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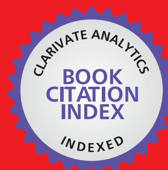

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Molecular Biodiversity Inventory of the Ichthyofauna of the Czech Republic

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1. Introduction

The term biological diversity (biodiversity) covers the variability of life on Earth. In 1989, the World Wildlife Fund defined biodiversity as "the richness of life on Earth – millions of plants, animals and microorganisms, including the genes which they carry, and complex ecosystems that create the environment" (Primack et al., 2001).

The degree of biodiversity was dynamically changing during the centuries and millennia, according to available resources it was gradually growing. In the course of evolution the periods of intensive speciation and relative "speciation rest" were alternating, with five major episodes of mass extinction (Wilson, 1989; Raup, 1992). The worst extinction event took place at the end of the Permian period, 250 million years ago (Primack et al., 2001), the most recent and also the best known mass extinction event occurred in the late Cretaceous period, i. e. 65 million years ago (Freeland, 2005). From then on, the rate of speciation was in equilibrium with the rate of extinction, or was even higher. Currently, the rate of extinction is 100-1000 times higher, namely almost exclusively in consequence of human activity. Therefore a lot of experts call the present situation the sixth mass extinction (Primack et al., 2001). At present, only approximately 1.5 million species are described, of which the majority is represented by insects and plants (Wilson, 1992). According to various estimates the number of non-described species amounts to 10 million or even 30-150 million (Hammond, 1992).

In recent years, when the effort to map and at the same time to protect global biodiversity has been intesifying, it is becoming clear that the existing morphological approach to species classification is not sufficient. Therefore, interest has focused on the methods of molecular genetics.

In 2003, a global project aimed at the mapping and protection of global biodiversity using a new taxonomic method called "DNA barcoding" was started. The key personality in this

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initiative was Canadian professor Paul D. N. Hebert from the Biodiversity Institute of Ontario (BIO), who proposed the creation of a global public library of DNA barcodes for all living organisms, also known as BoLD (Barcode of Life Database). The Barcode of Life Data Systems is an informatics workbench for the acquisition, storage, intercontinental comparison and publication of DNA barcode records (Fig. 1).

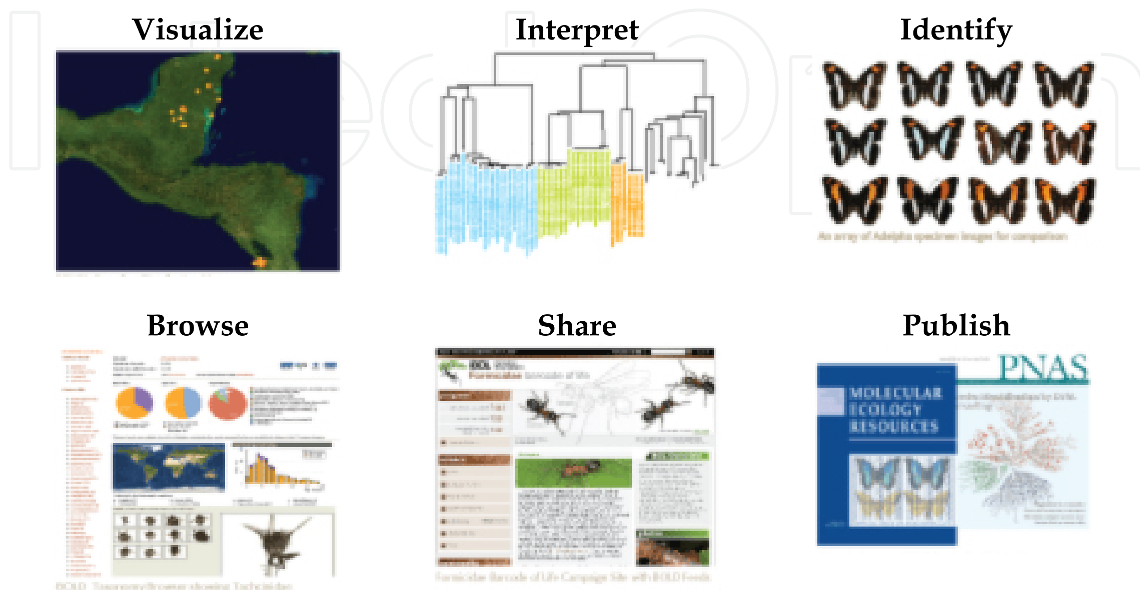

Fig. 1. Multifunctionality of the BoLD database (adopted from http://ibol.org/resources/barcode-library/)

At the same time it bridges a long-standing bioinformatics chasm between molecular, morphological and distributional data. BoLD is freely available to anyone interested in these problems, the identification and preservation of all life on Earth.

2. DNA barcoding

The basic assumption behind this method is that every biological species has a short sequence in its genome which is unique to that species, like a fingerprint to every human being. Its mutation rate should be sufficiently fast to enable the divergence of the sequences of closely related species and at the same time slow enough to enable the minimization of the differences between members of the same species. The implementation of a comparative sequence analysis of this carefully selected sequence should enable mutual identification of the individual species.

The term DNA barcoding is based on the analogy with EAN barcodes on goods which enable safe identification of the individual products (Hebert et al., 2003a). EAN barcode (Fig. 2) is composed of 10 digits at 11 positions, which gives 10^{11} possible combinations. The genetic code has only 4 letters (DNA bases) for one position, but the length of that code is incomparably longer. Only with the sequence length of 15 nucleotides, theoretically 4^{15} various combinations are available, which is a number of unique combinations far exceeding even the boldest estimates of the number of biological species living on Earth (Hebert et al., 2003a).

The technique of DNA barcoding provides a standardized method based on the mapping of a single gene in all the species on Earth (Hebert et al., 2003a). At present, the fragment of the gene for the first cytochrome c oxidase (COI) subunit appears to be the major marker for species of the animal kingdom. This segment is approximately 650 bp long and the protein it codes is part of the respiration chain. The results suggest that this global standard can also be easily obtained from phylogenetically very distant taxa by using a relatively small set of primers and that it is effective in the differentiation of closely related animal species belonging to a great number of groups of invertebrates and vertebrates (Hajibabaei et al., 2007; Hebert et al., 2003a, 2003 ; Hebert & Barett, 2005; Ivanova et al., 2007).

Fig. 2. EAN barcode (adopted from Brian Krueger).

Hebert et al. (2003b) proposed a limit of a 2% deviation in the barcode sequence for the identification of intraspecific variability. The intraspecific deviation only rarely exceeds 2% and in most cases does not reach 1% (Avise, 2000). They also proposed an experiential rule that the divergence between the species sequences should be 10 times higher than that within one species (Hebert et al., 2004).

For the identification of samples with degraded DNA, Hajibabaei et al. (2006) and Meusnier et al. (2008) proposed using shorter COI segments, the so-called "universal mini-barcodes" of a length smaller than 150 bp. Thus they provided a reliable solution for the identification of older tissues, badly preserved museum specimens, partly digested pieces of food in animal stomachs, etc. The results indicate that although the selection of a concrete position of the mini-barcode plays an important role concerning the success of identification, generally it can be stated that the mini-barcodes analysis provides virtually the same results as the specific full-barcode analysis.

2.1 Global progression of DNA barcoding

In April 2004, the Consortium for Barcode of Life (CBOL) was established (Schindel & Miller, 2005). It is an international collaborative effort of more than 160 member organizations from more than 50 countries on six continents (http://barcoding.si.edu, Hebert et al., 2004). CBOL´s mission is to develop the potential of DNA barcoding as a practical and financially affordable tool for taxonomic research, the study and preservation of biodiversity and the development of applications which will utilize the taxonomic information for the benefit of science and society.

Since 2004, a number of campaigns and projects striving to map global biodiversity of all life on Earth have been started (http://ibol.org/about-us/campaigns/):

- **Formicidae Barcode of Life –** a campaign aimed at barcoding all of the world's more than 12,000 ant species.
- **Bee Barcode of Life Initiative (Bee-BOL) –** a global effort to assembly the barcodes of all 20,000 bee species.

- **All Birds Barcoding Initiative (ABBI)** – the aim is to collect DNA barcodes of all of the approximately 10,000 known bird species. The genetic analyses made within the project show that there are hundreds of bird species which have not been described yet.
- **Trichoptera Barcode of Life –** the aim of the project is to barcode the world's approximately 13,000 species of caddisflies.
- **Coral Reef Barcode of Life –** a detailed study of fishes living at one site of the Great Barrier Reef.
- **Fish Barcode of Life Initiative (FISH-BOL)** – a project aimed at the mapping of global ichthyofauna involving 31,200 known fish species (Ward et al., 2009). This initiative also includes the Czech project „Molecular biodiversity inventory of the ichthyofauna of the Czech Republic" which has been introduced here.
- **All Fungi Barcoding** – a project associating initiatives mapping the global diversity of fungi.
- **HealthBOL** – an initiative coordinating the barcoding of vectors, pathogens, and parasites for the improvement of human health around the world.
- **Lepidoptera Barcode of Life** – a campaign mapping all butterflies, with an associated sub-campaign for selected families of Australia and North America.
- **Mammal Barcode of Life** – a project studying global mammal fauna is part of the initiative encompassing all vertebrates.
- **Mosquito Barcoding Initiative** – an international effort aimed at the identification of approximately 3,200 known mosquito species, the disease-bearing species being the priority.
- **Marine Barcode of Life campaign (MarBOL) –** a joint project of the Consortium for the Barcode of Life (CBOL) and the Census of Marine Life (CoML) is aimed at the comprehensive mapping of the diversity of the world's oceans.
- **Polar Barcode of Life campaign** – a campaign studying the biodiversity of the Arctic and Antarctic. It includes marine, freshwater and terrestrial ecosystems.
- **Shark Barcode of Life** – a project aimed at the barcoding of sharks. It plans for 1,000 marine and 100 freshwater shark species.
- **Sponge Barcoding Project** – it is the first global project studying diploblastic species using DNA barcoding. It covers the complete taxonomic range of Porifera.

