ForGen
Forensische Genetik und Rechtsmedizin
am Institut für Hämatopathologie GmbH

ForGen – Forensische Genetik und Rechtsmedizin am Institut für Hämatopathologie GmbH | Fangdieckstr. 75a | 22547 Hamburg

DREAL
Auvergne-Rhone-Apleb
5 place Jules Ferry
69006 Lyon
Frankreich

ForGen - Forensische Genetik und Rechtsmedizin am Institut für Hämatopathologie GmbH
Priv.-Doz. Dr. Nicole von Wurmb-Schwark
Fangdieckstr. 75a, 22547 Hamburg
Tel: +49 (0) 40 524 72 36-600
Fax: +49 (0) 40 524 72 36-610
Mail: info@forensik-hh.de
URL: http://www.forensik-hh.de

Hamburg, den 28.12.2018

Forensic genetic analysis of biological samples with animal origin (SU0170-18)

Subject:	Species identification
Order from:	11.09.2018
Receipt of order form:	13.09.2018
Receipt of biological samples:	13.09.2018

According to a written order and our mutual agreement, a

forensic-genetic expert opinion

is to be prepared

1. Question / task

I. Please provide molecular genetic analysis and - if possible - species classification.

II. Is there any genetic material on the trace carriers that is suitable for investigation?

III. if yes, belong the samples do a dog, wolf or other representative of Canidae?

2. Background

In the past, ForGen has examined biological samples from France to determine whether they were traces of wolves or dogs or simply members of the Canidae family. In several cases, it was not possible to clearly identify them as dogs or wolves. The results partly provided indications of hybridization. Neither degree nor time of any hybridization was given. The results were heavily criticized and especially the existence of hybrids was negated.

In a study carried out by another laboratory in France, completely different samples were analyzed during their examination and hardly any evidence of hybridization was found using other genetic markers and evaluation methods.

Therefore, a comparative study (identical samples in two different laboratories) should identify and, if possible, explain any differences.

The investigations and statistical calculations and evaluations took place in the following period:

17.11.2018	**to**	20.12.2018

3.1 Sample material

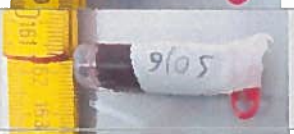
The samples to be tested were shipped in a normal package and were neither refrigerated while sending nor dried before shipment.

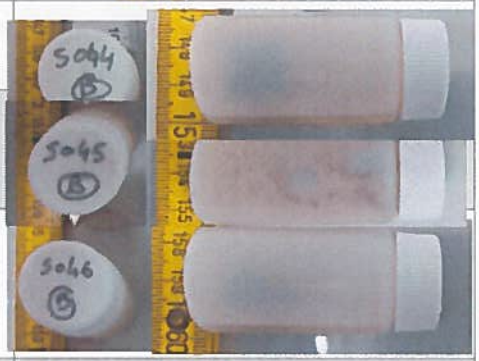
Picture 1: Samples to be investigated as they have arrived at the lab.

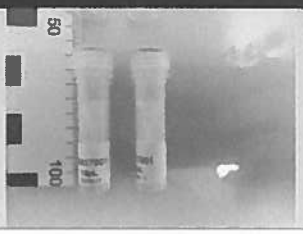
All samples were cooled immediately upon receipt and all those samples, DNA-extracts and PCR-products were also all stored in a refrigerator during processing and analyzing when possible.

Altogether 25 different samples were investigated:

Table 1: Samples analyzed in this report. Given are the German lab-number, ONCFS-affiliation, kind of sample, picture of corresponding sample and short description of abnormalities (remarks).

#	FG # (	ONCFS-#	Kind of sample	Remarks	Picture of samples
1	616-18	ZG-48-18-S002	Native blood	Liquid-coagulated, severely degraded	
2	617-18	ZG-48-18-S004	Native blood	Liquid-coagulated, severely degraded	
3	618-18	ZG-48-18-S006	Native blood	Marking smeared Liquid-coagulated, severely degraded	
4	619-18	ZG-48-18-S007	Native blood	Liquid-coagulated, severely degraded	
5	620-18	ZG-48-18-S008	Native blood	Liquid-coagulated, severely degraded	
6	621-18	ZG-48-18-S009	Native blood	Marking smeared Liquid-coagulated, severely degraded	
7	622-18	ZG-48-18-S010	Native blood	Liquid-coagulated, severely degraded	
8	623-18	ZG-48-18-S012	Native blood	Marking smeared, different to others Liquid-coagulated, severely degraded	
9	624-18	ZG-48-18-S016	Native blood	Liquid-coagulated, severely degraded	
10	625-18	ZG-48-18-S017	Native blood	Liquid-coagulated, severely degraded	
11	626-18	ZG-48-18-S019	Native blood	Marking smeared, unreadable Liquid-coagulated, severely degraded	
12	627-18	ZG-48-18-S025	Native blood	Liquid-coagulated, severely degraded	
13	628-18	ZG-48-18-S027	Native blood	Marking smeared Liquid-coagulated, severely degraded	
14	629-18	ZG-48-18-S028	Native blood	Liquid-coagulated, severely degraded	

#	FG # (	ONCFS-#	Kind of sample	Remarks	Picture of samples
15	630-18	ZG-48-18-S033	Native blood	Liquid-coagulated, severely degraded	
16	631-18	ZG-48-18-S044	tissue	Severely decomposed	
17	632-18	ZG-48-18-S045	tissue	Severely decomposed	
18	633-18	ZG-48-18-S046	Tissue	Skin with fur Severely decomposed	
19	634-18	ZG-48-18-S055	Tissue	Muscle tissue, decomposed	
20	635-18	ZG-48-18-S056	tissue	Muscle tissue, decomposed	
21	636-18	ZG-48-18-S057	tissue	Muscle tissue, decomposed	
22	637-18	P481701	DNA Extract	Low volume, unknown DNA concentration, lid not properly closed	
23	638-18	P481701	DNA Extract	Low volume, unknown DNA concentration, lid not properly closed	
24	639-18	U481701	DNA Extract	Low volume, unknown DNA concentration, lid not properly closed	

#	FG # (	ONCFS-#	Kind of sample	Remarks	Picture of samples
25	640-18	U481701	DNA Extract	Low volume, unknown DNA concentration, lid not properly closed	

3.2 Vergleichsmaterial (VM) bzw. –daten (VD)

None.

