Forensic Science International: Genetics 4 (2009) 49–54

Contents lists available at ScienceDirect

Forensic Science International: Genetics

journal homepage: www.elsevier.com/locate/fsig

# Forensic analysis of dog (*Canis lupus familiaris*) mitochondrial DNA sequences: An inter-laboratory study of the GEP-ISFG working group

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ARTICLE INFO

*Article history:*
Received 16 February 2009
Received in revised form 20 April 2009
Accepted 20 April 2009

*Keywords:*
Non-human forensic genetics
*Canis lupus familiaris*
mtDNA
Collaborative exercise
GEP-ISFG

ABSTRACT

A voluntary collaborative exercise aiming at the mitochondrial analysis of canine biological samples was carried out in 2006–2008 by the Non-Human Forensic Genetics Commission of the Spanish and Portuguese Working Group (GEP) of the International Society for Forensic Genetics (ISFG). The participating laboratories were asked to sequence two dog samples (one bloodstain and one hair sample) for the mitochondrial D-loop region comprised between positions 15,372 and 16,083 using suggested primers and PCR conditions, and to compare their results against a reference sequence. Twenty-one participating laboratories reported a total of 67.5% concordant results, 15% non-concordant results, and 17.5% no results. The hair sample analysis presented more difficulty to the participants than the bloodstain analysis, with a high percentage (29%) failing to obtain a result. The high level of participation showed the interest of the community in the analysis of dog forensic samples but the results reveal that crucial methodological issues need to be addressed and further training is required in order to respond proficiently to the demands of forensic casework.


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doi:10.1016/j.fsigen.2009.04.008

## 1. Introduction

Dogs have become ubiquitous in human environments and are the most commonly reported animals at crime scenes and in forensic science, either as evidence or perpetrators. Therefore, progress in forensic genetics that will enable the development of tools for analysis in this species is a pertinent issue [1]. Moreover, the most commonly found animal evidence at crime scenes are shed hairs, from which nuclear STR loci can often not be reliably typed for unambiguous identification of individuals. In human forensic investigations, mitochondrial DNA (mtDNA) haplotyping is currently used in a diverse range of situations, especially when DNA is scarce or degraded. Recently, several studies have initiated the development of this marker for forensic applications in the canine group, predominantly dogs [2–4]. Although the correlations between mtDNA haplotypes and breeds or types of dogs are weak [5], the potential value of this method in forensic analysis can be considerable for the exclusion of individual dogs as non-contributors of forensic evidence [6,7].

Facing these demands, the GEP-ISFG approved the creation of a specific working section on forensic genetic problems involving the analysis of non-human materials.

This work reports the results of the first collaborative exercise of the Non-Human Forensic Genetics Commission of the GEP-ISFG and summarizes the methodologies employed by the participating laboratories. The first phase of the exercise consisted in the mitochondrial haplotyping of a blood sample and was proposed, discussed and agreed at the GEP-ISFG at the 11th Annual Meeting, held at Madrid, Spain (1–2 June 2006), aiming at the familiarization of the working group with sequencing based canine mtDNA haplotyping (http://www.gep-isfg.org/ISFG/Portugues/Grupos_de_trabalho/Genetica_forense_nao_humana/propuesta_2006_port.php). A second phase was launched in 2008 (http://www.gep-isfg.org/ISFG/Portugues/Grupos_de_trabalho/Genetica_forense_nao_humana/exercicio2008.php) and consisted, for the laboratories who had already participated in the first phase, in the analysis of a canine hair sample. Participants joining the exercise at this point were asked to analyse and report results on both the blood sample and hair samples. A detailed list of participants and affiliations can be found in Appendix A. This exercise was part of a step-by-step approach that aimed firstly at assessing the quality of the genetic profiling, therefore involving only methodological and typing issues in this phase. For that reason, the realistic samples that would mimic crime scene specimens such saliva traces on clothing were not under scrutiny.