The existing projects proceed successfully and have already brought the first results regarding various groups of plants and animals, e. g., birds (Hebert et al., 2004), gorillas (Thalmann et al., 2004), tropical beetles (Monaghan et al., 2005), spiders (Barrett & Hebert., 2005), ants (Fisher, 2006), flowering plants (Kress et al., 2005), amphibians (Vences et al., 2005), fishes (Ward et al., 2005), etc.

Currently, as many as 1,371,809 DNA barcodes are identified and stored, which corresponds to approximately 113,435 denominated species (as of September 30, 2011). The collaborating scientific teams have set a goal by 2015 to collect 5 million barcodes/specimens for 500,000 species.

2.1.1 Fish DNA barcoding

In 2005, the FISH-BOL initiative was established. It is an expression of the global effort to develop and coordinate a standardised library of sequences for all fish species. For this

purpose a guideline containing DNA barcodes, photographs of the studied specimens, geospatial coordinates of the locations of the finds, etc. was issued. It also contains references to type specimens, information on species distribution, nomenclature, author taxonomic information, literature citations, etc. Thus, it complements and enhances the information resources available so far – the genome databases (GenBank, EMBL, DDBJ), including the internet encyclopaedia FishBase.

Barcoding in fish has multiple usages, of which we select only some:

- Identifying endangered and protected species (Ward et al., 2008)
- Identifying historical and museum material (Meusnier et al., 2008)
- Identifying new and cryptic species and possible fusions of existing taxa, and insight into phylogenetic relationships (Pyle et al., 2008)
- The development of a reference library for known species (Ratnasingham & Hebert, 2007)

Fishes are the most diversified group of vertebrates; at present, there are about 30,000 known species, including 15,758 (53.3%) marine species, 13,779 (46.4%) freshwater species and 86 (0.3%) brackish species (Ward et al., 2005). By September 2011, DNA barcodes were obtained from 8,293 species (27%) in the world, and from 503 species (25%) in Europe (http://www.fishbol.org/progress.php).

Some of the best-known projects utilizing the DNA barcoding in fishes are:

1. “Barcoding Marine Fishes: A Three-Ocean Perspective Project”. The goal of this project is to obtain DNA barcodes of all marine species in the Pacific, Indian and Atlantic oceans. Participating countries include Canada, Australia, South Africa and Portugal.
2. “Planetary Biodiversity Inventories – Catfishes”. It incorporates 300 participants from 40 countries whose work is taking an inventory of catfishes (Siluriformes).
3. “The Cypriniformes Tree of Life Initiative” (CToL), an American initiative aimed at the collection and analysis of DNA samples and reference specimens of nearly all North American freshwater species (~ 1100 species).

2.1.2 The state of the molecular biodiversity inventory of the ichthyofauna of the Czech Republic and introduction of the project

The study and recognition of genetic diversity in the Czech Republic has an approximately twenty-year history. Although the methods of biochemical genetics which were used in the past brought significant knowledge, only the application of molecular-genetic methods allowed significant detailed recognition and disclosure of the genetic diversity of species at the level of populations (Lusk & Hanel, 2008). Existing information about the genetic diversity of the individual species of the native ichthyofauna of the Czech Republic was and still is insufficient. The result of the research project VaV – SM/6/3/05 “Genetic diversity of endangered fish species - the essential basis for effective biodiversity protection” brought a breakthrough in the recognition of genetic diversity, especially of the protected and endangered species. The project was conducted in 2005-2007 in cooperation between our department and the research workers from the Institute of Animal Physiology and Genetics, v.v.i., Liběchov. This allowed the presentation of the first taxonomic approaches to some species of the Czech ichthyofauna (Bartoňová et al., 2008; Mendel et al., 2005; Mendel et al.,

2008a; Papoušek et al., 2008a, 2008b; Vetešník et al., 2007). Other taxonomic studies of our team are included in the proceedings "Biodiversity of fishes in the Czech Republic VII", which paid attention to the problems of the genetic diversity of some protected and rare fish species of the Czech Republic. Apart from the study of actual intra-species diversity, attention in this book was also paid to the distribution or specification of the occurrence of the studied species (Bartoňová et al., 2008; Lusk et al., 2008a, 2008b; Lusková et al., 2008a, 2008b; Mendel et al., 2008b, 2008c; Papoušek et al., 2008b).

The main goal of the presented project is the mapping and inventory making of all Czech ichthyofauna using the DNA barcoding technique. In addition to that, in the case of archived specimens forming new museum type series a comprehensive approach was used (morphology and nuclear genome analysis).

A study of this type has been lacking in the Czech Republic. It is unique due to its comprehensiveness using both the classical and the newest taxonomic tools and due to its future potential – the intercontinental mapping of the diversity of life on Earth. Thanks to our cooperation with the Biodiversity Institute of Ontario the Czech Republic became actively involved in the international iBOL project (International Barcode of Life Project), namely in its part which was already mentioned above – FISH-BOL (Fish Barcode of Life Initiative). Thus, the Czech study took the side of already finished or currently running studies which take place within whole countries and continents, e. g.:

- Fishes of Australia (Ward et al., 2005)
- Barcoding of Canadian freshwater fishes (Hubert et. al., 2008)
- DNA barcoding of fish of the Antarctic Scotia Sea (Rock et al., 2008)
- Freshwater fishes from Mexico and Guatemala (Valdez-Moreno et al., 2009)
- Aquarium Imports (Steinke et al., 2009)
- Fishes of Alaska and the Pacific Arctic (Mecklenburg et al., 2011)
- Amazon fishes (Ardura et al., 2010)
- DNA Barcoding of Indian Marine Fish (Lakra et al., 2011)
- Fishes of Japan (Zhang & Hanner 2011)
- Freshwater Fishes of North America (April et al., 2011)
- Barcoding Fishes of Eastern Nigeria (Nwani et al., 2011)
- Fishes of Argentina (running project)
- Fishes from South China Sea (running project)
- Etc.

More information can be found at: http://www.boldsystems.org/views/projectlist.php

3. Material and methods

The inventory and subsequent cataloguing were focused on recent indigenous and non-indigenous fishes and lampreys living in the natural waters of the Czech Republic. It concerned 11 orders, 17 families and 72 species. The selection of collection localities covered the main distribution areas of the studied taxa according to comprehensive surveys and the most recent taxonomic and phylogenetic studies (Baruš & Oliva, 1995; Hanel & Lusk, 2005; Kottelat & Freyhof, 2007; Janko et al., 2007; Mendel et al., 2008d, etc). The collection of samples was divided into three levels:

- classification of the first level is based on the hydrologic position of the Czech Republic (Fig. 3)

Concerning hydrologic division, the Czech Republic belongs to three sea drainage areas (the North Sea, Baltic Sea and Black Sea drainage areas).

Fig. 3. Collection localities – 1st level of classification.

- classification of the second level (Fig. 4)

The hydrologic network of the Czech Republic was further divided into six areas, which for the most part correspond with the division of state administration: I. the Ohře River basin (the North Sea drainage area, the actual Ohře River basin and parts of other tributaries of the Elbe), II. the Vltava River basin (the North Sea drainage area, without the Ohře and the Elbe River basins), III. the Elbe River basin (the North Sea drainage area, without the Ohře and the Vltava River basins), IV. the Dyje River basin and the lower course of the Morava River, V. the Morava River basin (the Black Sea drainage area, the upper and middle courses of the Morava River), VI. the Odra River basin (the Baltic Sea drainage area).

Fig. 4. Collection localities – 2nd level of classification.

- classification of the third level

The selection of the sampling localities for the given species was further specified according to up-to-date information concerning its distribution, according to the specific features of the given species (for example, the Natura 2000 localities, etc.) and with regard to the

elimination of "family sampling". In the case of threatened and critically endangered fish species (*Pelecus cultratus*, *Zingel zingel*, *Zingel streber*, etc.) the database of the tissue samples collected by our workplace in the past was used.

The capturing of adult specimens was done using electro-fishing gear (direct pulsed current) in collaboration with the Czech and Moravian Fishing Unions and the representatives of the Agency for Nature Conservation and Landscape Protection of the Czech Republic. Approximately 1,400 specimens were taken for subsequent molecular-genetic analyses. From the captured specimens (about five individuals per location) a part of the pectoral fin (fin border) was taken and then they were released back into the stream. Only an indispensable number of complete type specimens were taken for morphological description and museum collections.

The identification of the individual species was made on the background of the study of morphological characteristics of the species using a multilocus and two-genome comparative approach (analysis of both mt and nDNA markers). Thus some limitations of the DNA barcoding method were eliminated, e. g. a) occurrence of hybrids; b) maternal contribution to COI marker as only one part of the information necessary for a valid description of a species. For reasons of saving space we do not present here the morphological characteristics of the individual species and the detailed results of the analyses of the nuclear marker – 1st intron of the S7 r-protein (RP1).