4. Results

4.1 Pretestings

None.

4.2 Genetic testing

Several methods/procedures were applied for investigation: handling of biological samples according to standard operation procedure as they are routinely applied in our laboratory for all forensic investigations, semi-automatic DNA extraction, PCR amplification of 16 STR-markers and of two gonosomal markers for sex determination (SRY and Amelogenin), fragment analysis via capillary electrophoresis on an ABI Prism Genetic Analyzer and STR-fragment determination using Genemapper software 4.1 followed by biostatistical analysis for origin determination (cluster/association analysis), DNA sequencing of different fragments of mitochondrial DNA (cytochrome for species identification and hypervariable region for origin and maternal heritage) and subsequent sequence analysis and comparison of obtained sequences to published data in an international database (NCBI Blast). See topic 6 for further information.

4.2.1. Mitochondrial DNA typing

First, a fragment of the mitochondrial Cytochrome region was amplified and sequenced to perform **species determination**. Then a part of the **mitochondrial control region** was amplified and sequenced to determine the maternal lineage and the mitochondrial haplotype:

Tab. 2: Results of mitochondrial DNA sequencing for species identification (mtDNA cytochrome) and determination of Haplotype if possible (mtDNA hypervariable region). Given are German lab number, French affiliation, results of species fragment blasting and of HV region blasting with corresponding haplotype.

#	FG #	ONCFS #	Species testing, mtDNA Cytochrome	Determination of haplotype, mtDNA HV region	mtDNA haplotyping
1	616-18	ZG-48-18-S002	Canis lupus Bulgaria Canis lupus Mongolia4 Canis lupus Mongolia3	**Canis lupus Mongolia3** **Canis lupus Mongolia 4** **Canis lupus familiaris Kimberly 2**	A(129) (Pang et.al, 2009)
2	617-18	ZG-48-18-S004	same	**Canis lupus familiaris GON_23** **Canis lupus familiaris GON_19** **Canis lupus familiaris BOK_13**	A(129) (Pang et.al., 2009)
3	618-18	ZG-48-18-S006	same	**Canis lupus Mongolia 3** **Canis lupus familiaris CF30** **Canis lupus familiaris H18**	A(129) (Pang et.al., 2009)
4	619-18	ZG-48-18-S007	same	***Canis lupus dingo DW*** ***Canis lupus dingo DY*** ***Canis lupus dingo DB***	D2 (Duleba et.al.,2014)
5	620-18	ZG-48-18-S008	same	**Canis lupus familiaris GON_23** **Canis lupus familiaris GON_19** **Canis lupus familiaris BOK_13**	A(129) (Pang et.al, 200
6	621-18	ZG-48-18-S009	same	**Canis lupus familiaris GON_23** **Canis lupus familiaris GON_19** **Canis lupus familiaris BOK_13**	A(129) (Pang et.al, 200
7	622-18	ZG-48-18-S010	same	**Canis lupus Mongolia 3** **Canis lupus familiaris CF30** **Canis lupus familiaris Xiasi8**	A(129) (Pang et.al, 200
8	623-18	ZG-48-18-S012	same	**Canis lupus Mongolia 3** **Canis lupus familiaris CF30** **Canis lupus familiaris Xiasi8**	A(129) (Pang et.al, 200
9	624-18	ZG-48-18-S016	same	**Canis lupus familiaris GON_23** **Canis lupus familiaris GON_19** **Canis lupus familiaris BOK_13**	A(129) (Pang et.al, 200
10	625-18	ZG-48-18-S017	same	No results	---
11	626-18	ZG-48-18-S019	same	**Canis lupus familiaris GON_23** **Canis lupus familiaris GON_19** **Canis lupus familiaris BOK_13**	A(129) (Pang et.al, 200
12	627-18	ZG-48-18-S025	same	**Canis lupus Mongolia 3** **Canis lupus familiaris CF30** **Canis lupus familiaris Xiasi8**	A(129) (Pang et.al, 200
13	628-18	ZG-48-18-S027	same	**Canis lupus Mongolia 3** **Canis lupus familiaris CF30** **Canis lupus familiaris Xiasi8**	A(129) (Pang et.al, 200
14	629-18	ZG-48-18-S028	same	**Canis lupus Mongolia 3** **Canis lupus familiaris CF30** **Canis lupus familiaris Xiasi8**	A(129) (Pang et.al, 200
15	630-18	ZG-48-18-S033	same	**Canis lupus Mongolia 3** **Canis lupus familiaris CF30** **Canis lupus familiaris Xiasi8**	A(129) (Pang et.al, 200
16	631-18	ZG-48-18-S044	No results	No results	---
17	632-18	ZG-48-18-S045	No results	No results	---
18	633-18	ZG-48-18-S046	No results	No results	---
19	634-18	ZG-48-18-S055	Canis lupus Bulgaria Canis lupus Mongolia4 Canis lupus Mongolia3	No results	---
20	635-18	ZG-48-18-S056	same	**Canis lupus Familiaris A50** **Canis lupus familiaris GREY-RJB/15/02524** **Canis lupus familiaris DD14** **Fragment slightly shorter due to DNA degradation**	A(129) (Pang et.al, 20
21	636-18	ZG-48-18-S057	Same	**Canis lupus Familiaris A51** **Canis lupus familiaris GREY-RJB/15/02525** **Canis lupus familiaris DD15** **Fragment slightly shorter due to DNA degradation**	A(129) (Pang et.al, 20

