Results of a collaborative study on DNA identification of aged bone samples

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A collaborative exercise with several institutes was organized by the Forensic DNA Service (FDNAS) and the Institute of the Legal Medicine, 2nd Faculty of Medicine, Charles University in Prague, Czech Republic, with the aim to test performance of different laboratories carrying out DNA analysis of relatively old bone samples.

Eighteen laboratories participating in the collaborative exercise were asked to perform DNA typing of two samples of bone powder. Two bone samples provided by the National Museum and the Institute of Archaeology in Prague, Czech Republic, came from archeological excavations and were estimated to be approximately 150 and 400 years old. The methods of genetic characterization including autosomal, gonosomal, and mitochondrial markers was selected solely at the discretion of the participating laboratory.

Although the participating laboratories used different extraction and amplification strategies, concordant results were obtained from the relatively intact 150 years old bone sample. Typing was more problematic with the analysis of the 400 years old bone sample due to poorer quality.

The laboratories performing identification DNA analysis of bone and teeth samples should regularly test their ability to correctly perform DNA-based identification on bone samples containing degraded DNA and potential inhibitors and demonstrate that risk of contamination is minimized.

The quality and reliability of DNA typing results produced by research and forensic laboratories are limited by the amount and condition of the samples processed, presence of inhibitors, sample collection and storage until analysis, and the practices of the laboratory. Due to frequently limited quantity and quality of DNA in bone samples, even low levels of cross-contamination can become a serious problem for obtaining reliable results. Thus, special attention must be paid to both the procedures and the interpretation of data. Errors can occur and, therefore, laboratories should test their competence through proficiency tests (internal and/or external) and collaborative exercises (1-4). Aged bone samples are among the most difficult biological samples for DNA-based analyses (5,6), and the laboratory should have adequate testing capabilities to analyze these types of samples. It is not sufficient to rely on the analysis of standard reference materials or typical participation in proficiency tests or collaborative exercises of more ideal sample types. While desirable, human osteological material is not considered a typical standard reference material and is not readily accessible to serve as a material for proficiency tests (4,7-10). To address this testing deficiency, the organizers of the collaborative exercise described herein obtained sufficient quantities of two old bone samples that could be distributed and analyzed among a number of laboratories. The purpose of the exercise was to determine whether concordant results could be obtained from two common samples in different laboratories that use varied extraction procedures, different commercial short tandem repeat (STR) kits, different in-house mitochondrial DNA (mtDNA) protocols, and different laboratory-specific interpretation guidelines.
MATERIALS AND METHODS

Sample preparation

The initial step of the sample preparation was the selection of appropriate samples for the collaborative exercise (CE) according to the following six criteria. First, to avoid potential ethical issues, the bone specimens had to be at least 150 years old archeological material, without any identity link to a known person (11), and already subjected to scientific examination (anthropology, archeology, etc.). Second, only the middle parts of long bones were used as test samples (12). Third, the sample preparation (ie, decontamination and cleaning) had to follow the protocol specified previously (5,13). Fourth, the bone specimens had to be converted to homogenous bone powder using a liquid nitrogen grinding mill (14,15) before distribution. Fifth, the bone samples had to be successfully typed by at least two commercial kits to select samples that are typable before distributing them to participating laboratories. Sixth, to assure the correctness of the results, the bone powder had to be quality control checked for typability and contamination before the dispatch of the samples (3).

The above criteria are based on the previous experience and published work of the organizing laboratory.

Collaborative exercise design

Participating laboratories obtained two different samples, Sample 1 and Sample 2, which had been successfully analyzed by the organizing laboratory. Sample 1 was approximately 400 years old, with degraded DNA and difficult to type. Sample 2 was approximately 150 years old and well-preserved, with relatively intact DNA suitable for standard typing procedures. The age of the specimens was determined by archeologists based on the burial pattern and artifacts found at the excavation site (16,17). Laboratories received 600 mg (Sample 1) and 150 mg (Sample 2) of bone powder prepared from cuttings from the compacta of the respective femurs. The surface of the femurs was cleaned using a rotary sanding tool (Dremel, Racine,WI, USA). Following the removal of surface material, additional 2-3 mm