The preparation of the DNA barcodes conformed to the standardized protocols developed by the CCDB Centre, the FISH-BOL initiative and the BoLD database (http://www.ccdb.ca/pa/ge/research/protocols; Ratnasingham & Hebert, 2007; Hajibabaei et al., 2005; deWaard et al., 2008).

DNA isolation was performed by using a commercial kit - the Genomic DNA Mini kit (KRD) – according to the instructions for use. Cocktails of primers from the genetic study (Ivanova et al., 2007) were used for PCR amplification of the COI gene fragment (Fig. 5). For sequencing PCR, mainly M13F and M13R primers (Messing, 1983) were used, in a small number of cases the primers described by Ivanova et al. (2007) were used.

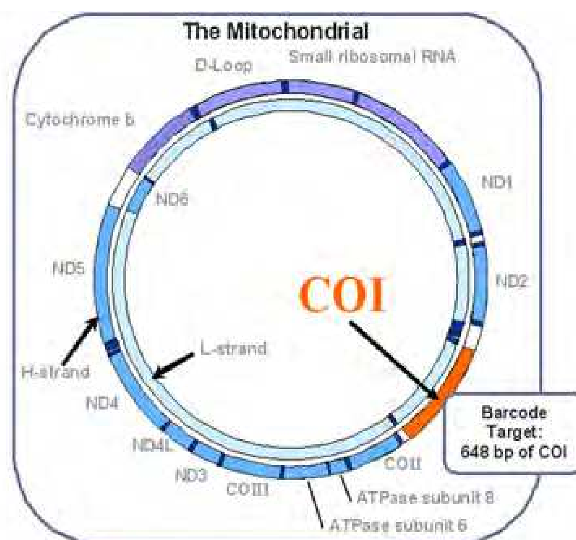

Fig. 5. Mitochondrial DNA – the area of the COI marker – adopted from http://www.barcodeoflife.org.

At the Canadian workplace, the subsequent steps were performed according to the above mentioned protocol. At the Czech workplace, the PCR products were re-purified by precipitation with PEG/Mg/NaAc solution (26% Polyethylene glycol, 6.5 mM $MgCl_2 . 6H_2O$, 0.6 M $NaAc.3H_2O$) and subsequently used for the sequencing reaction using BigDye™ Terminator Cycle Sequencing kit v 1.1. (Applied Biosystems), performed according to the manufacturer´s instructions. The products of the sequencing reaction were re-purified with ethanol precipitation. For the COI sequencing, the instrumentation of the Canadian BIO workplace (ABI 3730XL; Applied Biosystems) was used and for test and complementary COI sequencing and nuclear marker analysis also the instrument ABI PRISM 310 (Applied Biosystems) at the Czech workplace was used. Also, commercial sequencing (Macrogen, Korea) was used in optimized samples. The analyzed fragment of mitochondrial DNA from each sample was sequenced from both directions. Chromatograms were adjusted and developed using the computer module SeqManII (part of the program package Lasergene v. 6.0; DNASTAR Inc.) and MEGA software.

The developed sequences were visually checked for errors and subsequently compared using the ClustalW algorithm. The correct taxonomic classification of the sequences was confirmed using comparison with the GenBank and BoLD databases. An electropherogram depicting the order of nucleotides in the sequence was obtained through sequencing. With its subsequent processing the DNA barcode was produced (Fig. 6).

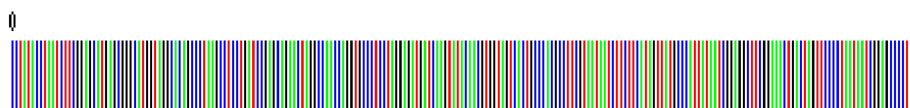

Fig. 6. DNA barcode.

The individual steps of the barcoding process are shown in Figure 7.

The morphologic characteristics were identified in selected adult specimens to enable the establishment of a reference type series. A holotype was selected as a nomenclatoric type and other specimens from the described group were assigned to it as the documentary material (paratypes).

For the purpose of the cataloguing of the taxa of the ichthyofauna of the Czech Republic using the DNA barcoding method, the basic data concerning each specimen were collected and completed, including the body length (SL), weight and sex, photo documentation, locality description (including the GPS coordinates), date of collection and the person who caught or identified the specimen, the catalogue number and place of storage. The COI sequences of a length of at least 500 bp and the sequences of used PCR primers were recorded as well. For further details see the instructions of the National Museum and the standardized protocol of the BoLD database (http://www.boldsystems.org/docs/boldmas.html; Ratnasingham & Hebert, 2007).

In all archived specimens of each species a sequencing of RP1 marker from nuclear genome was also performed in order to exclude the presence of hybrid specimens. The correct nuclear identification is ensured by sequence comparison with the results of various studies from both domestic and foreign workplaces (e. g., He et al., 2008; Mayden et al., 2008; Mendel et al., 2008d; etc.) and international initiatives (Cypriniformes Tree of Life; Assembling the Tree of Life; etc.)

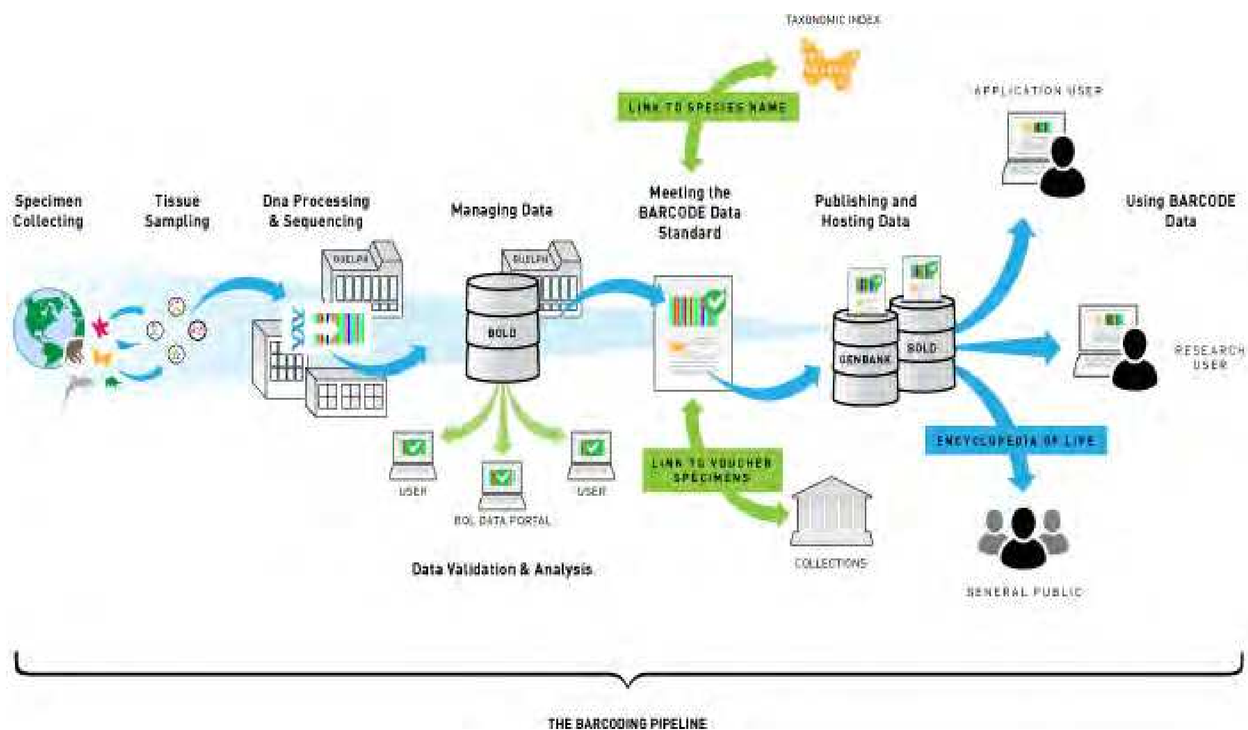

Fig. 7. The barcoding pipeline (http://www.barcodeoflife.org/content/about/what-dna barcoding).

The platform of the BoLD system (http://www. barcodinglife.org) with three identification-statistical modules - MAS, IDS and ECS - was used for the organization of the results. The obtained sequences are saved by the CBOL consortium primarily in the BoLD datasystem and then automatically sent, within INSDC (International Nucleotide Sequence Database Collaboration), to the interconnected databases: GenBank, EMBL and DDBJ. For more information see the standardized protocol of the CBOL consortium: http://www.dnabarcoding.ca/pa/ge/research/protocols/.

4. Results

An analysis of the COI gene sequences of 500-652 bp in length was performed on 820 individuals from 67 species, which represent 93.1% of the Czech ichthyofauna (Table 1). The map of sampling distribution (Fig. 8) clearly shows that we have evenly sampled the whole territory of the Czech Republic. Altogether, 109 localities were sampled. The number of individuals per species ranged from 1 to 31, with an average number of 12 individuals per species. Out of a total number of 72 species, the following species, which occur very rarely, were not analysed: *Micropterus salmoides*, *Romanogobio banaticus*, *Romanogobio belingi*, *Ameirus melas* and *Acipenser baeri*. Only one or two individuals were analysed in some critically endangered (*Zingel zingel*, *Zingel streber*, *Sabanejewia balcanica*, *Eudontomyzon mariae*, *Pelecus cultratus*, *Ballerus sapa*, *Gymnocephalus schraetser*), and endangered (*Misgurnus fossilis*) species. Neither insertions/deletions nor stop codons were found, thus encouraging the view that all amplified sequences represent functional COI gene sequences.