#	FG #	ONCFS #	Species testing, mtDNA Cytochrome	Determination of haplotype, mtDNA HV region	mtDNA haplotyping
22	637-18	P481701	Canis lupus familiaris HXHdog Canis lupus familiaris CTCdog Canis lupus Mongolia5	Canis lupus CLU73 Canis lupus Sweden3 Canis lupus familiaris 20	(F1 (Matsumura et.al., 2009))
23	638-18	P481701	same	Canis lupus CLU74 Canis lupus CLU67 Canis lupus Sweden3	(F1 (Matsumura et.al., 2009))
24	639-18	U481701	same	Canis lupus CLU73 Canis lupus Sweden3 Canis lupus familiaris 20	(F1 (Matsumura et.al., 2009))
25	640-18	U481701	same	Canis lupus CLU74 Canis lupus CLU67 Canis lupus Sweden3	(F1 (Matsumura et.al., 2009))

4.2.2 Genetic typing of sex markers and polymorphic STR-markers.

After mitochondrial DNA analysis, typing of nuclear DNA followed using two different, independent multiplex-PCRs:

Tab. 3: Results of sex determination (SRY locus and Amelogenin loci) and STR typing.

#	Sex SRY amg	PEZ1	FHC 2054	FHC 2010	PEZ5	PEZ 20	PEZ 12	PEZ3	PEZ6	PEZ8	FHC 2079	FH 2087	PHC 2137	PEZ 15	WTF	FHC 2508	FHC 2361
1	Y/xy male	96/ 104	155	227/ 231	100/ 114	173/ 177	305	153/ 159	182/ 190	226/ 229	296	109/ 121	153/ 161	203/ 227	163	104/ 112	144
2	Y/xy male	112/ 124	147/ 151	231/ 235	100/ 112	177/ 181	270/ 296	144/ 156	182/ 190	233/ 242	292/ 296	121	161/ 179	235	175	96/ 112	138/ 142
3	-/X fem	112	151/ 155	231/ 235	100	177	305	153/ 159	178/ 190	229/ 233	296	117/ 121	153/ 161	199/ 203	163	104/ 112	138/ 144
4	Y/xy male	118	143/ 171	227/ 235	108	173	254/ 292	153	190	226/ 247	266/ 272	113/ 117	161	203/ 235	163	104/ 112	138/ 164
5	-/X fem	104/ 116	179	235	112	173/ 177	292	144/ 153	178/ 190	234/ 242	296	117/ 121	161/ 179	203/ 235	163	112	138
6	Y/xy male	116	151	235	108/	173/ 177	267/ 274	150/ 156	190	234	296	117/ 121	153/ 161	203/ 235	163/ 175	96/ 112	138
7	Y/xy male	96/ 108	147/ 151	227/ 235	96	—	291/ 305	144/ 156	186	226/ 234	296	109/ 121	179	203	163/ 175	96/ 112	138
8	-/X fem	112/ 116	155/ 175	231/ 235	100/ 112	177	267/ 274	144/ 153	190/ 198	226/ 234	280/ 296	117	153/ 161	235	163	112	138/ 144
9	-/X fem	116/ 120	151	227/ 235	92/ 100	173/ 177	274/ 305	153/ 156	178/ 198	226/ 234	296	109/ 117	153	203	163	112/ 116	138/ 144
10	Y/xy male	112	151	235	92/ 116	—	259	144/ 153	174/ 182	238	266/ 296	109/ (121)	153/ 179	203	163/ 175	112/ 116	144
11	-/X fem	108/ 116	147/ 151	227/ 235	108/ 112	177	274/ 291	156	178/ 190	234/ 242	296	117/ 121	153/ 179	203/ 235	163	96/ 112	142/ 144
12	-/xy male	112/ (124)	147/ 151	231	100/ 112	177	274/ 305	150/ 159	178/ 190	226/ 230	280/ 292	109/ 121	161	199/ 203	163	104/ 112	142/ 144
13	Y/xy male	96	147/ 151	231/ 235	92/ 108	173/ 177	267	144/ 156	190	226/ 235	296	109/ 117	153/ 161	203/ 235	163	112	138/ 140
14	Y/xy male	120	151	231/ 235	108/ 118	177	274/ 291	150/ 153	178/ 190	230/ 234	296	109/ 121	153/ 179	203/ 235	163	112	142/ 144
15	(Y)/X fem	116	151	219/ 235	108/ 112	(173) /177	274/ 291	153/ 156	170/ 190	226/ 234	296	117/ 121	161/ 179	203/ 235	163/ 175	96/ 112	138/ 142
16	Y/xy male	120	166	227/ 239	92/ 100	—	—	—	170	242	277	142	—	—	—	111	—
17	Y/xy male	96		227/ 239	102/ 110	185	299	—	190	—	—	—	—	—	—	—	—
18	Y/xy male	100/1 (116)	—	227/ 239	---	181	301	---	190	252	280/ 284	---	---	---	---	---	
19	Y/xy male	112	176	227/ 239	96/ 100	177	267	—	174/ 190	238	280/ 296	—	—	—	—	—	—
20	Y/xy male	96/ 116	143/ 159	235	100	---	260	156	174	259/ 267	296	109/ 121	153/ 179	203/ 235	163/ 175	112	138/ 144
21	Y/xy male	100	—	—	—	—	260/ 311	156	---	251	266	117/ 121	179	---	163	---	—
22	Y/xy male	112	151	231/ 235	108	169/ 178	265/ 271	156	190	229/ 242	266/ 274	117/ 121	157/ 165	---	159	97/ 104	134
23	/y	135/ 139	151	227/ 239	100	209	275/ 305	125/ 153	—	236/ 252	281/ 282	117	—	—	—	---	---
24	y/xy male	112/ 120	—	231/ 285	100	178	—	147/ 167	---	---	266/ 273	117/ 121	157/ 165	199/ 203	159	97/ 104	134
25	/y	107	147	219/ 231	110	—	—	—	—	—	—	—	—	—	—	—	—

