CONVENTION ON INTERNATIONAL TRADE IN ENDANGERED SPECIES
OF WILD FAUNA AND FLORA

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PROJECT TIGRIS ID: RESULTS

This document has been submitted by the Czech Republic in relation to agenda item 36 on Asian big cats.*

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Project Tigris ID: Results

Summary

The project TigrisID was funded by the Czech Republic and implemented by the Forensic DNA Service Laboratory (FDNAS) between 2018 to 2021. Under the project, two methods have been developed for species identification of tiger biological material in different types of products and for determination of tiger individual profile using STR loci. **Two types of analytical kits for species and individual identification of tigers** have been developed and are **available free of charge** to CITES authorities of other countries on request. **FREE testing of tiger samples** is offered by the Forensic DNA Service Laboratory in Prague, Czech Republic. It can include testing of a product of unknown origin in any investigated case as well as an individual identification and determination of the DNA profile of any tiger individual. Additional samples of tigers (or other big cat species) are needed for further research that continues with the follow-up **project PantheraID** (the Charles University in Prague, morphological and genetic identification of big cats). Please contact team members if you are interested in analytical kits, can provide any samples (tiger or other big cat species), wish to have your sample analysed or are interested in cooperation.

Contacts

Researcher: **Forensic DNA Service** (Budínova 67/2, 180 00 Prague 8, http://www.fdnas.cz)
Director/Genetic expert: **Daniel Vanek, Ph.D.; daniel.vanek@fdnas.cz**
Supervision:
- **Ondrej Kloucek, Ph.D.**; ondrej.kloucek@mzp.cz, Ministry of the Environment of the Czech Republic (CITES MA CZ)
- **Pavla Rihova, M.Sc.**; wildlife enforcement and forensic expert for CITES MA CZ, pavla.rihova@natur.cuni.cz, Environmental Forensic Sciences Centre, Charles University

TigrisID project sponsor: Ministry of Interior of the Czech Republic (project number VH20182021028 - Applied research on the genetics of selected species of animals protected under the international convention of CITES)

Project objectives

The goal of the TigrisID project was to develop and validate new forensic tools for DNA-based identification of *Panthera tigris* biological material. The project included the development of STR analytical kits for the species and individual identification of tigers, perform population study for unrelated tigers, reference database development, and verification of methods for the species identification of *Panthera tigris* in heavily processed materials such as broths, tiger glue, tiger wine, boiled tiger bones, etc.

Collecting samples for the purpose of the project

For the purpose of the research, tiger samples taken during inspections of tiger breeding facilities (private breeders, circuses), obtained from zoos, and samples from investigated cases were used. The samples came from the Czech Republic, Slovakia, Russia, Finland, Ireland, France, Germany, the UK, and Lithuania. A total of **424 tiger samples were obtained**. DNA of 384 tiger individuals was successfully isolated (these profiles have been entered into the reference database). Reference materials of other related feline species were also collected and analysed (*Panthera leo*, *Puma concolor, Panthera onca, Panthera pardus*, and *Felis catus*).

Sampling kits for different types of samples have been developed which included hair, blood, saliva, raw soft tissue, hard tissue (bones, teeth, claws), droppings. Each sampling kit contains a **primary sampling protocol** with the following data:

- ID number of the kit,
- Animal species and Individual identification (microchip number or other),
- Description of the sample, date of sampling,
- Conditions during sampling (temperature, humidity…),
- Sample stabilization method (drying, cooling, freezing, DNA stabilization solution),
- Animal owner's name,
- Name and signature of the officer or person who provided sampling,
- Case number,
- Photo documentation,
- Date and time of delivery to the laboratory,
- Notes.

Detailed guidelines with descriptions of methods of sampling and sample storage were developed (**TigrisID: Guidelines and Sampling Protocols**). These were provided to the CITES Secretariat (also widely distributed within partner institutions, zoos, EU-TWIX members, Interpol, etc.).

TigrisID project methods used and results

DNA analysis is one of the key forensic methods. For human biological material, the method is standardized worldwide and well managed in terms of the quality of the results. However, the usage of animal DNA analysis for forensic purposes is still evolving and will require a great deal of research and effort to harmonize approaches, standardize the methods used and ensure the quality of results. The first recommendations in the field of animal DNA analysis were published in 2005. In 2011, the International Society for Forensic Genetics (ISFG) issued a set of 13 recommendations that also consider the requirements of enforcement authorities. Within the TigrisID project, it was found that some recommendations are difficult to apply in practice (e.g., ensuring a sufficient number of samples from unrelated individuals in rare endangered species), so it was proposed to modify them and add new points (will be published).