Family	Species	N	No of haplotypes	Intraspecies difference range (%)
Acipenseridae	*Acipenser gueldenstaedtii*	3 (+1)	1 (+1)*	0 (4.484)
	Acipenser ruthenus	4	1	0
	Acipenser stellatus	5	1	0
Anguillidae	*Anguilla anguilla*	5	5	0.154 – 3.494
Balitoridae	*Barbatula barbatula*	27	13	0 – 5.967
Centrarchidae	*Lepomis gibbosus*	10	1	0
Cobitidae	*Cobitis sp.*	3	2	0 – 0.677
	Misgurnus fossilis	1	1	-
	Sabanejewia balcanica	1	1	-
Cottidae	*Cottus gobio*	21	4	0 – 0.773
	Cottus poecilopus	15	6	0 – 2.681
Cyprinidae	*Abramis brama*	29	4	0 – 0.617
	Alburnoides bipunctatus	14	5	0 – 0.617
	Alburnus alburnus	26	4	0 – 0.308
	Aspius aspius	14	2	0 – 0.308
	Ballerus ballerus	3	1	0
	Ballerus sapa	2	1	0
	Barbus barbus	25	2	0 – 0.163
	Blicca bjoerkna	14 (+1)	4 (+1)**	0 – 0.308 (4.316)
	Carassius carassius	16	5	0 – 1.266
	Carassius gibelio	27	6	0 – 0.621
	Carassius langsdorfii	3	1	0
	Ctenopharyngodon idella	3	1	0
	Cyprinus carpio	10	2	0 – 0.31
	Gobio gobio	27 (+1)	3 (+1)***	0 – 0.308 (11.420)
	Gobio obtusirostris	5	3	0 – 0.308
	Hypophthalmichthys molitrix	3	1	0
	Chondrostoma nasus	25	5	0 – 0.308
	Leucaspius delineatus	5	2	0 – 3.808
	Leuciscus idus	13	4	0 – 0.621
	Leuciscus leuciscus	21 (+5)	2 (+1)†	0 – 0.161 (0.933)
	Pelecus cultratus	2	1	0
	Phoxinus phoxinus	25	10	0 – 3.973
	Pseudorasbora parva	22	7	0 – 1.084
	Rhodeus amarus	18	7	0 – 1.244
	Romanogobio vladykovi	7	3	0 – 0.618
	Rutilus rutilus	31	7	0 – 0.795
	Scardinius erythrophthalmus	16	1	0
	Squalius cephalus	28	4	0 – 5.252
	Tinca tinca	24	3	0 – 1.521
	Vimba vimba	4	1	0
Esocidae	*Esox lucius*	22	4	0 - 0.772
Gasterosteidae	*Gasterosteus aculeatus*	2	1	0
Gobiidae	*Neogobius melanostomus*	5	1	0
	Proterorhinus semilunaris	6	2	0 – 1.087
Ictaluridae	*Ameiurus nebulosus*	2	1	0
Lotidae	*Lota lota*	16	3	0 – 0.308

Percidae	*Gymnocephalus baloni*	1	1	-
	Gymnocephalus cernua	16	5	0 – 0.798
	Gymnocephalus schraetser	2	1	0
	Perca fluviatilis	29	5	0 – 0.772
	Sander lucioperca	16	1	0
	Sander volgensis	2	1	0
	Zingel streber	1	1	-
	Zingel zingel	1	1	-
Petromyzontidae	*Eudontomyzon mariae*	2	2	0.197
	Lampetra planeri	14	3	0 – 0.308
Salmonidae	*Coregonus maraena*	4 (+1)	1 (+1)††	0 (1.558)
	Coregonus peled	5	4	0 – 0.618
	Hucho hucho	2	1	0
	Oncorhynchus mykiss	24	5	0 – 0.308
	Salmo salar	3	2	0 – 0.154
	Salmo trutta	31	4	0 – 1.087
	Salvelinus fontinalis	16	4	0 – 0.617
	Thymallus thymallus	16	2	0 – 0.155
Siluridae	*Silurus glanis*	11	1	0
Umbridae	*Umbra krameri*	5	1	0

Table 1. List of species analysed in the current study. N - number of studied individuals (further individuals with haplotypes shared with another species in parentheses). No of haplotypes - number of COI haplotypes found (further haplotypes shared with another species in parentheses). * - shared haplotype with *Acipenser ruthenus*. ** - shared haplotype with *Abramis brama*. *** - shared haplotype with *Romanogobio vladykovi*. † - shared haplotype with *Leuciscus idus*. †† - shared haplotype with *Coregonus peled*. Intraspecies difference range - range of intraspecific genetic distances excluding haplotypes shared with another species (maximum genetic distance including shared haplotypes in parentheses).

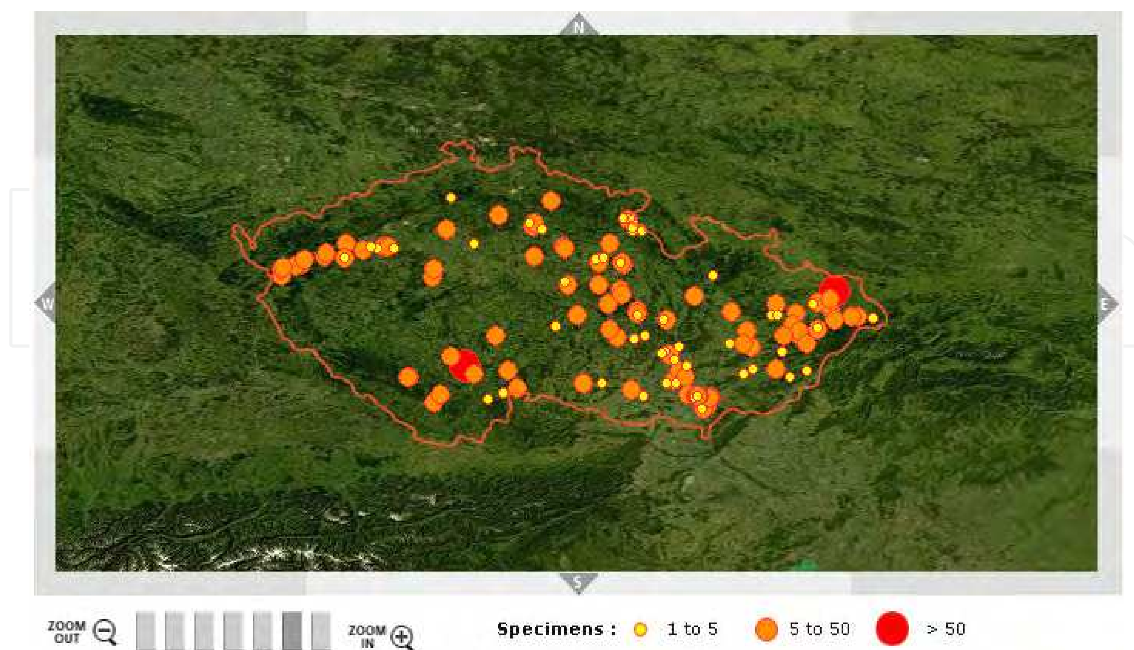

Fig. 8. Overview of sampling localities in the Czech Republic. The borders of the Czech Republic are schematically drawn in red.

As we expected, we found a growing genetic divergence with raising taxonomical level. On the intra-species level, the distances ranged 0-5.97% (Fig. 9), not including inter-specific shared haplotypes (hybrids, misidentifications etc.). Intra-specific diversities exceeding 5% were detected in *Barbatula barbatula* (the highest value of 5.97% was found here), and in *Squalius cephalus* (0-5.25%), values exceeding 2% were found in *Phoxinus phoxinus* (0-3.97%), *Anguilla anguilla* (0.15-3.49%), *Leucaspius delineatus* (0-3.81%) and *Cottus poecilopus* (0-2.68%). In these six mentioned species, there was an apparent deep divergence of the lineages that had been assigned to a single species. Two or three (*B. barbatula, S. cephalus)* lineages were distinguished in each species, both from the phylogenetic tree (Fig. 10) and the list of variable sites (Fig. 11). Two lineages can also be distinguished in the following species, although they possess lower genetic distances: *Tinca tinca* (0-1.52%) and *Carassius carassius* (0-1.27%). In *Rhodeus amarus*, a single individual diverges from all other, which raises the mean intra-species diversity from 0.46 to 1.09%.

