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Cytochrome b gene (*cytb*) in analysis of anonymous biological traces and its application in veterinary diagnostics and animal conservation

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Institutions and agencies which are to enforce veterinary and animal protection and conservation regulations require means enabling identification of species origin of meat, trophies and other biological material. In this study a partial DNA sequence of cytochrome b gene (*cytb*) was used to identify three different anonymous samples: (1) the hair from the bird's nest, (2) the blood stain found in the forest on snow cover and probably left by poachers, and (3) the fragment of the muscle from exotic reptile, provided by the Warsaw Zoological Garden. The sequences derived were compared with the sequences registered in GenBank, and species origin of samples was determined. The partial sequence of *cytb* adopted in the study proved to be useful for identification of animal species. Out of three samples subject to identification, one was found to belong to the goat, one to elk and another one to *Python molurus,* the latter two included in the list of protected species issued by *Convention on International Trade in Endangered Species of Wild Fauna and Flora* (CITES).

KEY WORDS*:* animal conservation / cytochrome b / species identification

Veterinary and forensic science laboratories frequently encounter samples lacking any morphological detail allowing their identification. Moreover, a highly degraded nature of samples collected in wild area or stored under non-sterile conditions often make impossible their identification based on conventional methods or even on ampli-

fying a large fragment of gene. There is a need, therefore, to work out a simple method allowing to determine the origin of anonymous biological trace(s) and check whether the species is included in the *Convention on International Trade in Endangered Species of Wild Flora and Fauna* (CITES) list, or whether the product(s) being sold is (are) not derived from a particular species of endangered wildlife. According to the report drawn up by TRAFFIC (the wildlife trade monitoring network) upon the order of the European Commission, Poland has been ranked the third among the candidate countries with respect to the number of animal individuals protected by Washington Convention and retained at the border [htttp://www.wwf.pl/handel.php]. It is also noteworthy that in the *Sixth Framework Programme for Research, Technological Development and Presentation of the UE* the improvement of safety of life and food has also been considered as the most significant current need [Anonymous 2002]. The food safety includes all conditions and actions which have to be respected on each stage of producing and controlling the quality of food. In analyses of old, trace and degraded samples molecular tests based on mitochondrial DNA (mtDNA) are recommended [Parson *et al.* 2000, Budowle *et al*. 2003]. The cytochrome b gene of mtDNA has been found to be a powerful indicator for identifying the species with DNA analysis techniques [Zehner *et al.* 1998, Parson *et al.* 2000] also used in studies of molecular evolution [Kocher *et al.* 1989, Montgelard *et al.* 1997, Prusak *et al*. 2004] and legal medicine [Barlett and Davidson 1992]. Moreover, mtDNA is of higher stability and occurs in a much higher number of copies than nuclear DNA.

In the present study a partial cytochrome b gene sequence was used for identification of three anonymous biological samples, representing different fields of animal conservation and sanitary logistics, *i.e*. expanding knowledge about biology of wild species ("ornithological trace"), observing game regulations ("forest trace"), and controlling international trade of endangered species ("zoological trace"). Analysed fragment of cytochrome b gene was 273 bp long. Hsieh *et al*. [2001, 2003] showed that the gene fragment of that size is sufficient for discriminating between even closely related species.

Material and methods

Biological material

The material covered hair, blood stain and soft tissue (muscle) – sample 1, 2 and 3, respectively.

Sample 1 further referred to as "ornithological trace" was the hair from an unidentified bird nest knocked-off the tree during the night gale and found near the road to Jastrzębiec (Central Poland).

Sample 2 further referred to as "forest trace" was blood collected from the blood stain (1.5 m. in diameter) found on the snow in the Notecka Forest (northwest Poland), probably left by poachers.

Sample 3 further referred to as "zoological trace" was fragment of the dead rep-

tile's soft tissue (muscle) supplied by the Warsaw Zoological Garden and remaining unknown to the authors.

Moreover, the blood withdrawn from the Polish White Improved goat maintained close to the bird's nest from which sample 1 originated was used as a comparative sample.

Morphological analysis of the nest

The components of the nest were examined according to the standards of hair forensic examination. At first, all kinds of material were isolated from the nest under binocular microscope. Then the slides with the specimen were observed in glycerin medium under light microscope with 10 × objective. To characterize the specimens (especially hairs) morphologically the terminology proposed by Ogle and Fox [1999] was applied.

DNA extraction, PCR amplification and cycle sequencing

Total genomic DNA from the hair, blood stain and reptile's muscle was extracted according to the standard organic procedure [Wilson *et al*. 1995]. For each sample specific conditions of extraction were improved. Genomic DNA from both blood samples ("forest trace" and control goat) was extracted using Wizard Genomic DNA Purification Kit (PROMEGA).