1. Quantification and quality of isolated DNA

The determination of the quantity of DNA isolated from the examined sample is essential in the process of DNA forensic analysis for subsequent amplification by multiplex PCR (optimal amount of DNA should be used). For DNA quantification, a multiplex qPCR reaction with TaqMan probes was used.

Data about the quality of isolated DNA make it possible to detect the presence of inhibitors, or to determine the level of DNA degradation and, according to the results, to propose the appropriate subsequent analysis steps (removal of inhibitors, usage of PCR mini amplicons, etc.).

2. DNA profiling

Within animal identification genetics, a distinction is made between species and individual identification. **Species identification** is used to determine the species of an animal when morphological determination is not possible (products, tissues, medicine…). **Individual identification** serves to confirm the identity of a particular individual, to determine the parentage or to determine whether the examined product of biological origin (claws, teeth, skin, medicine, etc.) comes from an individual whose reference sample has been entered into the database.

Tiger species identification (*Ptig Qplex*)

Mitochondrial DNA analysis is more appropriate for species identification of products or modified biological materials than nuclear DNA, as it is 10-100 times more abundant in cells. Due to the larger number of copies of mitochondrial DNA, species determination is possible even in samples with nuclear DNA degraded due to chemical or thermal processing (e.g., tanning, production of medicinal products). The major advantage of using mitochondrial DNA is high sensitivity of species determination even when only picograms of DNA are available. The only disadvantage is the inability to distinguish hybrids (e.g., tigon = offspring of a male tiger and a female lion) due to transfer of mitochondrial DNA through the maternal line.

Primers for amplification of mitochondrial genes encoding cytochrome b, cytochrome oxidase I, and 12S rRNA were designed and tested for tiger species identification. Primers were continuously tested on forensic samples of tigers (excrements, hair, bone (native/processed), tissue, tanned/untanned skin, and blood). This activity included testing for the PCR robustness and tolerance to common inhibitors as well as bioinformatics analysis of the resulting sequences. Primers for species identification were continuously tested on biological materials sampled from other feline species. The target sequence for amplification is located in the D-loop region of the mtDNA.

The procedure was validated for QuantStudio™ 5 thermocycler system (Applied Biosystems, USA) and QuantStudio Design and Analysis Software

v1.4.3. (Applied Biosystems, USA). As part of the testing, the laboratory participated in a proficiency test organized by ENFSI APST (European Network of Forensic Science Institutes, Animal, Plant and Soil Traces expert working group).

As part of the output of the project, the **specific analytical kit for RT-PCR species-specific determination of *Panthera tigris* called *Ptig Qplex*** was developed. The analytical kit is intended for analysis of tiger biological material in of unknown origin and with complex composition (like tissues, products, medicine…). It allows to detect minute amounts of DNA and thus provide evidence of possible illegal trade. The analytical kit not only provides information about the presence of tiger DNA but also delivers information about DNA concentration and the presence of inhibitors using internal amplification control.

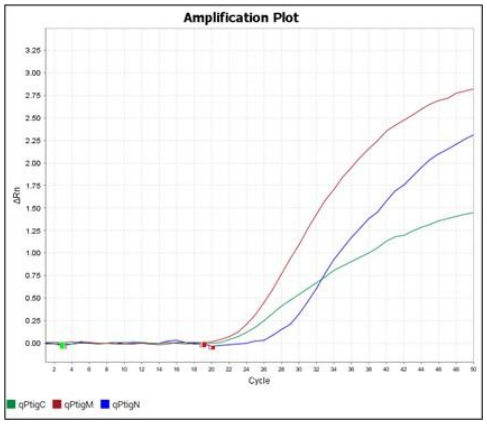

Figure 1: Analytical kit *Ptig Qplex* - amplification plot for tiger sample
qPtigM - red curve for mitochondrial DNA (indicates the presence of *Panthera tigris* DNA in the test sample),
qPtigN - blue curve for nuclear DNA (determines the concentration of nuclear DNA in the examined sample),
qPtigC - green curve for internal amplification control (determines the purity of the isolated DNA).

Developed analytical kits for tiger species-specific determination can be obtained from the laboratory Forensic DNA Service on request (only for wildlife enforcement purposes, free of charge).