## 2. Materials and methods

A blood sample was collected during a routine medical examination at the Veterinary Hospital of the University of Las

**Table 1**
Individual strategies employed by the participating laboratories in the DNA quantification, extraction, amplification, sequencing and sequence edition of a dog blood sample (BH) and a dog hair sample (HS).

| Laboratory | Sample | DNA extraction | Quantification | Polymerase | Post-PCR purification | Sequencing chemistry | Post-sequencing reaction purification | Sequencer | Sequence edition |
|---|---|---|---|---|---|---|---|---|---|
| 1 | BS<br>HS | ProtK/Phe-Chlo/ QIAamp[a] | Not performed | AmpliTaq Gold[a] | Microcon-100[e] | BigDye[B] | Centri-Sep[C] | ABI 310[I] | Sequencher[K]<br>BioEdit[L] |
| 2 | BS<br>HS | QIAamp[a]<br>Invisorb[b] | Agarose minigel/ BrEt | Taq Polymerase[q] | Centricon-100[c] | Not performed | Not performed | Not performed | Not performed |
| 3 | BS | QIAamp[q] | Spectrophotometry | AmpliTaq Gold[l] | QIAquick[t] | BigDye[B] | EtOH/NaAc precipitation | ABI 3130[I] | Chromas Pro[M] |
| | HS | | | | | | Montage SEQ96[D] | ABI 3130[I] | BioEdit[L]<br>ChromasPro[M]<br>ClustalW[N] |
| 4 | BS | Differential lysis/ Phe-Chlo Centricon-100[c] | Not performed | AmpliTaq[m] | Centricon-100[c] | BigDye[B] | EtOH/MgCl precipitation | ABI 3130[I] | SeqScape[S] |
| | HS | Differential lysis/ Phe-Chlo Amicon[d] | Microchip-electrophoresis | | MinElute[u] | | DyeEx[E] | | |
| 5 | BS<br>HS | Chelex[f]<br>ProtK/Phe-Chlo | Agarose minigel/BrEt | Platinum Taq Polymerase[n] | Wizard[v] | Unspecified dye terminators | EtOH/NH4Ac precipitation | MegaBACE 1000[J] | Mega 3.1[P]<br>Not performed |
| 6 | BS<br>HS | Chelex[f]<br>ProtK/Chelex[f] | Not performed | Multiplex PCR Kit[o] | ExoSAP-IT[w] | BigDye[B] | Sephadex[F,O] | ABI 3130[I] | Manual edition |
| 7 | BS<br>HS | ProtK/Phe-Chlo Microcon-100[e] | Not performed | AmpliTaq[m] | Microcon-100[e] | BigDye[B] | Sequencing Reaction Cleanup Kit | ABI 3100[I] | Sequencher[K] |
| 8 | BS<br>HS | ProtK/Phe-Chlo ProtK/Chelex[f] ProtK/Phe-Chlo Microcon-100[e] | Not performed | DNA Polimerasa[p] | Wizard[v] | BigDye[B] | Isopropanol/ EtOH precipitation | ABI 3100[I] | Sequencing Analysis[R] |
| 9 | BS<br>HS | ProtK/Phe-Chlo Microcon-100[e] | Not performed | AmpliTaq Gold[l] | Microcon-100[e] | BigDye 3.1[B] | EtOH/NaAc precipitation | ABI 310[I] | SeqScape[S] |
| 10 | BS<br>HS | ProtK/Phe-Chlo Tissue & Hair Extraction Kit[g] | Not performed | AmpliTaq Gold[l] | MinElute[u] ExoSAP-IT[w] | BigDye[B] | DyeEx[E] | ABI 310[I] | SeqScape[S] |
| 11 | BS<br>HS | ProtK/Phe-Chlo Centricon-100[c] | Agarose minigel/ BrEt Quantifiler[j] | AmpliTaq Gold[l] | QIAquick[t] | BigDye[B] | Centri-Sep[C] DyeEx[E] | ABI 310[I] | SeqScape[S] |