Sex determination: SRY x/; Amelogenin /x or xy; ---: no results; (xx) signals not clear, male: possible male sex, fem: possible female sex, given are the non-normalized base pairs of specific fragments after capillary electrophoresis, for association or clusteranalysis data are normalized using the respective positive control values as references.

4.3 Interpretation of STR-results and summary of all genetic results

Table 4: Summary on results. Given are the groups that show the highest genetic similarities to the investigated sample and if possible also the values in percent (xx). Dogs are grouped in FCI acknowledged groups e.g. Molossers or herding dogs (group one, Hütehunde), wolf groups in the database used here are: W92 Russian wolves, zoo wolves (animals from zoos in Austria and Germany), Jackals (from the Ukraine), W96 Latvian wolves, WFR Samples from typical French wolves.

#	FG #	ONCFS #	Clusteranalysis	Summary of results
1	616-18	ZG-48-18-S002	01 – Hütehunde (65) , WFR – wolves (45), W92-Russian wolves (40) , Zoo wolves (35), Jackals (25)	**Wolf** according to species identification, maternal line **wolf** or **Mongolian wolf**, probably **male Wolf** or hybrid, **rather typical wolf** in France, dog excluded
2	617-18	ZG-48-18-S004	WFR – Wölfe (60), 02 – Molosser (60), W96 - W96 Latvian wolves (40), Zoo wolves (35)	**Wolf** according to species identification, maternal line **wolf**, probably **male Wolf** or hybrid, rather wolf, dog excluded
3	618-18	ZG-48-18-S006	01 - Hütehunde(60) , WFR – Wolves (45), W92 – Russian wolves (35), Zoo wolves (35)	**Wolf** according to species identification, maternal line **wolf or Mongolian wolf**, probably **female Wolf** or hybrid, rather typical wolf in France, dog excluded
4	619-18	ZG-48-18-S007	Unknown, 01 shepherd, Jackal, WFR - wolves, W92 – Russian wolves	**Wolf** according to species identification, maternal line **DINGO**, STR analysis difficult since we do not have enough Dingos in our database for reliable comparison. Thus, main result of STR analysis is unknown followed by dogs, Jackals and wolves, probably **male**
5	620-18	ZG-48-18-S008	01 – (60), WFR – wolves (45) W92 – Russian wolves (35), Timberwolf Jackal (30)	**Wolf** according to species identification, maternal line **wolf or Mongolian wolf**, probably **male Wolf** or hybrid, rather typical wolf in France, dog excluded
6	621-18	ZG-48-18-S009	01 – shepherd (55), WFR - wolves(45), W92 – Russian wolves (35), Jackal (25)	same
7	622-18	ZG-48-18-S010	WFR – Wölfe, W92 – Russian wolves 08 - Apportierhunde	**Wolf** according to species identification, maternal line **wolf or Mongolian wolf**, probably **male Wolf** or hybrid, dog excluded, no significant high genetic similarity to any group which usually points to mixtures. Thus, the most likely explanation here would be a mix of members of the canidae
8	623-18	ZG-48-18-S012	WFR – wolves (55), W92 – Russian wolves (50), 01 – shepherd, Zoo wolves (35), Jackal (35)	**Wolf** according to species identification, maternal line **wolf**, wolf or hybrid, rather typical **wolf** in France, or **Mongolian wolf**, probably female, dog excluded
9	624-18	ZG-48-18-S016	W92 – Russian wolves (55), WFR – wolves (45), 01 - shepherd , Zoo wolves (40), Jackal (35)	**Wolf** according to species identification, maternal line **wolf or Mongolian wolf**, probably **female Wolf** or hybrid, dog excluded
10	625-18	ZG-48-18-S017	WFR – wolves, W96 - W96 Latvian wolves, Zoo wolves , 02 – Molosser, Jackal	**Wolf** according to species identification, haplotyping not possible, STR results weak and often homozygous, pattern shows similarity to the typical French **wolf**, probably **male**, dog excluded
11	626-18	ZG-48-18-S019	WB – baltic wolves (40), WFR – wolves (40), 01 – shepherd (65) Zoo wolves (30)	**Wolf** according to species identification, maternal line **wolf or Mongolian wolf**, probably **female Wolf** or hybrid, dog excluded Low genetic similarities to the reference groups in the database which again can point to mixtures
12	627-18	ZG-48-18-S025	WFR – wolves (55), 01 – shepherd (55), W92 – Russian wolves (35), Zoo wolves (30)	**Wolf** according to species identification, maternal line **wolf**, wolf or hybrid, rather typical **wolf** in France, or **Mongolian wolf**, probably **male**, dog excluded
13	628-18	ZG-48-18-S027	01 – Shepherd (60), W92 – Russian wolves (45), WFR – wolves (40), Jackal (35)	**Wolf** according to species identification, maternal line **wolf**, wolf or hybrid, rather typical **wolf** in France or **Mongolian wolf**, probably **male**, dog excluded
14	629-18	ZG-48-18-S028	01 – shepherd (60), W92 – Russian wolves (50), WFR – wolves (35), Fox (40)	**Wolf** according to species identification, maternal line **wolf**, wolf or hybrid, rather typical **wolf** in France or **Mongolian wolf**, probably **male**, dog excluded, only individual with high similarity to fox, also high values for wolves
15	630-18	ZG-48-18-S033	01 – shepherd (60), WFR - wolves(55), W92 – Russian wolves (45), Jackal (40)	**Wolf** according to species identification, maternal line **wolf or Mongolian wolf**, probably **female Wolf** or hybrid, rather typical wolf in France, dog excluded
16	631-18	ZG-48-18-S044	Too weak/profile not sufficient	Canine, **wolf** specific allele, thus rather wolf or hybrid and no dog, probably **male**
17	632-18	ZG-48-18-S045	same	Canine, probably male
18	633-18	ZG-48-18-S046	same	Same

