtained between Salmoniformes and Acipenseriformes without hybrids. When comparing the Acipenseriformes members between them, the genetic distance had a maximum value of 0.08 (between *A. stellatus* and both *A. ruthenus* and sterbe hybrids). In the case of Salmoniformes, the maximum value for the genetic distance (0.2) was obtained for the pair *T. aeliani* and *O. mykiss*.

4 Discussions

The DNA barcoding technique is useful for restocking activities, when correct species assignment is crucial in order to maintain the population structure in the targeted rivers, this aspect being important not only for sturgeons, but also for salmonids. The socio-ecological importance is clear in the fish markets samples, where correct species labelling ensures not only compliance with the laws, but also a significant increase in customers' trust in the desired products.

The vast majority of the samples labelled as *Salmo trutta* were classified as *S. farioides* when they were compared with sequences from the databases. The phylogenetic analysis showed that proper identification of these specimens is problematic since more than one species clustered within the same group. *S. farioides* – the Adriatic brown trout (Marić and Rakočević, 2015) or Balkan brook trout (Kottelat and Freyhof, 2007) is reported to be present in the streams of the Adriatic

Fig. 3. Plot of the matrix pairwise genetic distances for all the Acipenseriformes species (a), all the Salmoniformes species (b). Darker colour indicates higher genetic difference between the groups.

basin, mainly in the Eastern part, in Croatia, Bosnia-Herzegovina, Montenegro, Albania and Greece (Kottelat and Freyhof, 2007; Marić and Rakočević, 2015), but also reported as an alien species in Serbia (Simonović *et al.*, 2015). The identification of these samples as *S. farioides* is possibly due to the relatively short fragment of the COI gene considered as barcode – thus some polymorphic sites that could have been important for proper molecular diagnostic, might have been omitted in the PCR step. We do not exclude the possibility that the databases could be erroneous, as it was reported before (Meier *et al.*, 2006; Collins and Cruickshank, 2012; Faisal *et al.*, 2012; Ardura *et al.*, 2013). Also, it is well known that brown trout shows a great eco-morphological diversity (Stelkens *et al.*, 2012) which might lead to an erroneous morphological identification. There still are ambiguities regarding the taxonomy of salmonids, so there is possible that the barcode sequences from databases might belong to a different *Salmo spp.*

For one sample coming from a fish farm and labelled as *H. hucho* resulted in a 100% identity match with *L. delineatus* (sunbleak) in GenBank (Table 1). The sunbleak is a member of the *Cyprinidae* fish family, distributed in Europe and Asia, that reaches a maximum length of 9 cm (Kottelat, 1997), as opposed to the huchen, a salmonid that has an average length of 60–120 cm.

Considering the phylogenetic tree topology, all of the *L. delineatus* sequences, except one, were placed in a separate clade from the salmonids. We assume that the identity assigned by the BLAST analysis is erroneous, as suggested by the placement of one sequence of *L. delineatus* in a group with *H. hucho*. This might be also the case for a sequence of *A. nudiventris* retrieved from GenBank which was actually placed in the *A. stellatus* group.

Another sample morphologically identified as *S. trutta* received maximum similarity and identity with *S. labrax*

(Table 1), and the phylogenetic trees support this assumption considering that *S. labrax* sequences were clustered together with various other *Salmo spp.* sequences. The Black Sea salmon is known to live in the rivers draining into the Black Sea, but also several rivers from Macedonia (Kottelat and Freyhof, 2007). This sample came from the Cerna River, a Danube tributary from the south-western part of Romania. Although Freyhof (2011) mentions it as a native species in Romania, *S. labrax* is reported to be present especially on the marine coast of the Black Sea and sporadically in the Danube River. In Romania, *S. labrax* is endemic and considered to be critically endangered (Oțel, 2007). In the recent years sporadic catches of individuals that morphologically have been identified as *S. labrax* have been reported in different Danube Romanian tributaries, like Bistrița Aurie River, Argeș River (Freyhof *et al.*, 2013) and possibly Jiu River (Paraschiv and Suciu, 2016). Although, we do not exclude the possibility of catching *S. labrax* in the Cerna River, outside of the historical range of distribution of this species, a clear conclusion regarding the identity of our sample cannot be drawn. Unfortunately, only two sequences of *S. labrax* were deposited in the GenBank, this aspect hampering a more detailed analysis.

The cases of two fish market samples, which were identified as *O. mykiss* and *S. fontinalis*, but sold as *S. trutta*, along with the ones that did not have a species label, could represent a fraud attempt for the fish marketers to sell aquaculture individuals as wild ones. It is well known that fish coming from the wild has a much higher market price and that the Romanian consumers prefer wild brown trout. Both *S. fontinalis* and *O. mykiss* are rarely found in the wild in Romania and mostly in fish farms, raised especially for human consumption (Dudu *et al.*, 2011a; Georgescu *et al.*, 2011). Regarding the salmonids specimen identification, COI has been found useful for *S. salar*, *O. mykiss*, and *S. fontinalis* specimens.