**Table 1** (*Continued*)

| Laboratory | Sample | DNA extraction | Quantification | Polymerase | Post-PCR purification | Sequencing chemistry | Post-sequencing reaction purification | Sequencer | Sequence edition |
|---|---|---|---|---|---|---|---|---|---|
| 12 | BS<br>HS | ProtK/Phe-Chlo<br>Microcon-100[e] | Agarose/SYBR Green | AmpliTaq Gold[l] | ExoSAP-IT[w] | BigDye[B] | Isopropanol/ EtOH precipitation | ABI 3130[I] | Chromas Pro[M]<br>Manual edition |
| 13 | BS<br>HS | ProtK/Phe-Chlo | Spectrophotometry | AmpliTaq Gold[l] | Purification Kit 250[x] | BigDye[B] | Sephadex[F,O] | ABI 3730[I] | Mega 3.1[P]<br>Mega 4[P] |
| 14 | BS<br>HS | Maxwell 16[h] | Not performed | AmpliTaq Gold[l] | PSI Clone[y] | BigDye[B] | Centri-Sep[C] | ABI 310[I] | Manual edition |
| 15 | BS<br>HS | ProtK/Phe-Chlo<br>ProtK/Phe-Chlo<br>Microcon-100[e] | Spectrophotometry | Taq Polymerase[q] | Microcon-100[e] | BigDye[B] | AutoSeq G-50[G] | ABI 310[I] | SeqScape[S] |
| 16 | BS<br>HS | ProtK/Phe-Chlo<br>Centricon-100[c] | Not performed | AmpliTaq Gold[l] | Microcon-100[e]<br>Montage PCR[z] | BigDye[B] | Centri-Sep[C] | ABI 310[I] | SeqScape[S] |
| 17 | BS<br>HS | QIAamp[a]<br>ProtK/Chelex[f] | Not performed<br>Spectrophotometry | Taq Polymerase[r] | Microspin[A] | BigDye[B] | EtOH/NaAc precipitation | ABI 3130[I] | Chromas Pro[M]<br>BioEdit<br>Chromas Pro[M] |
| 18 | BS<br>HS | ProtK/Phe-Chlo<br>Centricon-100[c] | Agarose minigel/BrEt | AmpliTaq[m]<br>Taq Polymerase[q] | ExoSAP-IT[w] | BigDye[B] | EtOH/EDTA precipitation | ABI 310[I] | SeqScape[S] |
| 19 | BS<br><br>HS | Chelex[f]<br><br>ProtK/Phe-Chlo | Spectrophotometry<br><br>Nucleic Dot Metric[k] | Taq Polymerase[q]<br><br>High fidelity[s] | Wizard[v] | BigDye[B] | EtOH/NaAc precipitation<br>EtOH/NaAc/ EDTA precipitation | ABI 3130[I] | BioEdit[L] |
| 20 | BS | FTA Purification Reagent[i] | Not performed | AmpliTaq Gold[l] | Wizard[v] | BigDye[B] | EtOH/NaAc precipitation | ABI 310[I] | DNAMAN[Q] |
| 21 | BS | ProtK/Phe-Chlo<br>Microcon-100[e] | Agarose Minigel/ BrEt Fluorimetry | Taq Polymerase[r] | Microcon-100[e] | | SQE 384[H] | ABI 3130[I] | Chromas Pro[M] |

[a] QIAamp DNA Extraction Kit (QIAGEN, Hilden, Germany).
[b] Invisorb Spin Forensic Kit (Invitek GmbH, Berlin, Germany).
[c] Centricon-100 columns (Millipore, Billerica, MA, USA).
[d] Amicon-Ultra-30 kDa columns (Millipore).
[e] Microcon-100 columns (Millipore).
[f] Chelex (Bio-Rad, CA, USA).
[g] Tissue and Hair Extraction Kit (Promega Corporation, WI, USA).
[h] Maxwell 16 DNA Purification Kit (Promega).
[i] FTA Purification Reagent (GIBCO-BRL).
[j] Quantifiler DNA Quantification Kit (AB, Applied Biosystems, Foster City, CA, USA).
[k] Nucleic dot metric quantitation kit (VH Bio, UK).
[l] AmpliTaq Gold (AB).
[m] AmpliTaq (AB).
[n] Platinum Taq Polymerase (Invitrogen, CA, USA).
[o] Multiplex PCR Kit (QIAGEN).
[p] DNA Polimerasa (Inbio-Highway, Tandil, Argentina).
[q] Taq Polymerase (Promega).
[r] Taq Polymerase (Bioline, London, UK).
[s] High Fidelity (Fermentas, MD, USA).
[t] QIAquick PCR Purification Kit (QIAGEN).
[u] MinElute PCR Purification Kit (QIAGEN).
[v] Wizard Genomic DNA Purification Kit (Promega).
[w] ExoSAP-IT (USB, Ohio, USA).
[x] Purification Kit 250 (QIAGEN).
[y] PSI Clone PCR Purification Kit (AB).
[z] Montage PCR Clean-Up Kit (Millipore).
[A] Microspin (GE Healthcare, UK).
[B] BigDye (AB).
[C] Centri-Sep (Princeton Separations, NJ, USA).
[D] Montage SEQ96 (Millipore).
[E] DyeEx (QIAGEN).
[F] Sephadex G-50 (GE Healthcare, UK).
[G] AutoSeq G-50 (GE Healthcare).
[H] SQE 384 (Millipore).
[I] ABI 310, ABI 3130, ABI 3730 (AB).
[J] MegaBACE 1000 (GE Healthcare).
[K] Sequencher (Gene Codes Corporation, MI, USA).
[L] BioEdit (Ibis BioSciences, Carlsbad, CA, USA).
[M] Chromas Pro (Technelysium PTY, Australia).
[N] ClustalW (EMBL-EBI).
[O] SeqScape Software v2.5 (AB).
[P] Mega 3.1 and 4 (http://www.megasoftware.net/author.html).
[Q] DNAMAN (Lynnon Corporation, Quebec, Canada).
[R] Sequencing Analysis Software (AB).
[S] SeqScape Software (AB).