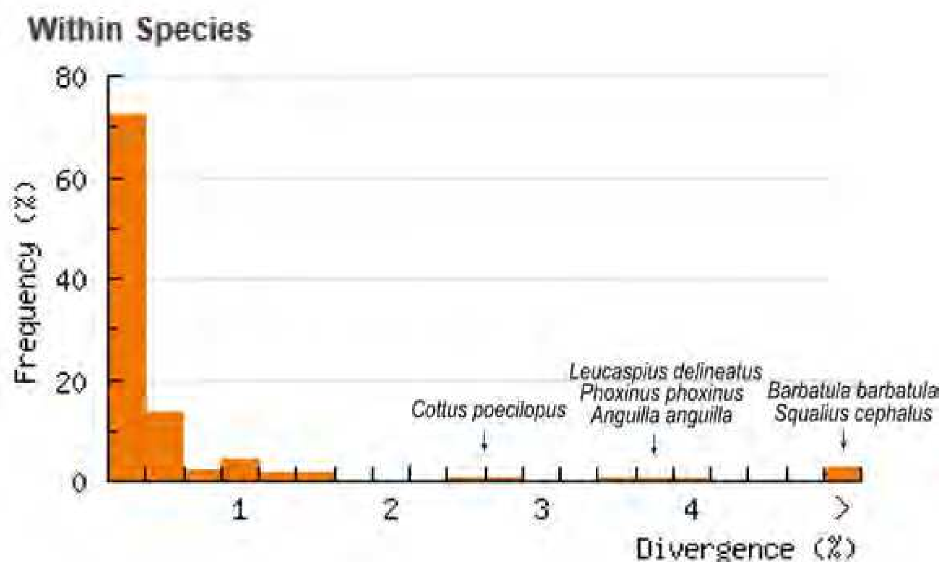

Fig. 9. Plot of an average intraspecific divergence. Species that exceeded 2% are listed.

An analysis of distribution of the nearest-neighbour distance (NND, the minimum distance between a species and it closest – usually congeneric – relative, Table 2) has shown that only a single species pair exhibited NND lower than 1%, that being namely: *Leuciscus leuciscus* and *L. idus*. The NND averaged 9.67%, which was 40 times higher than the mean within species distance (ca. 0.25%) and 14 times higher than the mean maximum intraspecific distance (0.70%).

We have detected altogether nine cases of shared barcode haplotypes: one *Acipenser gueldenstaedtii* individual possessed an *A. ruthenus* haplotype, one *Blicca bjoerkna* individual possessed an *Abramis brama* haplotype, one *Gobio gobio* individual possessed a *R. vladykovi* haplotype, one *Coregonus maraena* possessed a *C. peled* haplotype, and five *Leuciscus leuciscus* carried a *L. idus* haplotype.

A phylogenetic tree based on all sequences longer than 500 bp, employing the Kimura-2-parametr (K2P) model is available on http://www.ivb.cz/projects-molecular-

biodiversity-inventory-of-the-ichthyofauna-of-the-czech-republic.html. Subsequently, we selected the representatives of all species or intra-species lineages with intra-species distance at least three times higher (1.176%) than the mean distance within the species (0.392%; Hubert et al., 2008). Based on these sequences, a reduced phylogenetic tree was constructed and put through a bootstrap analysis of 1000 replications. The reduced phylogenetic tree (Fig. 10) documents strong bootstrap support both on the level of genera and on family level. Only the most numerous family Cyprinidae has exhibited moderate support. The *Neogobius* and *Proterorhinus* genera were not supported in forming a single clade of the Gobiidae family.

The phylogenetic tree supports the most recently proposed changes in the scientific taxonomical nomenclature. According to recent taxonomical opinions and studies (Froese & Pauly, 2010; Kottelat & Freyhof, 2007; Perea et al., 2010), *A. sapa* and *A. ballerus* have been currently sorted into the genus *Ballerus*; *Abramis bjoerkna* now belongs again to the *Blicca* genus, *L. cephalus* into *Squalius* and it is suggested that *Aspius aspius* be included into *Leuciscus*. All these proposales are reflected in the tree, as the above mentioned species always cluster together.

Order	Family	N	< 0.1	0.1 - 1.0	1.0 - 2.7	> 2.7
Acipenseriformes	Acipenseridae	3				3
Cypriniformes	Cobitidae	3				3
	Cyprinidae	30		2*	2**	26
	Balitoridae	1				1
Scorpaeniformes	Cottidae	2				2
Salmoniformes	Salmonidae	8			2***	6
Petromyzontiformes	Petromyzontidae	2				2
Esociformes	Umbridae	1				1
	Esocidae	1				1
Perciformes	Gobiidae	2				2
	Percidae	8				8
	Centrarchidae	1				1
Gadiformes	Lotidae	1				1
Anguilliformes	Anguillidae	1				1
Siluriformes	Siluridae	1				1
	Ictaluridae	1				1
Gasterosteiformes	Gasterosteidae	1				1
Totals:		67	0	2	4	61

Table 2. Summary of Czech ichthyofauna diversity and distribution of genetic distance of each species analysed to nearest neighbour (in per cent). N - number of species in family.
* - *Leuciscus idus* and *Leuciscus leuciscus* (0.62%). ** - *Blicca bjoerkna* and *Vimba vimba* (2.51%).
*** - *Coregonus peled* and *Coregonus maraena* (1.4%).