All the sturgeon hybrids received maximum identity and similarity scores for their maternal species. Considering the maternal inheritance of the mitochondrial genome, these specimen identifications are as expected, because each sturgeon hybrid was found in the clades of their corresponding maternal genitor species. In sturgeons the phenomenon of site heteroplasmy in mtDNA has been previously encountered (Dudu *et al.*, 2012) due to the possible presence of paternal mtDNA along with the maternal mtDNA known as paternal leakage (Guo *et al.*, 2006). All the COI sequences obtained here did not show this phenomenon. The DNA barcoding technique does not allow the interspecies hybrids identification and it is not useful for the molecular diagnosis of the species that easily hybridize, which is the case of sturgeons. There was no indication about the species name or about their genetic status as pure or hybrid for the sturgeon samples taken from the fish markets. For all the five specimens, the market label was *sturgeon*, and so we can either state that the samples are sturgeon hybrids (as it might be the case for several of our samples), or they are pure sturgeon species. Either way, all the fish markets' samples are marketed as aquaculture ones, which barcoding cannot identify as correct/accurate (Cline, 2012).

One other issue detected in sturgeons is that the barcodes of *A. gueldenstaedtii*, *A. baerii*, *A. persicus*, and *A. naccarii*, along with some of their hybrids were placed in the same monophyletic clade. *A. gueldenstaedtii* is a Ponto-Caspian species for which a wild population is still found in the Lower Danube. The Russian sturgeon has a high phenotypic variability; different morphs being identified. Also, this species is closely related with *A. persicus*, *A. naccarii* and *A. baerii*. Previous studies based on morphometric, meristic and genetic data have proposed the existence of cryptic lineages (pure *A. gueldenstaedtii*, a form *A. baerii*-like and a form *A. naccarii*-like) within the species (Birstein *et al.*, 2005). The present study has shown the limitation of DNA barcoding when it is used to differentiate between these sturgeon species. Furthermore, for some of the salmonids and acipenserids samples, the 400+ bp COI sequences were inadequate for accurate specimen identification. This is not a singular case since there are other studies that dealt with this problem (Birstein *et al.*, 2009).

In order to add more accuracy to the specimen identification steps using barcoding, Rach *et al.* (2008) suggested DNA barcoding character-based approaches, while Ghadimejad *et al.* (2009) suggested that this technique could represent a working alternative especially for identifying individuals that are part of the *A. gueldenstaedtii* group. Furthermore, it is known that the congeneric sturgeon species have similar morphological characteristics and most of them inhabit the same or adjacent areas (Peng *et al.*, 2007), therefore interspecific hybridization can often occur (Ludwig *et al.*, 2002). The morphological differentiation between pure sturgeon species and their hybrids is difficult and COI is not of much help. By combining DNA barcoding with nuclear markers analyses (Ludwig, 2006; Dudu *et al.*, 2011b; Li *et al.*, 2015) the rate of correct specimen identification could increase and it could be helpful to combat illegal sturgeon or caviar trade. Recently, Bayesian fingerprinting has been proposed as an alternative to DNA Barcoding technique (Yang and Rannala, 2016). This method could be applied on various types of tissues, even on fish eggs (Lewis *et al.*, 2016).

5 Conclusions

This study showed that there are still some issues in identifying the correct species of the tested samples, a more accurate identification being possible at the genus level when barcoding is used as the primary identification technique. Furthermore, the barcodes have proven to be unsuccessful when used to differentiate between pure species and their hybrids and between closely related species in terms of phylogeny. This is a real problem, especially when dealing with species that have a high capacity to hybridize in the wild and in aquaculture. Also, the online databases that deal with barcodes collection are not always reliable when older entries are used as their accuracy in terms of species identification might be low.

Finally, although our results point out that the designated DNA barcodes show a low degree of interspecies variation and the classification is possible only at the genus level, this technique is still relatively cheap, fast and useful in proper identification of possibly incorrectly labelled market products. Furthermore, the food tracing and species identification programs are developing more and more each year, both in terms of technology and biological samples used for analysis.

Supplementary Material

Supplementary Material 1. List of all the sequences obtained in the laboratory. Highlighted are the sequences that were considered reliable from taxonomic and technical points of view.

Supplementary Material 2. List of sequences from GenBank and BOLD databases used for the phylogenetic analysis.

Supplementary Material 3. List of the genetic distances between and within the members of Acipenseriformes and Salmoniformes.

Supplementary Material 4. Plots with the genetic distances identified within Acipenseriformes (a) and Salmoniformes (b).
Supplementary Material 5. More detailed phylogenetic trees inferred by using the Maximum Likelihood (ML) methods (a) for salmonids and (b) for acipenserids based on the Tamura 3-parameter model. For the ML analysis, the tree with the highest log likelihood is shown. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site. With light blue we depicted those samples that were considered reliable from taxonomic and technical points of view, while in orange we depicted the samples which were considered mislabelled after database query. The phylogenetic trees depict the position of our investigated samples among all the DNA COI sequences with maximum Identity scores in both GenBank and BOLD.

The Supplementary Material is available at <https://www.alr-journal.org/10.1051/alr/2017018/olm>.

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