**Table 2**
Summary statistics of the materials and methods employed by the participating laboratories.

| Materials and methods | % | | |
|---|---|---|---|
| | BS | HS | Total |
| DNA extraction | | | |
| ProtK/phenol–chloroform methods | 57 | 58 | 58 |
| QIAamp[a] | 14 | 5 | 10 |
| ProtK/Chelex[f] | – | 16 | 8 |
| Chelex[f] | 14 | – | 8 |
| Other | 14 | 21 | 18 |
| DNA quantification | | | |
| Not performed | 52 | 42 | 48 |
| Agarose minigel/ethidium bromide methods | 24 | 21 | 23 |
| Spectrophotometry | 19 | 16 | 18 |
| Other | 5 | 21 | 13 |
| Polymerase | | | |
| AmpliTaq Gold[l] | 48 | 47 | 48 |
| Taq Polymerase[q] | 14 | 16 | 15 |
| AmpliTaq[m] | 14 | 11 | 13 |
| Taq Polymerase[r] | 10 | 5 | 8 |
| Other | 14 | 21 | 18 |
| Post-PCR purification | | | |
| Microcon-100[e] | 29 | 21 | 25 |
| ExoSAP-IT[w] | 14 | 21 | 18 |
| Wizard[v] | 19 | 16 | 18 |
| QIAquick[t] | 10 | 11 | 10 |
| Centricon-100[c] | 10 | 5 | 8 |
| Microspin[A] | 5 | 5 | 5 |
| Other | 14 | 21 | 18 |
| Sequencing chemistry | | | |
| BigDye[B] | 90 | 89 | 90 |
| Not performed | 5 | 5 | 5 |
| Unspecified dye terminators | 5 | 5 | 5 |
| Post-sequencing reaction purification | | | |
| EtOH precipitations (EDTA, MgCl, NaAc, Isopropanol, NH4Ac) | 48 | 37 | 43 |
| Centri-Sep[C] | 19 | 16 | 18 |
| DyeEx[E] | 5 | 16 | 10 |
| Sephadex[F,O] | 10 | 11 | 10 |
| Not performed | 5 | 5 | 5 |
| Other | 14 | 16 | 15 |
| Sequencer | | | |
| ABI (310, 3730, 3100, 3100 Avant, 3130, 3130 XL)[I] | 90 | 89 | 90 |
| MegaBACE 1000[J] | 5 | 5 | 5 |
| Not performed | 5 | 5 | 5 |
| Sequence edition | | | |
| SeqScape[S] | 33 | 37 | 35 |
| BioEdit[L] | 5 | 21 | 13 |
| Chromas Pro[M] | 19 | 5 | 13 |
| Manual edition | 10 | 11 | 10 |
| Mega (v3.1, v4)[P] | 10 | 5 | 8 |
| Sequencher[K] | 10 | 5 | 8 |
| Not performed | 5 | 11 | 8 |
| Other | 10 | 5 | 8 |

Refer Table 1 for explanation of table footnotes.

Palmas de Gran Canaria, Spain. Bloodstains were prepared with 50 μl of blood applied to stain cards and dried at room temperature. One bloodstain was sent to the participating laboratories. The participants were asked to sequence a specific region of the canine mitochondrial D-loop and to identify polymorphic positions relative to a given reference sequence (GenBank accession NC_002008) [8]. Two primer pairs were suggested for amplification of two overlapping fragments: A (15341–15804); primer AF 5′-TTACCTTGGTCTTGTAAACC and primer AR 5′-CTGAAGTAAGAACCAGATGCC ($T_a$ = 58 °C); and B (15746–16107): primer BF 5′-CATACTAACGTGGGGGTTAC and primer BR 5′-CCATTGACTGAATAGCACCTTG ($T_a$ = 60 °C). The hair samples were collected from another dog also during a routine medical examination at the Veterinary Hospital of the University of Las Palmas de Gran Canaria, Spain. Three anagen hairs without roots were sent to each participant.