The DNA amplification was performed with primers suggested by Parson *et al.* [2000]. The sequences of the forward and reverse primers were extended by a universal primer sequence (-21) M13 at the 5'end. The PCR reaction was conducted in a GenAmp PCR System 9600 Thermal Cycler (AB, USA), as follows: 94°C for 2 min. (denaturation) and next 94°C for 30s, 50°C for 45 s, 72°C for 45s – 35 cycles. In order to improve sequencing efficiency, the PCR amplification was carried out in two steps. First step was conducted in a volume of 50 μl in a reaction mixture composed of 50-100 ng of genomic DNA, 200 μM of each dNTP, 1 × PCR buffer (AB, USA), 1.5 mM $MgCl_2$, 1μM of each primer, and 1.5 U DNA Taq Gold polymerase (AB, USA). In the second step 4 μl of 1000 × diluted PCR products were used which then were amplified under the same conditions except the amount of primers (0.1 μM) and polymerase (1 U). The amplification products were purified, sequenced and analysed as described earlier by Prusak *et al*. [2004].

Species identification

Sequence homology was evaluated by importing a 273 bp fragment of the consensus sequences obtained in the present study into the BLAST query window [Altschul *et al*. 1990, 1997]. A non-redundant search was launched applying default settings of the software package [Smith *et al*. 1996].

Results and discussion

The universal primers of L14840 and H15153 (nomenclature according to the human mtDNA reference sequence given by Anderson *et al*. [1981]) were used to amplify part of cytochrome b gene from the three unknown samples: the hair from bird's nest, blood stain and reptile's muscle. The results showed that primers amplified only one-size fragment from a target sample. However, mitochondrial DNA fragments have been found in the nuclear genome of humans [Fukuda *et al*. 1985]. In the present study no sequence behaving like a pseudogene (no sequence with two different bases detected in one position or distinctly differing in number of substitutions – Li *et al*. [1985]) was found, leading to the conclusion that all sequences were of mitochondrial origin. Sequences obtained from the samples analysed were deposited in GenBank under the accession numbers AY840102, AY840106, AY840103 for sample 1, 2 and 3, respectively, and were used to identify corresponding species by aligning to cytochrome b gene sequence entries in nucleotide database GenBank applying the BLAST software package according to Smith *et al.* [1996]. As a result, sequence pairs were presented, consisting of the query sequence and a database entry in combination with a similarity value reporting the significance of the match as described by Altschul *et al*. [1990].

The "ornithological trace" (sample 1) was a hair from the bird's nest. The structure of the nest remained intact. The morphological analysis performed according to the routine forensic procedures showed that the nest was built with several types of biological material. Four out of six materials described were of plant origin while the remaining two comprised two types of animal hair (Tab. 1). Morphological identification of animal anonymous hairs requires for each species standard samples of both fur and protective hair of various body regions. Therefore, recognition of the origin of a hair based on its morphology is laborious and complicated. In light of this, in the present study the molecular test based on sequence analysis of partial cytochrome b gene on randomly chosen type II single hair has been carried out. The derived sequence for the "ornithological" trace completely matched (100% similarity) with the sequence from GenBank belonging to the goat (Tab. 2). Moreover, the sequence derived was compared with that of a goat from the flock situated close to the site on which the nest was found. Again, 100% homology occurred.

The "forest trace" (sample 2) was the blood from the blood stain found on snow. The snow fixed the sample, but its dark red colour indicated that it was not a fresh stain, but probably frozen and unfrozen several times. Besides the central spot from which the sample has been collected, there were small blood spots dispersed all around the central one. The observed features indicated that the animal was killed by poachers. Suspecting the posible cell damage and small DNA content of the sample, the authors assumed that more successful in PCR amplification will be the use of partial sequence of mitochondrial DNA. On the basis of GenBank, searching the "forest trace" was assigned with a sequence match of 97.8% – Table 2. The closest record was found to belong to the elk (*Alces alces*), the species protected in Poland.

The "zoological trace" (sample 3) was the fragment of muscle of a dead reptile provided by the Warsaw Zoological Garden. The animal has been brought there by a

Table 1. Description of types of biological material from the nest as based on Ogle and Fox [1999] standard classification

Type of biological material	Description
Type I	- animal dark brown hair; loose curl form; length above 20; shaft diameter 140-150 µm; medulla partially double, fragmentary; opaque; pigment distribution peripheral; ovoid bodies absent; cortical fusi absent; root growth stage telogen
Type II	- animal colourless hair; loose curl form; length above 30; shaft diameter 95-105 µm; medulla single, continuous, opaque; pigment distribution uniform; ovoid bodies absent; cortical fusi absent; root growth stage telogen
Type III	- thin, small, grey vegetable curved fibres, length below 1 cm
Type IV	- fragments of plants with irregular dense aggregations, length below 20 cm
Type V	- irregular, grey vegetable fibres, aggregations of tiny fibers with round or pointed extremities, length below 0.5 cm
Type VI	- irregular, brown woodan parts, length below 2 cm