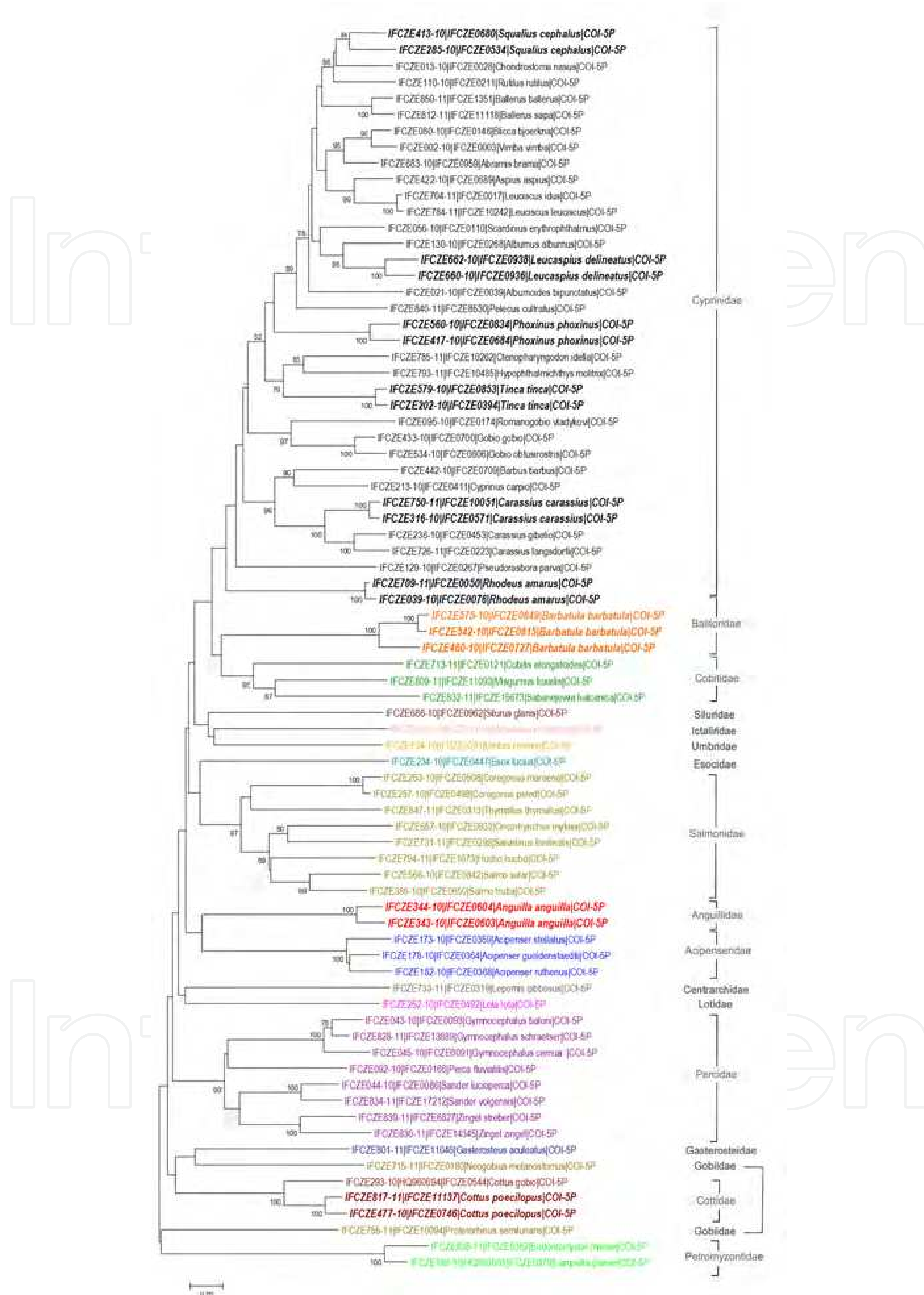

Fig. 10. K2P/NJ tree of representatives of each species, or lineage within species. Bootstrap values are listed near the nodes, only values ≥75% are shown. Families are listed in colour. Species showing an internal divergence more than three times higher than the mean distance within the species are listed in bold italics.

```
[                                   1222222 2223333333 4444445555 5555555666 66]
[                                2389223345 6890112379 0113680015 6667899011 22]
[                                5150035870 5081362408 9250645810 2581038469 25]
#IFCZE0815|Barbatula_barbatula   ACCCACCGCG GCCTAGGAAC CGTAAGGATC TGCACCCGCG GA
#IFCZE0556|Barbatula_barbatula   .......... ........G. .......... .......... ..
#IFCZE0802|Barbatula_barbatula   .......... .......... ...G...... .T........ ..
#IFCZE0809|Barbatula_barbatula   .......... .......... ....G..... .......... ..
#IFCZE11111|Barbatula_barbatula  .......... ...C...... .......... .......... ..
#IFCZE0555|Barbatula_barbatula   G.......T. ....GA..GT ...G..A... .......... A.
#IFCZE0835|Barbatula_barbatula   ........T. ....GA..GT ...G...... .......... A.
#IFCZE0849|Barbatula_barbatula   ........T. ....GA..GT ...G..A... .......... A.
#IFCZE11031|Barbatula_barbatula  ........T. ....GA..GT ...G..A... .....G.... A.
#IFCZE11071|Barbatula_barbatula  ........T. A...GA..GT ...G..A... .......... A.
#IFCZE0726|Barbatula_barbatula   .TGTCTAT.A ATA..AAG.. TAC..A.GCT CTTCT.TAGA A.
#IFCZE0727|Barbatula_barbatula   .TGTCTAT.A ATA..AAG.. TAC..A.GCT CTTCT.TAGA AG
#IFCZE0730|Barbatula_barbatula   .TGTCTAT.A ATA..AAG.. T.C..A.GCT CTTCT.TAGA A.

[                              122222233 3333333444 4444445555 56666]
[                             4600144700 2223457000 0368991235 71235]
[                             7028747447 2581020036 9334797023 10242]
IFCZE0830|Squalius_cephalus   CAGAATTATG GGAGACACTA GGAACCAATT ACGAA
IFCZE0680|Squalius_cephalus   .......... ...A..G... A.....G... G....
IFCZE0785|Squalius_cephalus   .......... ..G....... .......... .....
IFCZE0534|Squalius_cephalus   TCAGGCCGAA AA.AGT.TCG .AGGTAGGCC .TAGG

[                                    111 2222333333 4444455556 6]
[                             3448899099 3679144456 0389903561 4]
[                             7032512836 2515903627 3643655953 3]
#IFCZE0682|Phoxinus_phoxinus  GGCGGCCTGT CAATATCCCA CCGATCTTTT C
#IFCZE0684|Phoxinus_phoxinus  A...A..... T....CA... ..A....... .
#IFCZE0686|Phoxinus_phoxinus  A...A..... T.G..CA... ..A....... .
#IFCZE0767|Phoxinus_phoxinus  ....A..... T...GCA..G ..A....... .
#IFCZE0805|Phoxinus_phoxinus  A...AT.... T....CA... ..A....... .
#IFCZE0816|Phoxinus_phoxinus  AA..A..... T....CA... ..A....... .
#IFCZE0127|Phoxinus_phoxinus  ..TTATT.A. TG.CGCGTT. TT.CCGCCA. T
#IFCZE0255|Phoxinus_phoxinus  ..TCATTCA. TG.CGCGTT. TT.CCGCCAC T
#IFCZE0551|Phoxinus_phoxinus  ..TT.TT.A. TG.CGCGTT. TT.CCGCCAC T
#IFCZE0834|Phoxinus_phoxinus  ..TTATT.A. TG.CGCGTT. TT.CCGCCAC T
#IFCZE0914|Phoxinus_phoxinus  ..TTATT.AG TG.CGCGTT. TT.CCGCCAC T

[                                      1111222 2333334455 5556]
[                                   4780037013 7122895612 4572]
[                                   9926938378 7958877640 1015]
#IFCZE0936|Leucaspius_delineatus    GGTGTCCCGT CGGGGATAAA CTAA
#IFCZE0938|Leucaspius_delineatus    AACACTATAG TAAAAGCGTG TAGG
```

```
[                                  1222333 3444455556 666]
[                               2247128114 7013802380 012]
[                               5605796030 0059426001 438]
#IFCZE0248|Anguilla_anguilla    AGCTCAACAA GCTCGTCTGG CTA
#IFCZE0603|Anguilla_anguilla    GA........ .......... A..
#IFCZE0771|Anguilla_anguilla    .A........ .......... A..
#IFCZE0604|Anguilla_anguilla    ..TCT..TGG ATCTACTCAA .CG
#IFCZE0772|Anguilla_anguilla    ..TCTGGTG. ATCTACTCAA .CG

[                                   2223333 344445556]
[                                1560490147 702394561]
[                                0548181300 397067329]
#IFCZE0187|Cottus_poecilopus     AAGAAAAATA CATATTGCC
#IFCZE1132|Cottus_poecilopus     G......... .........
#IFCZE11133|Cottus_poecilopus    .......... T........
#IFCZE11137|Cottus_poecilopus    ....G..... .........
#IFCZE0746|Cottus_poecilopus     .CAG.GGGAG .GG.CCAAT
#IFCZE0750|Cottus_poecilopus     .CAG.GGGAG .GGGCCAAT

[                         111223345]
[                         089041210]
[                         076249259]
#IFCZE0264|Tinca_tinca    TACGTAGCG
#IFCZE0853|Tinca_tinca    ........T
#IFCZE0394|Tinca_tinca    CGTACGAT.

[                                2222334 56]
[                             2550277256 54]
[                             0563917550 09]
#IFCZE0048|Rhodeus_amarus     CTCCCACCCC GC
#IFCZE0050|Rhodeus_amarus     G......... ..
#IFCZE0494|Rhodeus_amarus     GCT....... ..
#IFCZE0633|Rhodeus_amarus     G......... A.
#IFCZE0822|Rhodeus_amarus     G...T..... ..
#IFCZE10047|Rhodeus_amarus    G.......G. ..
#IFCZE0076|Rhodeus_amarus     G..T.GTT.T .T

[                                  1223344]
[                                 66272428]
[                                 20012077]
#IFCZE0571|Carassius_carassius    CATATTGC
#IFCZE10150|Carassius_carassius   G.......
#IFCZE0392|Carassius_carassius    ..CGCCAT
#IFCZE0821|Carassius_carassius    ..C.CCAT
#IFCZE10051|Carassius_carassius   .GCGCCAT
```

Fig. 11. Summary of variable sites in the COI gene in haplotypes of nine species showing an internal divergence more than three times higher than the mean distance within the species. Numbers show variable position of nucleotide in whole 652 bp sequence. Dots represent nucleotide identical with first sequence.

5. Discussion

This study presents the first molecular screening of the whole Czech ichthyofauna. Indeed, the position of our project, „The Ichthyofauna of the Czech Republic" (abbreviated IFCZE in the BoLD database), is in a certain sense unique. Table 3, which compares genetic divergences on various taxonomic levels in projects spanning countries across continents from North America to Asia, Australia and Africa, contains only a single project representing the European ichthyofauna – Czech project. It is the first and currently the only public project in BoLD database dealing with the barcoding of European ichthyofauna on this scale.

Among the analyzed species, there was no species endemic to the Czech Republic. Aside from indigenous species, further 18 non-indigenous species introduced in the past to the Czech ichthyofauna were also tested. One species is considered to be regionally extinct in the wild, 12 species are considered to be critically endangered, five to be endangered and six to be vulnerable according to the IUCN classification (Lusk et al., 2011). Also included in the project was the *Umbra krameri*, while its indigenity to the Czech Republic is currently disputed.

The amplification of the 5′ region of the COI gene (up to 652 bp) was successful for all 820 tested individuals, while utilizing a single primer set for amplification of all tested species. No nuclear copies of the COI gene were detected, neither frame shifts nor mutations causing the emergence of stop codons. By means of DNA barcoding, all tested species could be successfully distinguished. The DNA barcodes were acquired for 67 species. An example of such a barcode record can be seen in Figure 12.

	CAN	MEX	NorthAm.	IND	PHL	NGA	AUS	**CZE**	
	min%								
within species	0	0	0	0	0	0	0	**0**	
within genus	0	0	0	0.10	10.0	1.80	0	**0**	
within family	2.67	1.804	0.185	0.20	5.50	0.46	1.39	**0**	
within order	14.25	17.24	14.53	8.00	16.20	15.52	9.55	**18.07**	
within class	17.49	17.88	16.42	-	-	18.46	14.33	**18.36**	
	mean dist%								
within species	0.27	0.45	2.52	0.30	0.60	0.30	0.39	**0.39**	
within genus	8.37	5.10	14.10	6.60	11.70	1.90	9.93	**5.00**	
within family	15.38	13.57	16.00	9.91	17.67	17.19	15.46	**14.85**	
within order	20.60	23.38	21.50	16.00	24.80	21.62	22.18	**24.62**	
within class	24.57	25.34	24.80	-	-	25.40	23.27	**24.87**	
	max dist%								S.E.
within species	7.42	2.11	15.45	0.30	9.30	22.38	14.80	**11.61**	**0.013**
within genus	19.33	16.67	23.68	11.90	15.30	36.61	20.63	**11.20**	**0.067**
within family	23.22	28.48	29.92	23.10	22.60	24.82	35.7	**24.46**	**0.011**
within order	29.44	28.77	30.95	23.40	31.50	28.12	37.52	**28.84**	**0.013**
within class	31.20	31.16	37.50	-	-	31.90	37.39	**30.77**	**0.004**
species	190	76	605	98	23	59	207	**61**	
genus	85	56	134	78	21	33	122	**51**	
family	28	32	36	53	17	19	?	**17**	
order	20	11	18	24	9	8	?	**11**	
class	2	2	2	4	-	2	?	**2**	

Table 3. Comparison of genetic divergences (K2P model used) within various taxonomic levels among selected freshwater and marine fishes BoLD projects. (CAN-Barcoding of Canadian freshwater fishes, MEX-Freshwater Fishes of Mexico, NorthAm-Freshwater Fishes of North America, IND-Marine Fishes of India, PHL-Freshwater Fish of Taal Lake, Philippines, NGA- Barcoding Freshwater Fishes of Eastern Nigeria, AUS-Marine Fishes of Australia, CZE-Ichthyofauna of the Czech Republic).