Tiger individual identification (*Ptig STRplex*)

In the case of individual identification, it is necessary to determine the DNA profile of the individual. The DNA profile consists of the results of genetic testing of polymorphic loci, which can be Short Tandem Repeats (STR), point mutations (SNP - Single Nucleotide Polymorphism) or insertions and deletions (InDells), or insertions (InNulls). In human identification genetics, STR analysis is the gold standard, and ISFG recommendations also recommend the usage of STR (also recommended by European Network of Forensic Science Institutes - ENFSI). Because sufficient amounts of isolated DNA cannot be expected in all forensic samples, it is more efficient to amplify selected STR loci in one or more multiplex reactions. With respect to the ENFSI recommendations, STRs with tetranucleotide repeats and good informativeness were preferred when selecting STR polymorphic regions. Another advantage of STR analysis is the sensitivity in the tens of picograms of DNA and the relative ease of operation because capillary electrophoresis, which is used to separate and visualize amplified DNA fragments, is a common available method in forensic laboratories.
Different STR loci were selected and tested for individual identification of tigers. STR loci in which low polymorphism was found were excluded from the original multiplexes. After testing, a total of 11 selected STR loci (Short Tandem Repeats) were selected, divided into 2 tiger STR multiplexes. Both multiplexes include molecular system for gender determination and also contains an allelic ladder that allows precise allele calling and exchange of results.

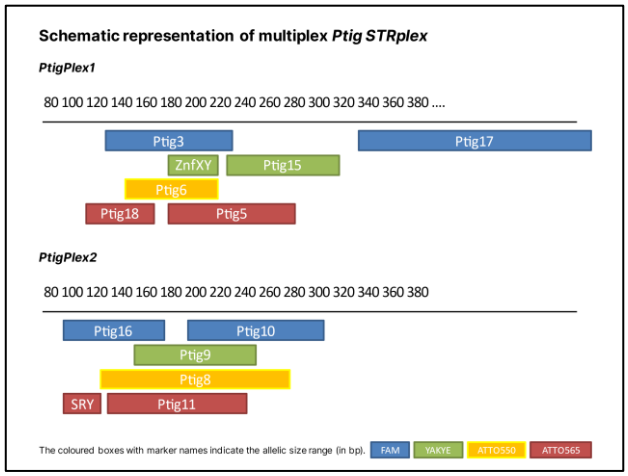

Figure 2: Schematic representation of STR loci in 2 distinct multiplexes of *Ptig STRplex*.

As part of the validation, the systems came through endurance testing (sensitivity, presence of inhibitors, degraded DNA) and were successfully tested on a broad variety of samples including hair, teeth, claw, droppings, tiger wine, and various artefacts of traditional medicine.

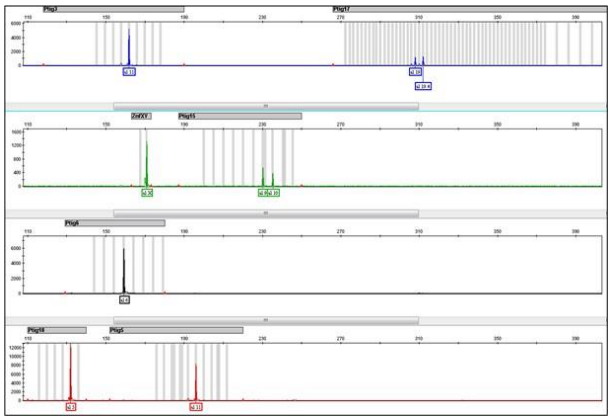

Figure 3: An example of DNA profile generated using *Ptig STRplex 1* – electrophoregram.

Allelic ladders required for subsequent fragment analysis of PCR products were analysed on a SeqStudio 3200 Genetic Analyzer. The protocols were validated on Eppendorf MasterCycler Nexus gradient (PCR DNA amplification) and SeqStudio 3200 Genetic Analyzer (fragmentation analysis of PCR products). Allelic ladders and corresponding bins were created for the individual STR loci for the GeneMapper5 Software.

As part of the output of the project, the **specific analytical kit for individual identification of *Panthera tigris* individuals/specimens called *Ptig STRPlex*** was developed. The kit is sensitive enough even for small amounts of input DNA (starting from 1 pg DNA) and contains sufficient reagents to determine 10 individual tiger DNA profiles.

Newly developed analytical kits for tiger individual identification can be obtained from the laboratory Forensic DNA Service on request (only for wildlife enforcement purposes, free of charge).

The DNA profile of the tiger individual can be used to establish a link between a sample of unknown origin and a reference sample or a database record. DNA profiles of tiger samples can be also used for parentage testing.