| Laboratory code | Laboratory type† | DNA extraction chemistry | STR kits (autosomal) | X-STR typing | mtDNA typing |
|-----------------|------------------|--------------------------|---------------------|--------------|-------------|
| 1               | Organizing laboratory | A                         | A1, B               | YES          | YES         |
| 2               | Government       | B, C                     | C, D1               | NO           | NO          |
| 3               | University       | C                         | A2, B               | YES          | YES         |
| 4               | Government       | B, D                     | E2                  | NO           | YES         |
| 5               | Government       | E                         | F1, F2              | NO           | NO          |
| 6               | Government       | A, D                     | NO                  | NO           | YES         |
| 7               | Government       | F                         | A1, E2              | YES          | NO          |
| 8               | Police           | E                         | B, F2, G1, G2       | NO           | NO          |
| 9               | Police           | E                         | B, E2               | NO           | NO          |
| 10              | Government       | D                         | A2                  | NO           | NO          |
| 11              | Private          | D                         | NO                  | YES          |             |
| 12              | Private          | D                         | B, E1               | NO           | NO          |
| 13              | Government       | C                         | F1, F2              | NO           | YES         |
| 14              | Government       | G                         | B, E2               | NO           | NO          |
| 15              | University       | B                         | D2, E1              | NO           | YES         |
| 16              | Private          | D                         | D3, D4              | NO           | NO          |
| 17              | Police           | A                         | B, E2               | NO           | NO          |
| 18              | Police           | C                         | B, E2               | NO           | NO          |
| 19              | University       | D                         | NO                  | NO           | YES         |

*Abbreviations: STR – short tandem repeat; mtDNA – mitochondrial DNA; DNA extraction chemistry codes – A: BTA PrepFiler (Life Technologies, USA), B: Phenol/chloroform, C: EZ1 DNA Investigator kit (Qiagen, Germany), D: QIAamp/DNeasy kit (Qiagen, Germany), E: Maxwell 16 (Promega Corporation, USA), F: QuickGene (Fujifilm, Japan), G: MagAttract DNA Mini M48 Kit (Qiagen, Germany). STR kit codes – A1: NGM, A2: NGM Select (Life Technologies, USA), B: MiniFiler (Life Technologies, USA), C: Investigator ESSplex SE (Qiagen, Germany), D1: PowerPlex 16, D2: PowerPlex 18D, D3: PowerPlex 16HS, D4: PowerPlex Fusion (Promega Corporation, USA), E1: Identifiler, E2: Identifiler Plus (Life Technologies, USA), F1: PowerPlex ES17, F2: PowerPlex ESX17 (Promega Corporation, USA), G1: MPX-5, G2: AUX-1 (Serac, Germany).
†All laboratories submitting results for X chromosome STRs used Investigator® Argus X-12 (Qiagen, Germany).
of the bone were ground away to remove potential contaminants. The cleaned fragment of approximately 2 x 8 cm was further cut into smaller pieces sized 3 x 6 mm. The bone fragments were then placed in a 50-mL tube and further cleaned by inversion for 30 seconds in 5% commercial bleach, 5 x inversion for 30 seconds in 30 mL of distilled water, and inversion for 30 seconds in 96% ethanol. The bone fragments were allowed to air dry completely before grinding. The bone powder was prepared by grinding the bone fragments in the presence of liquid nitrogen using the cryogenic mill SPEX Sample Prep 6770 FreezeMill (Spex CentriPrep, USA). All batches of bone powder were tested for potential contamination (single DNA profile by MiniFiler amplification), subsequently pooled, and divided in aliquots. The bone cleaning and grinding were performed by the organizing laboratory to minimize the possible variable effects of bleach (18) and temperature (19) on the results of the collaborative exercise. The laboratories were asked to perform DNA analysis of the samples with methods they routinely use for bone samples or to use the suggested extraction and typing protocol (Table 1). The suggested protocol recommended to use 50 mg of bone powder per silica-based DNA extraction as described by Vanek et al (20) or DNA extraction protocol as described in user’s manual of PrepFiler BTA Forensic DNA extraction kit (LifeTechnologies, USA). Participating laboratories provided a table with results and the original fragment analysis sample files (FSA files) with the printouts of the resulting electropherograms (EPGs).

RESULTS

DNA quantitation and STR typing

The results of DNA quantitation varied substantially across laboratories (Table 2). Five out of 19 participating laboratories did not quantify DNA extracts prior to polymerase chain reaction (PCR).

The STR types for the two bone samples were obtained by the CE organizers (Tables 3a and 3b). While the true types of these bones were unknown a priori, these STR results were assumed correct for the purpose of the collaboration. The only exceptions were the STR loci D5S818 and SE33 for Sample 1, and D5S818, TPOX, SE33, Penta D, and Penta E for Sample 2, where the consensus results based on the majority rule were considered the correct types. The TPOX, Penta D, and Penta E loci were not evaluated for Sample 1.