The barcoding of the Czech ichthyofauna has not ended yet: several further species (*M. salmoides*, *R. banaticus*, *R. belingi*, *A. baerii*, and A. *melas*) are currently being tested as well. Therefore, the analyzed sample set currently covers 67 of 72 species, or 93.1% of the Czech ichthyofauna. Certain species are present in all six investigated regions and were therefore analysed in greater numbers and in some cases exhibited greater haplotype variability (e.g. *B. barbatula*, 27 individuals/13 haplotypes or *P. phoxinus*, 25 individuals/10 haplotypes. For more details see Table 1.

Overall, the mean congeneric divergence was ca. 13 times higher than the mean conspecific divergence (5.00 vs 0.39%, Table 3). These values are in good concordance with the results of Valdez-Moreno et al. (2009), which studied the diversity of ichthyofauna in Mexico and Guatemala (5.10 vs 0.45%, Table 3). The divergence ratio was higher in studies dealing with the ichthyofauna of Australia (25x, Ward et al., 2005), Canada (31x, Hubert et al., 2008) and the Philippines (19x, Aquilino et al., 2011) (Table 3). Such a low divergence in our project is probably caused by overall more recent speciation of the freshwater species in comparison to their marine counterparts (which are included in the mentioned projects). This is also supported by the results of the marine project from India (27x, Lakra et al., 2011), China (50x, a running project) and Japan (59x, Zhang & Hanner, 2011).

The overlapping of conspecific and congeneric levels of divergence was minimal: only two genera have shown inter-specific distance between the nearest neighbours lower than 2.7%: *Leuciscus* – *L. leuciscus* and *L. idus* (0.62%) and *Coregonus* – *C. peled* and *C. maraena* (1.4%). Interestingly, in one case an inter-specific distance lower than 2.7% occurred between two non-congeneric species: *B. bjoerkna* and *Vimba vimba* (2.51%, Table 2). Zardoya & Doadrio (1999) and Cunha et al. (2002) observed the same effect, based on the sequencing of a different marker. All congeneric species always clustered together in the phylogenetic analysis. Furthermore, several species pairs (not always congeneric) have been shown to possess shared haplotypes. In most cases, a single individual of one species bore a haplotype typical of a close (sister) species. Such was the case of a single *B. bjoerkna* among *A. brama*, *G. gobio* among *R. vladykovi*, *C. maraena* among *C. peled*, and *A. gueldenstaedtii* among *A. ruthenus*. All these cases can be easily explained as simple hybridization, in the case of *Coregonus* even human-driven intentional hybridization, (with the second species being the maternal one due to the maternal inheritance of mtDNA), which was confirmed by the sequencing of the nuclear marker (first intron of the S7 ribosomal protein gene).

The last species pair, *L. leuciscus* and *L. idus*, represented an exception. We found five individuals classified as *L. leuciscus* bearing the haplotype of *L. idus*. These two species also presented the lowest mutual genetic distance from all analyzed species pairs (0.62%). We therefore conclude that in these two species, not only hybridization (as confirmed by the sequencing of S7) but also probably recent speciation might have taken place in the genetic similarity of both species.

Analyses have revealed a significant deep intraspecies divergence in several taxa. In most cases, those are species with large areas (Kottelat & Freyhof, 2007), such as *B. barbatula*, *L. delineatus*, *P. phoxinus* or *S. cephalus*.

The most pronounced example is the stone loach (*B. barbatula*). The DNA barcoding technique has revealed high intra-specific divergence (up to 5.97%, Table 1). We have tested 27 individuals from six regions and we found 13 haplotypes diverging into three lineages (Table 1, Fig. 10, Fig. 11). Šedivá et al. (2008) has described the presence of the lineage V in the Labe River basin and the sublineage V_a in the Morava River basin, both of the lineages belonging to the Danubian clade. Our results are in good concordance with these findings. Furthermore, we have distinguished a third, the most divergent lineage, occurring in the Odra River basin. This lineage possibly belongs to the Eastern clade similarly to previously detected individuals from the same basin in Poland (Šedivá et al., 2008).

BOLDSYSTEMS v2.5 | Management & Analysis

Ichthyofauna of the Czech Republic [IFCZE]

Barcode Identifiers

Barcode ID:	IFCZE510-10	**Sample ID:**	IFCZE0780
Identified As:	Silurus glanis		

COI-5P

Marker:	COI-5P	**GenBank Accession:**	HQ960905
Last Updated:	2010-12-10	**Translation Matrix:**	Vertebrate Mitochondrial

Sequencing Runs [2/2 Trace Files Successful]

Run Date	Run Site	Direction	Trace File	PCR primers	Seq Primer	Status
2010-12-05 20:23:52	Biodiversity Institute of Ontario	Reverse	IFCZE510-10 [C_FishF1t1,C_FishR1t1] _R.ab1	C_FishF1t1/C_FishR1t1	M13R	high qual
2010-12-05 16:03:50	Biodiversity Institute of Ontario	Forward	IFCZE510-10 [C_FishF1t1,C_FishR1t1] _F.ab1	C_FishF1t1/C_FishR1t1	M13F	high qual

View Trace Files | Download

Nucleotide Sequence

Residues:	652
Comp. A:	159
Comp. G:	123
Comp. C:	195
Comp. T:	175
Ambiguous :	0

Clear Sequence

```
CCTTTACCTAGTATTTGGTGCCTGAGCCGGAATAGTCGGCACAGCCTTAAGTCTCCTAATCCGAGCAGAGCTGGC
CCAACCTGGCGCCCTCCTAGGCGATGATCAAATTTATAACGTCATCGTTACTGCTCACGCCTTTGTAATAATCTT
CTTTATAGTAATACCAATTATGATCGGAGGGTTTGGAAACTGGCTTGTGCCTCTTATGATTGGGGCACCAGACAT
GGCTTTCCCCCGGATAAACAACATAAGCTTCTGACTTCTCCCTCCTTCATTCCTGCTACTACTAGCCTCCTCCGG
AGTCGAAGCGGGCGCAGGAACAGGATGAACCGTTTACCCCCCTCTTGCAGGAAACCTCGCCCACGCAGGTGCTTC
CGTAGACTTAACAATCTTTTCACTACACCTCGCAGGTGTGTCCTCCATCCTTGGGGCCATCAATTTCATTACAAC
TATTATTAACATAAAACCCCCAGCCATCTCACAATACCAAACACCTTTATTTGTGTGGGCCGTACTAATTACAGC
AGTGCTTTTACTCCTGTCCCTGCCAGTCCTGGCCGCAGGAATTACAATGCTCCTAACGGACCGAAATCTAAATAC
TACATTCTTTGACCCCGCAGGAGGCGGAGACCCAATCCTCTACCAACATCTT
```

Identify Sequence Using: Full Database | Species Database | Published DB | Full Length DB

Amino Acid Sequence

Residues: 230

```
LYLVFGAWAGMVGTALSLLIRAELAQPGALLGDDQIYNVIVTAHAFVMIFFMVMPIMIGGFGNWLVPLMIGAPDM
AFPRMNNMSFWLLPPSFLLLLASSGVEAGAGTGWTVYPPLAGNLAHAGASVDLTIFSLHLAGVSSILGAINFITT
IINMKPPAISQYQTPLFVWAVLITAVLLLLSLPVLAAGITMLLTDRNLNTTFFDPAGGGDPILYQHL
```

Illustrative Barcode

0 ... 320

321 ... 641

64651

Sample Report From LIMS

BOLDLIMS Report Available.

Logged in as : Jan Mendel | Build Time : 0.349 s | Render Time : 0.592 s

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Fig. 12. An example of barcode record of the species.

The second deepest divergence has been detected in chub (*S. cephalus*). We have tested 28 individuals and found four haplotypes, diverging into two lineages (Table 1, Fig. 10, Fig. 11), separated by 5.25%. The less abundant lineage occurred only in the North Sea drainage area (Labe and Vltava River basins), the second, more numerous lineage was found across all river basins. Seifertová et al. (2008) in their large study of phylogenetic diversity of chub across its European range distinguished four different haplogroups, corresponding to four lineages, i.e. the Western, Eastern, Aegean and Adriatic (*sensu*

Durand et al., 1999) ones, with the populations collected in the Czech Republic belonging to the Western Lineage. It is probable that our more prevalent lineage corresponds to the Western lineage, sharing its distribution pattern. It is unclear, but possible that the second of our lineages corresponds to the Eastern lineage. However, the Eastern lineage was not found by Seifertova et al. (2008) in the North Sea basin, but in populations from the Vistula River basin in Poland and in Finland. Therefore we cannot exclude the possibility that we have found a new, fifth lineage. A definite conclusion about the identity of lineages can not be drawn due to different markers utilized in both studies. The existence of other deeply divergent lineages of chub is also evident e.g. in Perea et al. (2010), who found two lineages of chub in their study of phylogenetic relationships in the Circum-Mediterranean subfamily Leuciscinae. It is clear that the taxonomy of *S. cephalus* needs to be further addressed; both on the level of additional samples, which we have already collected, and by the unification of utilized markers.