Ptig Qplex and *Ptig STRplex* are validated for commonly used instruments QuantStudio 5 and SeqStudio (Thermo Fisher Scientific, USA).
Ptig Qplex and *Ptig STRplex* are described in open-access *European Journal of Environmental Sciences* (Vanek D., Ehler E. and Vankova L. "Development of DNA quantitation and STR typing systems for *Panthera tigris* species determination and individual identification in forensic casework." *European Journal of Environmental Sciences* 11.2 (2021): 113-118.).
Legal protection of project results - Ptig Qplex and Ptig STRplex analytical sets are protected as a utility model (registered with the Industrial Property Office, CZ).

Processed materials

Experiments were performed to determine the **impact of the skin tanning processes on DNA degradation**. A strong correlation was found between the chemicals used to process the skin (especially formic acid) and DNA degradation and cross-linking. Procedures that use long-term maceration using formic acid are critical for DNA quality and quantity. Experiments investigating the effect of tanning processes and the various chemicals used for tanning will be continued as part of the follow-up project.
Experiments on **DNA degradation during long-term boiling** have shown that DNA (both mitochondrial and nuclear) is typeable even after long-term boiling. The study monitored the quality and quantity of DNA in bone samples (*Bos taurus*) that were boiled for 48 hours and sampled every hour. After 48 hours of boiling, the DNA is still present in a typeable form. However, in longer-processed 7-day solid tiger bone glue (cubic form), DNA is no longer detectable in

a species-specific form, but certain proteins are still present. In summary - liquid macerates, broths and soft products can usually be analysed. Methods for the identification of tiger DNA/proteins in heavily heat-damaged materials (long-term broths/glue cubes) must therefore be further investigated.
Results of the experiment have been published:
E. Tikalova, J. Votrubova, J. Kufnerova, D. Formanova, P. Rihova, L. Vankova, D. Vanek „Busting the myths: DNA typeability after 48 hours of boil“, *Forensic Science International: Genetics Supplement Series*, Volume 7, Issue 1, 2019, Pages 79-82, ISSN 1875-1768, https://doi.org/10.1016/j.fsigss.2019.09.031.

Analysis report

Genetic analysis protocol with the following information was developed:

- Laboratory number,
- Case number + authority,
- Description of the sample,
- Requirements: species identification/individual identification/parentage testing,
- Photo documentation,
- Processing time,
- Laboratory records (the range is in accordance with ISO 17025 and ILAC G-19):
  - Sampling from the original sample,
  - DNA isolation method, isolation date, notes,
  - DNA quantification method (standard/species specific),
  - Result of DNA quantification,
  - Presence of inhibitors, method of purification of isolated DNA to remove inhibitors,
  - Date of PCR, amplification conditions, post-amplification purification method,
  - Date of electrophoresis,
  - Method of analysis (sequencing/fragmentation analysis), parameters of analysis,
  - Sample designation code for insertion into the database,
  - Statistical evaluation,
  - Storage of the isolate and the original sample,
  - Signature of the expert.

3. Reference database

The database software *nhDNAdb* (non-human DNA database) for the **comparison of tiger DNA profiles** contains around 400 *Panthera tigris* DNA profiles generated from reference and casework samples. The database is used to store the results of tiger DNA analyses (DNA profiles), to compare the DNA profiles obtained from the forensic samples with records in the database (reference samples and traces) or to search for related DNA profiles for subsequent verification of the relationship (parent-offspring or siblings). Additional descriptive information can be attached to each inserted DNA profile (country of origin, type of sample, sample storage place, DNA sample storage place, description etc.). The database is currently designed for operations on the local user's computer in MS Windows with internet access and the IIS web server what is part of MS Windows. All functionalities are verified for browsers Chrome, Firefox, Safari, Opera, and Edge. The number of records is limited only by the physical limit of the MS Access database (about 1GB) which corresponds to about 500,000 DNA profiles. The structure of the register respects the ISO 17025:2018 standards and the ILAC-G19:2014 Directive. The data structures are based on the structures of the analogous Combined DNA Index System (CODIS) which supports a database of human DNA profiles and is operated by the US government (under FBI).
The database is owned by the Czech Republic (project sponsor) and at the moment, is accessible only to authorized persons from the Czech Republic (researchers dealing with the project, relevant government enforcement agencies). The user interface allows logging, recording activities of authorized users, data backup, comparing database profiles against each other, exporting individual records, exporting the number of records, etc. Comparison of data (tiger

profiles) from the database has already contributed to obtaining evidence in several investigations of illegal trade in tiger products in the Czech Republic. Comparison of data for samples from other countries can be made upon request from the relevant CITES authorities. Further development of the database is expected, including a possible extension of access. In the future, with wider use of the database (when used by multiple countries as a reference database), it would be appropriate **to transfer the database to a cloud environment**, which would require some financial budget.