#	FG #	ONCFS #	Clusteranalysis	Summary of results
19	634-18	ZG-48-18-S055	02 – Molosser, WFR wolves , W Zoo wolves, W96 – Latvian wolves	**Wolf** according to species identification, Wolf or hybrid, , maternal line wolf or Mongolian wolf, probably male, dog rather excluded since wolf specific allele is found,
20	635-18	ZG-48-18-S056	WFR, 01 – Shepherd, W92 russian wolves	**Wolf** according to species identification, Wolf or hybrid, rather typical wolf in France, maternal line wolf or Mongolian wolf, probably **male**
21	636-18	ZG-48-18-S057	Too weak/profile not sufficient	**Wolf** according to species identification, Haplotype like other wolves, probably male, thus wolf or hybrid, dog excluded
22	637-18	P481701	WFR, W92, WZoo, Jackal, 02 - Molosser	**Wolf (Mongolia)** or **dog** according to species identification **Wolf** according to haplotyping, different haplotype than samples above, possible Swedish origin, Thus, two methods pointing to wolf AND dog, combination: wolf possible, hybrid also, dog less
23	638-18	P481701	See above	See above
24	639-18	U481701	See above	See above
25	640-18	U481701	Too weak/profile not sufficient	See above, only mitochondrial data

*: Angabe der spezifischen Merkmale in Basenpaareinheiten (Rohdaten) nach Auftrennung in einem ABI3130 Genetic Analyzer; ()=Merkmale, die eine geringe Amplitude aufweisen oder nicht reproduzierbar bestimmt werden konnten. Diese werden in die Assoziationsanalyse nicht einbezogen. Signale in Klammern: Signalstärke sehr gering. SRY: Geschlechtsmarker, Y-Chromosom, da die Signale häufig homozygot vorliegen und dies aufgrund der schlechten DNA-Qualität durch allelic drop out entstanden sein könnte, wird eine Fehlerbreite von 10% angegeben.

5. Summary of the results and assessment of the given questions

A total of 25 samples were investigated; 15 blood samples (liquid, thickened, bad smell), six tissue samples (partly strongly rotten, highly degraded, bad smell) and four DNA extracts with mch less than ten µl content. Thus, DNA extracts had to be diluted with DNA-free water to obtain enough material to perform genetic testings. Additionally, not all vessels were neatly and securely closed (suggesting degradation or drying processes of samples) not properly and demonstrably labelled, so that further work was unnecessarily complicated.

After photo documentation and description the following methods were conducted:

First, a **short fragment of mitochondrial DNA** (Cytochrom) was analyzed for **species identification**. See Table 2. All 15 blood samples could be assigned to the **wolf** (**Canis lupus Bulgaria** and **canis lupus mongolia**). The same applies to three tissue samples.

The DNA extracts matched a sequence identical to **Canis lupus familiaris HXHdog**; **Canis lupus familiaris CTCdog** and **Canis lupus Mongolia5**. Thus, this analysis only allows the determination of "Canidae" but leaves it open whether it is DNA from wolf or a dog.

In the next step, **a part of the control region of the mitochondrial DNA** (D-loop) was investigated. **Three different sequences** (haplotypes/haplogroups) were identified after investigation of all 25 samples:

Of the 15 blood samples, 14 were evaluable. 13 samples can be assigned to **haplogroup A** (**129**). This sequence was found in **wolves** and **dogs** (Canis lupus familiaris GON_23, Canis lupus familiaris GON_19, Canis lupus familiaris BOK_13 and Canis lupus Mongolia 3, Canis

lupus familiaris CF30, Canis lupus familiaris Xiasi8). The assignment to the **Mongolian wolf** is conspicuous in almost all cases investigated.

One blood sample (ZG-48-18-S007) shows a clearly different sequences and matches DNA sequences from a **Dingo** (Canis lupus dingo DW, Canis lupus dingo DY, Canis lupus dingo DB), the corresponding **haplotype** is **D2**. The STR typing results point here to an unknown Canidae, dingo and jackal. The assignment here is more difficult because there are not enough dingos in our database to perform a reliable comparison analysis.

The DNA mitochondrial sequences from the DNA extract, which were already different in the species analysis, show another, third mitochondrial **haplotype (F1**). This indicates a **wolf origin** (Canis lupus CLU73; Canis lupus Sweden3; Canis lupus familiaris 20/ Canis lupus CLU74; Canis lupus CLU67; Canis lupus Sweden3). See table above for detailed information.