In the case that the participants would choose to use other primers, it was necessary that the region comprised between positions 15458 and 16039 was analysed and reported. PCR conditions were suggested as described by van Asch et al. [9]. For nomenclature of polymorphic positions, those described by Pereira et al. [10] were recommended. The proposed exercise did not raise questions from the participants, except with regards to nomenclature of ambiguous positions. The system described by Tully et al. [11] was then recommended in such cases. The participants were asked to fill a questionnaire regarding the materials and methods they chose to employ in processing of the samples.

Thirteen participants joined the first phase of the exercise and reported results on a canine blood sample (BS). Of those, all except two laboratories joined the second phase for the typing of a hair sample (HS) along with eight new participants. A total of 19 participants analysed both the blood and the hair sample, and two analysed only the blood sample. The present report integrates and discusses the results of both phases of the exercise. Individual strategies chosen by the participants for the DNA extraction, quantification, quantification, amplification, sequencing and sequence analysis are presented in Table 1. Frequencies of the different methods are summarized in Table 2.

## 3. Results and discussion

A summary of the results reported by the participants and the type of errors found therein are presented in Table 3. All non-concordant results were verified by manual inspection of electropherograms requested to the participants for that purpose and revealed deficient training in base calling. Approximately 90% of the participants reported the performance of blank DNA extraction and PCR amplification, and, when applicable, the sequencing of the fragments of interest in both forward and reverse directions.

For the BS analysis, 76% of the participant laboratories reported exactly the same five polymorphisms in comparison with the reference sequence (substitutions 15483C/T, 15627A/G and 15639T/A in fragment A and 15814C/T, 15912C/T in fragment B), therefore considered the consensus result. This sequence can be assigned to haplotype A1 (GenBank accession AF531654) [3], as noted by three participants. Only one participant did not attain any result. The non-consensus results derive from three types of errors: one lab failed to identify substitution 15814C/T although it was unambiguous in the solicited electropherogram, two labs wrongly reported substitutions (15639A/T instead of 15639T/A although correctly annotated by hand in the solicited electropherogram; and 15627T/G instead of 15627A/G), and the last reported a total 15 substitutions relatively to the reference sequence (including the consensus positions). This laboratory presented poor quality electropherograms and it is suspected that interpretation was only achieved through software-based sequence edition, regardless of background noise evident in visual inspection.

The HS analysis resulted in the exact identification of substitutions 15620T/C, 15627A/G, 15639T/A (fragment A), 15814C/T and 15955C/T (fragment B) by 58% of the participant laboratories (11). Since the GEP-ISFG working group demands that at least five participants are involved and at least 70% of them report exactly the result, this relatively low percentage does not allow for the result to be classified as consensus. In total, and in a high proportion contrastingly to the BS analysis, 32% of the laboratories could not attain a result. Two participants reported non-concordant results: one replicated a previous error in the BS

**Table 3**
Summary of results reported by laboratories participating in the Non-Human GEP-ISFG collaborative voluntary exercise, with percentages relative to the number of laboratories that performed the analyses (21 in the BS analysis and 19 in the HS analysis). Overall results are relative to the total of 40 analyses.

| Results | No. of labs | % | Type of error | |
|---|---|---|---|---|
| | | | Missing position | Wrong position |
| Non-concordant BS | 4 | 19 | 15814 C/T | – |
| | | | – | 15627 T/G |
| | | | – | 15639 A/T |
| | | | – | [a] |
| Non-concordant HS | 2 | 11 | 15955 C/T | 15627 T/G |
| | | | 15955 C/T | – |
| Concordant BS | 16 | 76 | | |
| Concordant HS | 11 | 58 | | |
| No result BS | 1 | 5 | | |
| No result HS | 6 | 32 | | |
| Non-concordant in both samples | 2 | – | | |
| No result in both samples | 1 | – | | |
| Concordant in BS and no result in HS | 3 | – | | |
| Non-concordant in BS and no result in HS | 2 | – | | |
| Total labs reporting errors/non-results | 8 | 38 | | |
| Overall results | | | | |
| Concordant | 27 | 67.5 | | |
| Non-concordant | 6 | 15 | | |
| No results | 7 | 17.5 | | |

[a] 15441 T/A, 15443 T/A, 15803 A/G, 15806 G del, 15807 C del, 15808 C del, 15810 T/A, 15822 C/T, 15827 A del, and 15847 T/C.