Sample Code	DB note	32A	DB44	7B	7C	BIRh18	DB66	DB52	BR6	DB1	BIRh1C	12F	BIRh37D	32F	DB23	SR63	IR10	IR12	IR22
3442	note1	200 / 200	212 / 214	227 / 227	158 / 166	242 / 246	198 / 208	219 / 219	134 / 156	158 / 158	126 / 128	231 / 235	203 / 203	173 / 173	247 / 247	194 / 194	136 / 136	182 / 182	217 / 221
3437		200 / 200	212 / 212	227 / 227	158 / 166	242 / 242	208 / 208	219 / 221	134 / 134	152 / 158	128 / 130	229 / 235	201 / 203	173 / 173	247 / 247	194 / 200	122 / 136	182 / 188	217 / 217
3433		200 / 200	212 / 214	227 / 227	158 / 158	242 / 242	204 / 208	219 / 221	134 / 134	152 / 158	128 / 128	231 / 231	201 / 203	173 / 173	247 / 247	194 / 194	136 / 136	182 / 188	217 / 221
3432		200 / 200	212 / 212	-/-	158 / 168	242 / 242	198 / 208	215 / 219	134 / 134	152 / 152	126 / 128	231 / 231	201 / 203	173 / 173	247 / 249	194 / 200	136 / 136	182 / 182	217 / 219

The DNA profiles of tigers held in captivity in the Czech Republic were also entered into the CITES Register maintained by the Ministry of Environment of the Czech Republic (CZ MA CITES), which serves to register live CITES specimens in the CZ and to monitor trade with them. Information about tiger genetic profiles can be used by enforcement authorities to individually identify tiger individuals, verify parentage, or obtain evidence of illegal handling of tigers or tiger products.

4. Population studies

The match between two DNA profiles ("track-track" or "track-reference" type) must be statistically evaluated to determine the weight of this match. Without statistical evaluation, results of the analysis should not be used for forensic purposes. For statistical evaluation, it is necessary to have data from a population study performed on a particular species. Population data obtained should be tested for compliance with Hardy-Weinberg equilibrium. The number of unrelated individuals for the population study should be higher than 100, which can be difficult to obtain in endangered species. Absence of population studies is a common mistake in the usage of genetics for forensic evidence. If population data is not available or did not pass the testing, it is not possible to statistically evaluate any match between samples and this fact must be explicitly stated in the assessment.

Alela	Ptig10	Ptig11	Ptig15	Ptig16	Ptig17	Ptig18	Ptig3	Ptig5	Ptig6	Ptig8	Ptig9
no_value	0.0056179	0	0.0166666	0	0.0505617	0.0055555	0	0.0055555	0	0.0056179	0.0055555
2	0	0	0	0	0	0.2833333	0	0	0	0	0
5	0	0	0	0	0	0.7111111	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0.8055555	0.0056179	0
6,1	0	0	0	0	0	0	0	0	0	0.0056179	0
7	0	0	0	0	0	0	0	0	0.1777777	0.2528089	0
8	0	0	0	0	0	0	0	0	0.0111111	0	0
8,1	0	0	0	0	0	0	0	0.05	0	0	0
9	0	0	0.6555555	0	0	0	0.1444444	0.2555555	0.0055555	0.0056179	0.4
9,1	0	0	0.0111111	0	0	0	0	0.0055555	0	0	0
10	0	0	0.3166666	0.3611111	0	0	0.0166666	0.3	0	0	0.1
11	0	0	0	0.2333333	0	0	0.35	0.3777777	0	0	0.0166666
11,3	0	0	0	0	0	0	0	0	0	0.0674157	0
12	0	0	0	0.1833333	0	0	0.4833333	0.0055555	0	0	0.0444444
12,3	0	0	0	0	0	0	0	0	0	0.0224719	0
13	0	0	0	0.2166666	0	0	0.0055555	0	0	0.4606741	0.2777777
13,3	0	0	0	0	0	0	0	0	0	0.1516853	0
14	0	0.7777777	0	0.0055555	0	0	0	0	0	0.0168539	0.1444444
15	0	0.2222222	0	0	0.0056179	0	0	0	0	0.0056179	0.0111111
17,2	0	0	0	0	0.0056179	0	0	0	0	0	0
19	0	0	0	0	0.0337078	0	0	0	0	0	0
19,4	0	0	0	0	0.2191011	0	0	0	0	0	0
21,2	0	0	0	0	0.0393258	0	0	0	0	0	0
22,2	0	0	0	0	0.0393258	0	0	0	0	0	0
22,4	0	0	0	0	0.0842696	0	0	0	0	0	0
23	0	0	0	0	0.0561797	0	0	0	0	0	0
23,2	0	0	0	0	0.0337078	0	0	0	0	0	0
23,4	0	0	0	0	0.0056179	0	0	0	0	0	0
24	0.1516853	0	0	0	0.0898876	0	0	0	0	0	0
24,4	0	0	0	0	0.0224719	0	0	0	0	0	0
25	0.1011235	0	0	0	0.0393258	0	0	0	0	0	0
25,2	0	0	0	0	0.1123595	0	0	0	0	0	0
26	0.4775280	0	0	0	0	0	0	0	0	0	0
26,2	0	0	0	0	0.0730337	0	0	0	0	0	0
26,4	0	0	0	0	0.0898876	0	0	0	0	0	0
28	0.1685393	0	0	0	0	0	0	0	0	0	0
29	0.0955056	0	0	0	0	0	0	0	0	0	0