Thus, it can be summarized that here with high probability many samples of **Mongolian wolves** and also of **possible hybrids** were examined. In most cases the mitochondrial DNA clearly points to the **Mongolian wolf.** We do not have these species in our database, so the STR analysis cannot give the result "Mongolian wolf" Instead, the next similar groups are displayed. Since the Mongolian wolf certainly differs from the European wolf (grey wolf) and it is also ascribed a strong similarity or kinship to the domestic dog, the results presented here explain themselves very well. They usually show high similarities to herding dogs and to the typical French wolf patterns. In particular also genuine mixtures of these animals or wolf hybrids would reach only low agreement values to the individual groups in the analysis. Thus, it can also be stated that no typical (pure) French wolf with its Italian origin has been examined here.

PD Dr. Nicole von Wurmb-Schwark
Fachabstammungsgutachterin DGAB
Spurensachverständige
Vorsitzende Bundesverband der Sachverständigen für Abstammungsgutachten e.V.

Dr. rer.nat Jan-Hendrik Modrow
Forensischer Genetiker

6. Methods and technical information

6.1 Methods and background

The following methods were used by the laboratory for trace analysis. All methods and procedures are verified and validated, embedded in an ongoing quality management system and currently accredited (unless otherwise described):

(7) DNA extraction (SAA_010):

DNA isolation is performed by digestion of the nucleated cells in several incubation and washing steps and the addition of magnetic beads. These bind the DNA molecules, which can then be washed and eluted again in various steps. The processing is carried out with an semi-automated extraction device from ThermoFisher.

(8) General information on DNA analysis, presentation, evaluation:

The DNA or DNA (deoxyribonucleic acid) is the carrier of the genetic material, which is arranged spirally in the cell nuclei on long molecular threads. It consists of individual building blocks containing 4 bases (adenine, thymine, cytosine, guanine). The arrangement corresponds to the genetic code.

At certain locations in the genome there are so-called short tandem repeats (STRs), areas of DNA that are characterized by specific sequences of 2 to 4 base pairs in length, which occur in repeats of 10 to 40. This number of repetitions is individually different and is codominantly inherited as a characteristic for each gene location. In mixer heredity, two different fragments (= alleles) are detectable. In pure inheritance, two identical fragments (alleles) are present. These short tandem repeats are all located in the so-called non-coding regions of the DNA, so that no gene location can be used to draw conclusions about possible diseases or malformations. 16 to 23 of these STR characteristics are analysed (SAA_14 and 15). One trait is always inherited from the mother, one from the father, so that pedigree reports can be carried out by means of the detection of STRs and with knowledge of the frequency of the individual genetic traits (SAA_019). In addition, an identity check is also possible. Since the different traits have different probabilities (i.e. they occur at different frequencies in the population), it can be calculated how likely it is that, for example, a certain biological trace originates from a certain person (SAA_020), if all their traits match those of the trace. In the case of so-called mixed traces, which were caused by more than one person, biostatistical statements on the origin or affiliation of a mixed trace can also be made using further calculation methods according to Schneider et al, 2006 (SAA_21).

(10) Fragment analysis (SAA_018):

By using specific, fluorescence-labelled primers, these relatively short DNA fragments can be amplified in a polymerase chain reaction (PCR) and determined in an automatic fragment analysis using capillary electrophoresis and laser detection in e.g. an AbiPrism3130 (Applied Biosystems).

(11) Dog-specific analyses (SAA_023):

In two different commercially available multiplex kits, STR traits specific for the canidae family can be amplified. In this report, Thermo Fisher's Stockmarks Canine for Dogs Kit is used to detect 10 of these traits. These traits also occur at varying frequencies, allowing simple identity and lineage analysis to be performed. The required frequency data for this are taken from a scientific doctoral thesis (Modrow, 2014, Kiel) and are continuously increased. Similar to humans, dogs also have specific frequency distributions which are specific for the different breeds. Therefore, an association analysis can be carried out with the obtained data in order to assign the dog to a specific breed. For this purpose, data for the corresponding breed must be available in the database. Breeds, which were not examined here, cannot be determined and/or assigned by this analysis. In addition, a PCR-supported sex determination is carried out. Furthermore, STR characteristics and an additional sex determination (amelogenin detection)