The DNA barcoding further discriminated two deep lineages in two other cyprinid species, *P. phoxinus* (lineages separated by 3.97%) and *L. delineatus* (lineages separated by 3.81%). In the most recent Red List of the Czech Republic, the minnow (*P. phoxinus*) is classified as vulnerable (Lusk et al., 2011). The priority task in the protection of minnow lies in the long-term sustenance of water quality, the segmentation of river bed and banks together with thoughtful fishing and angling management (Hanel & Lusk 2005). We have tested 25 individuals from six regions and we distinguished 10 haplotypes diverging into two lineages (Table 1, Fig. 10, Fig. 11). Lineage I is located in water courses flowing into the North and Black Sea drainage areas and the lineage II is located in the Baltic Sea drainage area (including the Bečva River). While the Bečva River currently belongs to the Black Sea drainage area, here we can consider an ancient proximity or identical origin of the two populations, as during the Pleistocene (e.g. the Elster glaciation) the waters of the Odra flowed towards the south into the drainage area of the river Bečva (Czudek, 1997). The systematics of the genus *Phoxinus* has not been solved yet.

The belica (*L. delineatus*) is currently classified among the critically endangered species in the Red List of the Czech Republic (Lusk et al., 2011). Apart from food competition and the continuous forcing out of original localities by invasive species, its vanishing is also probably influenced by the spread of a disease caused by the pathogen *Sphaerothecum destruens*, which came to Europe through an invasion the invasive fish species *Pseudorasbora parva* with the import of herbivorous fish fry (Gozlan et al., 2005; Hanel & Lusk 2005). Interestingly, both our lineages of the belica coexist in the same locality. Those five tested individuals came from one fish hatchery in the Ohře River basin. Currently, there is no detailed phylogenetic study of this species. Only Perea et al. (2010) dealt with the taxonomy and systematics of this genus, but based on two individuals only and in the context of general phylogenetic relationships and biogeographical patterns in the Circum-Mediterranean subfamily Leuciscinae. Our studies of this species continue in order to collect individuals from further localities. We also aim to evaluate hybrid events in the scope of uncovered lineages based on a nuclear marker.

Two separate lineages were also detected in eel (*A. anguilla)* (3.49%) and Siberian bullhead (*C. poecilopus*) (2.68%). The intra-specific diversity of eel on a molecular level has not been studied yet. Both of our two lineages were found in North Sea and Baltic Sea drainage areas. However, when performing a comparison of our sequences with other sequences stored in

BoLD, one of these lineages is identical to samples of the American eel (*A. rostrata*). Kottelat & Freyhof (2007) mention that the American eel has been stocked in Eastern Europe; our findings confirm their suspicion that the presence of isolated individuals of American eel cannot be ruled out in our free waters. In order to confirm these findings, further samples will be evaluated.

In the Siberian bullhead, Lusk et al. (2008a) distinguished two complexes based on the sequencing of the cyt *b* gene and mitochondrial control region: the "baltica" complex, which consists of the populations from the Baltic Sea drainage area, and the "danubialis" complex, which consists of the populations from the Black Sea drainage area. An existence of the lineages uncovered in this study further supports these findings: our tested populations from the Odra River basin fall into the "baltica" complex, the individuals from the Morava and Dyje River basins fall into the "danubialis" complex. Lusk et al. (2008a) also further discuss the necessity of a more complex genetic analysis (utilizing a nuclear marker in combination with morphological characteristics) in order to properly evaluate the possible taxonomic status of these complexes.

All these findings (visualized in a bootstrapped NJ tree, Fig. 10) are based on the sequencing of a single marker. Thus they are not *per se* a sufficient tool and criterion for a definitive taxonomical and phylogenetic consequences. For this reason, our project has employed a more complex approach: based on these findings, a sequencing analysis of a nuclear marker must be performed, and comparisons of the morphological characteristics of the lineages must be evaluated. Such complex evaluations will subsequently serve for the definitive confirmation of taxonomic conclusions, and also for the reference collection of the fish which will be stored in the National Museum of Natural History, Prague.

6. Conclusion

The presented study provides a clear example of the usefulness and suitability of DNA barcoding for the cataloguing of the biodiversity of the Czech freshwater fishes. The lineages newly uncovered in our study are being subjected to further detailed research in order to evaluate their correct taxonomical status and systematic position. Definitive results of the project, once obtained, can probably serve as a good starting point for subsequent comparatory phylogenetic studies in an Euroasian context. The presented project also enabled us to focus our attention also on taxa which have never been in the scope of molecular-genetic investigation (e.g. *L. delineatus, P. phoxinus, Gasterosteus aculeatus, A. anguilla, etc.*).

New taxonomically and systematically important knowledge may (and hopefully will) contribute to the updating of the background information for the monitoring of the NATURA 2000 system. It also brings crucial information and recommendations to the Agency for Nature Conservation and Landscape Protection of the Czech Republic. A new design of the species collection of type individuals, constructed on the basis of most recent molecular methods and including morphological data, as well as organizing a unified voucher, is also a great benefit for the National Museum of Natural Science in Prague.

By employing a complex approach (analysis of both the nuclear and mitochondrial genomes plus morphological analyses) we were able to eliminate the danger of including a hybrid individual as a representative of a particular species into the type series. Combined

preservation approaches (whole individuals stored in formaldehyde, fin clippings in ethanol, DNA isolates) targeted at individuals from the new type collections will also enable possible comparative DNA analyses in future, whenever needed. All the results are clearly and concisely assembled in the BoLD database, which allows very simple and quick on-line access to informations for the broadest scientific and for the general public.

The UN has declared the year, 2010, to be the International Year of Biodiversity. Let us all wish much success to all future projects and initiatives dealing with biodiversity; for every successful project will bring us closer to the ultimate goal of the knowledge and understanding of the biodiversity.

7. Acknowledgement

This study was carried out within the framework of research projects no. M200930901 supported by the Program of Internal Support for International Collaborative Projects of the Academy of Sciences of the Czech Republic and no. 206/09/P608 supported by the Grant Agency of the Czech Republic. We would like to thank our colleagues from the Biodiversity Institute of Ontario, University of Guelph for their financial, laboratory and informatics support, the Czech and Moravian Fishing Unions, the representatives of the water-resources authorities (pond management companies, river basin management agencies), the management of the Protected Landscape Areas, our colleagues from universities and other people for their help with sample collection.

Data accessibility

Cytochrome c oxidase subunit I gene sequences, trace files, digital images of fish and other metadata were submitted to Barcoding of Life Data systems (BoLD – http://www.barcodinglife.com) and were given BoLD Process ID’s IFCZE001-10 to IFCZE701-10 and IFCZE702-11 to IFCZE852-11, all being part of the project „The ichthyofauna of the Czech Republic (IFCZE). The complete NJ/K2P tree can be found on the website of IVB on http://www.ivb.cz/projects-molecular-biodiversity-inventory-of-the-ichthyofauna-of-the-czech-republic.html. Acquired DNA sequences are also being stored in GenBank with accession numbers HQ960417-HQ961093.

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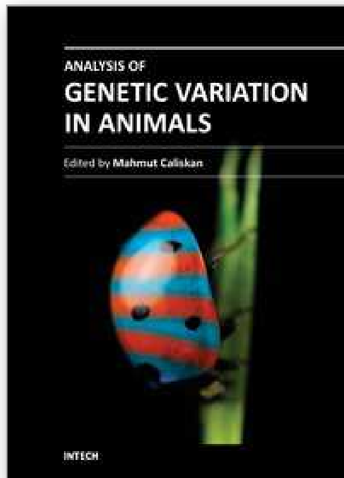

Analysis of Genetic Variation in Animals
Edited by Prof. Mahmut Caliskan

ISBN 978-953-51-0093-5
Hard cover, 360 pages
Publisher InTech
Published online 29, February, 2012
Published in print edition February, 2012

Analysis of Genetic Variation in Animals includes chapters revealing the magnitude of genetic variation existing in animal populations. The genetic diversity between and within populations displayed by molecular markers receive extensive interest due to the usefulness of this information in breeding and conservation programs. In this concept molecular markers give valuable information. The increasing availability of PCR-based molecular markers allows the detailed analyses and evaluation of genetic diversity in animals and also, the detection of genes influencing economically important traits. The purpose of the book is to provide a glimpse into the dynamic process of genetic variation in animals by presenting the thoughts of scientists who are engaged in the generation of new idea and techniques employed for the assessment of genetic diversity, often from very different perspectives. The book should prove useful to students, researchers, and experts in the area of conservation biology, genetic diversity, and molecular biology.

How to reference
In order to correctly reference this scholarly work, feel free to copy and paste the following:

Jan Mendel, Eva Marešová, Ivo Papoušek, Karel Halačka, Lukáš Vetešník, Radek Šanda, Milena Koníčková and Soňa Urbánková (2012). Molecular Biodiversity Inventory of the Ichthyofauna of the Czech Republic, Analysis of Genetic Variation in Animals, Prof. Mahmut Caliskan (Ed.), ISBN: 978-953-51-0093-5, InTech, Available from: http://www.intechopen.com/books/analysis-of-genetic-variation-in-animals/molecular-biodiversity-inventory-of-the-ichthyofauna-of-the-czech-republic

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