DNA profiles of **90 unrelated tiger individuals** were used to calculate the allele frequencies of STR loci included in *Ptig STRplex.* The resulting Combined discrimination capacity of 0.99999998 and Combined match probability: 1.083504×10^{-8} allows an efficient evaluation of database or sample-to-sample matches.

Tigers bred in captivity in Europe are highly related, so the number of unrelated individuals for the population study in TigrisID project was lower (90 unrelated individuals) than the number of samples actually obtained (424). For further research, analyses of tiger samples from other parts of the world would be needed.

If you can provide any tiger samples, please contact the project team members at the emails listed in the header of this report!

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Results of the project were presented at the Congress of the International Society for Forensic Genetics (Prague, September 2019), the ENFSI APST meeting (March 2019), Society for Wildlife Forensic Science Conference (Denver, June 2019), 2nd International Conference on Transnational Forensics (Caparica, November 2019).

Future Plans

- Research in identification of big cat species is currently ongoing (within the follow-up project ForWild focused on forensic wildlife research led by the Charles University in Prague and the Czech Academy of Sciences). In addition to tigers, **genetic research is now being extended to other feline species** (lion, leopard, etc.), **morphological research focused on the identification of big cat skulls, bones, claws, teeth** is also attached so the extended project will be called **PantheraID**.

- An additional analytical kit for *Panthera tigris* will be developed. It should enable to trace maternal or sibling lines or to distinguish a closely related individual from inbred lineages.

- Development of a **field test** (instrument + analytical kit) is also planned. This should enable simple and fast species identification (targeted at big cat species) for customs officers and wildlife officials inspecting suspicious artefacts (including medicinal products), mainly at airports and customs offices.

- Research into the possibility of analysing and determining species in **solid cubes** of bone glue (destroyed DNA) will continue.

- A higher number of DNA profiles in the database gives a higher chance of linking an unknown biological sample to a specific individual (reference sample), allows monitoring of other tiger lineages and populations, and increases the validity of the population study. Therefore, **FREE testing of *Panthera tigris* samples** is offered by the Forensic DNA Service Laboratory in Prague, Czech Republic. It can include testing of a product of unknown origin in any investigated case as well as an individual identification and determination of the DNA profile of any tiger individual. For easier exchange of samples and collaboration, we recommend possible partner institutions/laboratories to **register as a scientific institution and forensic research institution with the CITES Secretariat** (Charles University is currently in the process of registration).

- To promote the fight against poaching and illegal trade in tigers and tiger products, the Forensic DNA Service Laboratory can **provide *Ptig Qplex* and *Ptig STRplex* analytical kits FREE OF CHARGE** to DNA laboratories in other CITES member countries (tiger range countries and countries with tiger breeding facilities also). In exchange, we would expect the resulting DNA profiles to be sent to expand the existing database.

- In case of interest, we can also offer **training of laboratory staff in other countries** in the usage of developed analytical kits for species and individual identification of tigers, either in the form of training in the country (visits of Czech experts) or in the form of an internship in the laboratory in CZ.

- Any possible further **collaboration to combat the illegal trade in tigers, tiger parts and products**, including, for example, possible joint research projects, is also very welcome.