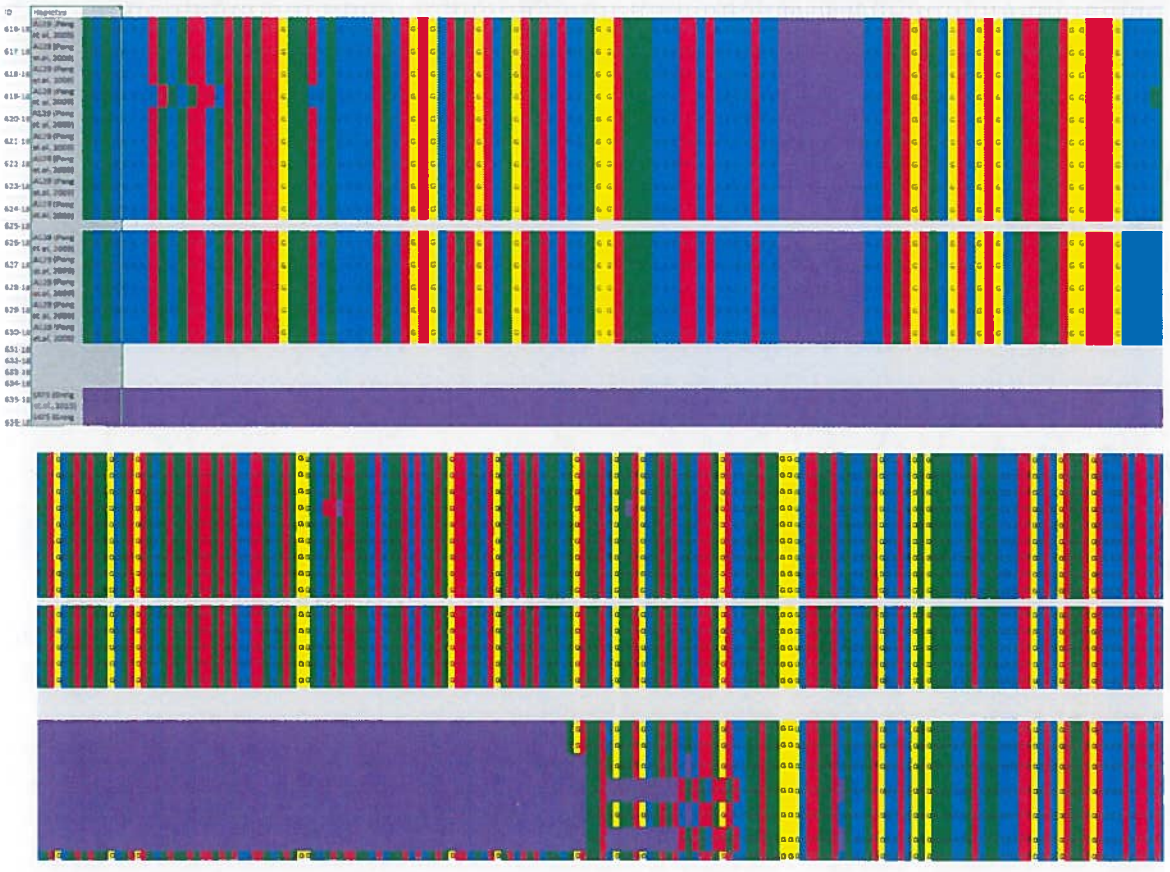
are investigated in an independent analysis based on the specifications of the ISFG canine working group (International Society of Forensic Genetics).
(15) Notes on forensic genetic analysis of samples from bitten or killed animals:
Our overall assessment of genetic identity with the wolf is based on the Washington Convention on International Trade in Endangered Species of Wild Fauna and Flora, Article II, and Mendelian inheritance rules as follows:
>75% it concerns with large probability a "pure-bred" wolf.
<75 % and >25 %: it is very likely a wolf-dog hybrid of the F1, F2, F3 or F4 generation or their backcrosses (B1-B4) or one of the dog breeds with high wolf similarity (Sallus Wolfhound and Wolfs-/Großspitz) or a hybrid of the same at the lower values.
<25 %: it is very likely not a direct wolf offspring, but a dog of the additionally indicated breeds.

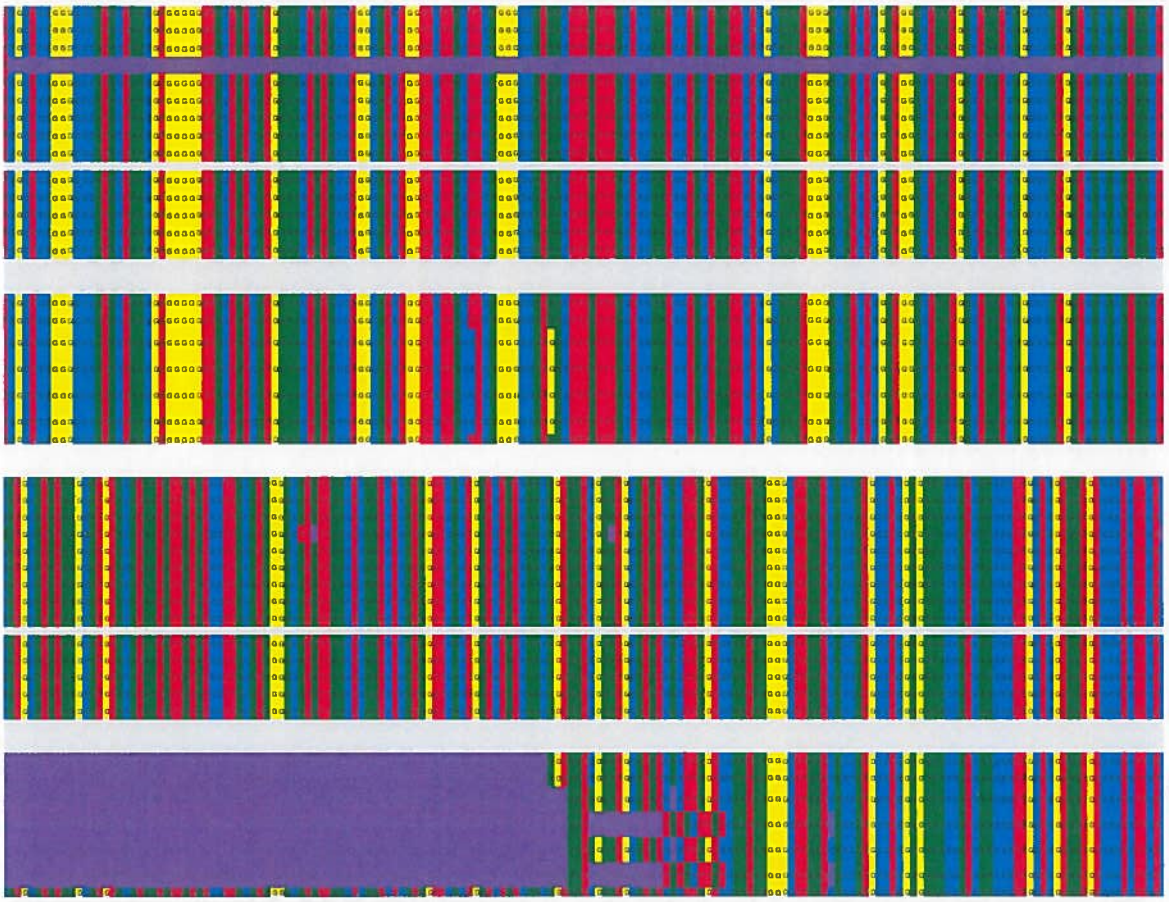
The calculation starts with the detection of traits in 6 gene varieties. If characteristics are detected in 6 to 7 gene varieties, a correction factor of 15 % is taken into account, which corrects the actual result; if characteristics are detected in 8 to 9 gene varieties, it is 10 %. This serves to avoid false-positive or negative species classifications. The mean value is given.
- The interpretation regarding a possible affiliation to the wolf refers to the examination of more than 1900 dogs from more than 150 breeds, in which in no case more than 35% genetic similarity to the wolf could be determined. The specific characteristic patterns of the dogs are compared with those of the wolves in an association study. The characteristic patterns originate from own investigations and literature references (n=2100, Broad Institute. 2014 Broad Institute, broadinstitute.com: http://www.broadinstitute.org/scientific-community/data, Ganco, L., et al. Genetic diversity analysis of 10 STR's loci used for forensic identification in canine hair samples. Forensic Science International: Genetics Supplement Series 2. 2009, p. 288-289.)
In addition, the analysis includes comparison with characteristics typical of the fox. For this purpose, too, own data have been compiled and in addition, the data from the literature have been used (n=68, A Multiplex PCR assay to differentiate between dog and red fox: Forensic Sci Int Genet 2011 Nov 29;5(5):411-4). Epub 2010 Dec 29,M Weissenberger, W Reichert, R Mattern/A marker set for construction of a genetic map of the silver fox (Vulpes vulpes):J Hered 2004 May-Jun;95(3):185-94,A V Kukekova, L N Trut, I N Oskina, A V Kharlamova, S G Shikhevich, E F Kirkness, G D Aguirre, G M Acland /Variation of short tandem repeats within and between species belonging to the Canidae family: Mamm Genome 1995 Jan;6(1):11-8 M Fredholm, A K Winterø).
All samples examined by us are fed into a database developed and maintained by ForGen. All crack samples are listed as group "cracks"; all wolf samples as group "wolves". The latter is further divided into "Baltic" and "Russian" and "Latvian" populations. Within the framework of an identity check and association analysis, new data are compared with the characteristic patterns existing in the database and similarity values are determined. This allows an assignment to the groups e.g. wolf (with subgroups), dog (with subgroups), jackals or fox or an assignment to a single sample ("match") with a complete match. In the latter case, it would also be possible to statistically assess the sample membership by determining the genotype frequency. If a partial sample matches an animal, this can also be calculated biostatistically. Since, however, the degree of kinship cannot be determined, especially among the wolf groups, such analyses can only be regarded as approximate values. We also do not determine the generations (F1, F2) since we do not know (in case of possible hybridization) the genetic composition of the elders. E.g. a mixture of e wolf and a wolfdog would possess more wolf similarities than a wolf-Labrador mix.