(misreported substitution 15627T/G instead of 15627A/G, and also failed to report substitution 15955C/T although it was unambiguous in the requested electropherogram) and the last participant could only detect substitution 15814C/T (failing to detect 15955CT in the sequence although visible in the requested electropherogram) and reported unsuccessful PCR amplification of fragment A. The high proportion of no results in the analysis of the hair samples could be explained by the inexperience of some laboratories in obtaining DNA from biological materials other than bloodstains. The quality of particular distributed hairs may also have determined the success of the analyses since at least one experienced laboratory failed to achieve a result. Establishing methodologies for DNA quantification (here performed by only half of the participants) is recommended, and could assist the laboratories in the selection and optimization of the extraction methodologies.

### *3.1. Frequency of human, canine and other non-human mtDNA analysis*

The participants were additionally asked to classify their yearly frequency of mtDNA analysis in humans, canines and other non-humans. The total of answers was not always equivalent to the number of participants since some questions remained unanswered. Regarding human mtDNA, almost half the laboratories reported very frequent ($\geq$100) analysis, not frequent ($<$50) by five laboratories, frequent (50–100) in four laboratories and one laboratory did not perform human mtDNA analyses. Canine mitochondrial DNA analysis was classified as not frequent by almost half of the participants, frequent by one participant, and the first time for the remaining. No participant reported very frequent analysis of canine mitochondrial DNA. Relatively to mtDNA analyses in other non-humans, almost a third of the participants did not perform frequent analyses, a quarter did not analyse other non-humans and three laboratories reported very frequent analysis (one of them in camelids). The participants were also asked to report any demand of canine mitochondrial DNA analysis in the forensic and private context. Seven answers classified forensic requests as not frequent and nine stated nonexistent. As for private consultancy requests, three participants classified their requests as not frequent and nine as inexistent. We concluded that non-human, and particularly dog mtDNA analyses were not frequently performed by the majority of the participating laboratories, and not frequently requested. The questionnaire also invited for observations on the exercise and further suggestions. No observations were made, although two participants suggested the creation of a canine mitochondrial database also including wolf sequences.

## 4. Conclusions

The collaborative exercises organised since 1982 by GEP-ISFG have proven to be extremely useful both to participants, in order to ensure and increase quality of their results, but also for the general interest of the scientific community (i.e. [12–18]). These exercises have addressed general quality control and quality assurance issues as well as validation of new technologies or types of markers. In concurrence with this collaborative strategy, the present study was satisfactory in the sense that the high level of participation shows the will of the laboratories to establish mtDNA forensic analysis of dog samples.

As for the overall results, only 67.5% of concordant results were obtained. The remaining fraction is divided between non-concordant results (15%) and no results (17.5%), concentrated in eight laboratories. The most common errors in the report of the results consisted in the non-standardized nomenclature of substitutions, followed by poor interpretation of electropherograms. The elimination of this type of error should be achieved by proper training in the interpretation of electropherograms, a fundamental requirement for forensic genetic analyses. Obtaining results from anagen hairs without roots proved more challenging as expected, with a large proportion of participants reporting no results. This was mainly due to lack of PCR amplification of either one or both fragments of the sequence under scrutiny, revealing difficulty in obtaining DNA from this type of samples.

The consistency of the dog mtDNA typing results among the participants is thus below the forensic practice requirements, and it is highly recommendable that laboratories interested in the analysis of non-human evidence increase their proficiency both in the technical aspects of sample processing and the reporting of the

results. A new exercise on canine mtDNA will be proposed in the near future to monitor the progress of the participating laboratories in method development. In due course, the launching of a dog mtDNA database in a secure basis for forensic use will further contribute to encourage the forensic use of non-human crime scene biological samples.

## Acknowledgements

The authors are grateful to Leonor Gusmão and two anonymous reviewers for helpful suggestions on the manuscript. BA is a grantee from FCT (SFRH/BDE/15581/2006). IPATIMUP is partially funded by the VI Framework Program "Programa Operacional Ciência e Inovação (POCI 2010).

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fsigen.2009.04.008.

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