Table 2. List of the samples investigated in this study and results of searching the GenBank database. The most homologous sequence entries with derived sequence are depicted by accession number, the name of the entry was added to the accession number

Investigated sample	Accession no.	Similarity (%)
„ornithological trace"	D84204.1, AB004069.1 *Capreolus pygargus*; AB004430.1, AB004073.1, AB004071.1 *Capreolus hircus*	100
„forest trace"	AF040383.1 *Alces alces*	97,8
„zoological trace"	U69854.1, AY014890.1, U69855.1 *Python molurus*	99

person who could not supply any documents confirming its origin. According to the Interpol the international illegal trade for endangered species gives the profit comparable to that of drug or weapon trade. As a result, many rare animal species, especially reptiles and amphibians, are endangered with extinction. It seems that molecular comparisons can give important support to reduction the illegal trade for rare species and their products. Based on partial cytochrome b gene analysis the muscle sample in question was identified as originating from *Python molurus* (can be attested to the database record of *Python molurus*) which is endangered species enlisted in appendix II of CITES and IUCN *Red List of Threatened Animals*. The three most concordant

(closest) records for *Python molurus* out of five existing in GenBank displayed 99% of similarity with the sequence derived in this study (Tab. 2.) They differed only by one nucleotide position.

Observed differences in nucleotide substitutions between the query and the subject sequence in the samples from both "forest" and "zoological" trace can be attributed to individual variation. A review of relevant literature indicates that similar polymorphisms of the cytochrome b gene can be found in wild populations, especially within species demonstrating a wide range of geographical distribution [Taberlet *et al*. 1992]. Moreover, Hsieh *et al*. [2001] reported that the intra-species sequence diversity ranges from 0.25 to 2.74% and that of inter-species from 5.97 (related species) to 34.83% (distant species).

Typing and identification of animal species based on direct sequencing is well established and widely used [Barlett and Davidson 1992, Parson *et al*. 2000, Hsieh *et al*. 2001]. Although more expensive than the other PCR-based methods it provides information for all positions of a target sequence. For this reason direct sequencing can be successfully used for differentiation between taxonomically close species or determining the origin of anonymous samples that is of particular interest in veterinary control and animal conservation. Moreover, the method uses mtDNA which, due to its high stability against environmental stress, may be helpful in analysing old samples or microstains when only traces of genetic material are available. Analyses shown in the present study represent only several possible applications of the method in conservation biology. It seems that there is a strong need in Poland for the research on identification procedures. This is especially important in the light of rising international activity and launching international programmes dealing with protection of global genetic resources and controlling the international transfer of biological material.

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Wykorzystanie polimorfizmu sekwencji genu cytochromu b do analizy anonimowych śladów biologicznych w diagnostyce weterynaryjnej i ochronie przyrody

S t r e s z c z e n i e

Na podstawie analizy polimorfizmu sekwencji genu cytochromu b mtDNA zidentyfikowano gatunek pochodzenia trzech anonimowych próbek – włosa z ptasiego gniazda, plamy krwi dzikiego zwierzęcia znalezionej w lesie na śniegu oraz fragmentu tkanki mięśniowej egzotycznego gada. Uzyskane sekwencje genu *cytb* (długości 273 pz) wykorzystano do określenia gatunku pochodzenia badanych próbek przez porównanie sekwencji własnych z sekwencjami opublikowanymi w bazie GenBank. Dla sekwencji genu *cytb* uzyskanej z plamy krwi znaleziono w GenBank sekwencję pochodzącą od łosia (97,8% podobieństwo). Dla tkanki mięśniowej gada najbardziej podobny zapis (99% podobieństwo) dotyczył pytona tygrysiego (*Python molurus*). Uzyskanie tak znacznego podobieństwa między sekwencjami własnymi a opublikowanymi w GenBank pozwala przyjąć, że badane anonimowe próbki pochodziły odpowiednio od łosia i pytona tygrysiego. Zaobserwowane nieznaczne różnice można przypisać zmienności wewnątrzgatunkowej. Natomiast sekwencja w genie *cytb* uzyskana z włosa pozyskanego z ptasiego gniazda była identyczna (100% podobieństwo) z sekwencją kozią opublikowaną w GenBank. Okazała się ona identyczna także z sekwencją w genie *cytb* uzyskaną dla włosa kozy ze stada utrzymywanego w pobliżu miejsca znalezienia ptasiego gniazda, z którego pozyskano badany włos.