The genetic results are evaluated biostatistically. A Bayesian model is used for cluster analysis. The algorithms used are the same as those used in well-known, commercially available and frequently used software programs (e.g. STRUCTURE, ADMIXTURE; PLINK or R).
(16) Analysis of mitochondrial DNA (mtDNA, not accredited):
In dogs, mitochondrial DNA (mtDNA) is used as a supplement to association analysis (nDNA) if, for example, not sufficient
(intact) nuclear DNA is present for the production of a genetic autosomal trait pattern. I.e. when DNA quality and quantity are not sufficient for an analysis of the nuclear DNA. Due to the maternal inheritance of mitochondrial DNA (mtDNA), this method can only be used to a limited extent and is therefore used by us in addition to the analysis of nuclear DNA. In the analysis of mtDNA, a 319 bp fragment and, if possible, a 740 bp fragment from the hypervariable region of the mtDNA genome are sequenced (based on Gundry, et al. 2007, Schneider, Seo and Rittner 1999). In the last step, the resulting sequence pattern is analyzed using alignment algorithms and compared with the NCBI database. This method is specific for the family of canidae due to the choice of primers and enables the generation of an mtDNA haplotype and the maternal assignment e.g. to dog or wolf. The haplotype assignment is based on the work of Thai et al, 2016.
In addition, a pair of primers selected from the publication Lopez-Oceja et al, FSI Genetics 23 (2016) 199-205, which leads to a 148 bp fragment of cytochrome B of mitochondrial DNA, can be used to determine species. This is possible if the trace to be examined contains only DNA of one species.
SAA: Status

6.2 Mitochondrial data of Sequencing of the hypervariable region

Shown is the allignment of mitochondrial data from the different samples investigated here.





6.3 Whereabouts

The trace carriers/solutions used for the present study shall be retained as follows in accordance with procedure FG_VA_008 Sample custody:

Material	Aufbewahrung Entsorgung
Originäre Spuren (z.B. Sektionsasservate)	1 Jahr nach erfolgter Analyse und Gutachtenausgang
Extrahierte DNA aus obigen Spuren	s.o.
Mundschleimhautabstriche/Blut als VM	Sofortige Vernichtung nach Gutachtenausgang
Extrahierte DNA aus VM	s.o.
Spurenträger (Gegenstände) als VM in Identifizierungsfällen	4 Wochen nach Gutachtenausgang
Extrahierte DNA aus obigen Fällen	5 Jahre
Weitere Spurenträger/Abstriche diverse ohne spezielle Vereinbarung	2 Jahre
Extrahierte DNA aus obigen Spuren	5 Jahre

These periods shall not apply in the event of objections on the part of the client, if certain agreements have been made or in the event of analyses within the framework of a homicide or other capital offences. These samples will be preserved on a long-term basis.