### TABLE 2. DNA quantitation chemistries used for bone sample analysis by the laboratories participating in Collaborative Exercise*

| Laboratory code | DNA quantitation chemistry | Sample 1 (ng/μl)* | Sample 2 (ng/μl)* |
|----------------|---------------------------|------------------|------------------|
| 1              | LM                        | 0.00173          | 0.078            |
| 2              | QQ                        | 0                | 0.0316           |
| 3              | QQ                        | 0                | 0.046            |
| 4              | QA                        | 0.00616          | 0.0415           |
| 5              | PP                        | 0.01065          | 0.01411          |
| 6              | LM                        | 0.000001347, 0.000002232 | 0.0075, 0.07618, 0.10835, 0.0126, 0.1345 |
| 7              | QU                        | 1.07, 1.27, 0.808 | 1.29, 12.6, 1.7  |
| 8              | QDA                       | 0.00126          | 0.0177           |
| 9              | QA                        | 0.089, 0.019, 0.005 | 0.084           |
| 10             | QA                        | 0                | 0.2              |
| 11             | QU                        | 0                | 10               |
| 12             | NA                        | x                | x                |
| 13             | QA                        | 0.0135, 0.018    | 0.868            |
| 14             | NA                        | x                | x                |
| 15             | LM                        | 0.06             | 0.8              |
| 16             | NA                        | x                | x                |
| 17             | QDA                       | 0                | 0.107            |
| 18             | QDA                       | 0.00201          | 0.121            |
| 19             | NA                        | x                | x                |

*Abbreviations: DNA quantitation chemistries codes – QQ: Quantiplex (Qiagen, Germany), QA: Quantifiler Human DNA Quantification Kit (Life Technologies, USA), QDA: Quantifier Duo DNA Quantification Kit (Life Technologies, USA), LM: laboratory made RT-PCR quantitation system, PP: Plexor HY System (Promega Corporation, USA), QU: Qubit (Life Technologies, USA), x: no quantitation.

*More numbers means quantitation performed several times.
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The negative controls provided by the participating laboratories did not show evidence of contamination. A situation where a laboratory failed to produce results for a specific STR locus or if only 1 allele at a heterozygous locus was obtained was not considered an error, but a partial result for the purpose of the study. Results with concordant calls, either complete or partial at a locus, with those of the CE organizing laboratory were considered correct (Tables 4a and 4b). Fifteen laboratories submitted results for autosomal STRs. Three laboratories obtained full and concordant profiles for Sample 1, while 13 of 14 laboratories obtained full and concordant profiles for Sample 2. The success rates for autosomal STR typing ranged from 4.5% to 100% for Sample 1 and from 77.3% to 100% for Sample 2. Success was based on the total number of loci a laboratory assayed in this study. Therefore, the percentage of success might be affected by use of more loci. For example, laboratories 17 and 18 both used the MiniFiler kit, which contains only 9 STRs, and had 100% success. Most other laboratories typed more loci and tended to have a lower percentage of success.

Only two laboratories provided results for X-STR loci for Sample 2 and both obtained the same results as the organizing laboratory (Table 5). No laboratory submitted X-STR results for Sample 1.

DISCUSSION

DNA quantitation and STR typing

The majority of participating laboratories quantified DNA extracts before performing PCR. The substantial differences that were obtained in DNA quantitation results could have resulted from the extraction efficiency (ie, chemistry), quantitation methodology (eg, using Qubit, which is not human specific, fluorometric vs real-time quantitative PCR, or using a mtDNA specific vs nuclear specific assay), and the number of loci assayed.

TABLE 3A. Results of autosomal short tandem repeat (STR) typing of Sample 1 by the laboratories participating in Collaborative Exercise

| Laboratory code | D8S1179 | D21S1153 | D3S1358 | D16S539 | D2S1338 | D19S433 | VWA | D18S51 | AME | FGA |
|-----------------|---------|-----------|---------|---------|---------|---------|-----|---------|-----|-----|
| 1               | XX      | 10.2,10.2| 30.2,30.2| 9.3,9.3 | 14,14   | 18,20   | 14   | 16,17  | 9.3 | 9.3 |
| 2               | 30.2,30.2| 10.2,10.2| 9.3,9.3 | 14,14   | 18,20   | 14,14   | 16   | 17     | 9.3 | 9.3 |
| 3               | 30.2,30.2| 10.2,10.2| 9.3,9.3 | 14,14   | 18,20   | 14,14   | 16   | 17     | 9.3 | 9.3 |
| 4               | 30.2,30.2| 10.2,10.2| 9.3,9.3 | 14,14   | 18,20   | 14,14   | 16   | 17     | 9.3 | 9.3 |
| 5               | 30.2,30.2| 10.2,10.2| 9.3,9.3 | 14,14   | 18,20   | 14,14   | 16   | 17     | 9.3 | 9.3 |
| 6               | 30.2,30.2| 10.2,10.2| 9.3,9.3 | 14,14   | 18,20   | 14,14   | 16   | 17     | 9.3 | 9.3 |
| 7               | 30.2,30.2| 10.2,10.2| 9.3,9.3 | 14,14   | 18,20   | 14,14   | 16   | 17     | 9.3 | 9.3 |
| 8               | 30.2,30.2| 10.2,10.2| 9.3,9.3 | 14,14   | 18,20   | 14,14   | 16   | 17     | 9.3 | 9.3 |
| 9               | 30.2,30.2| 10.2,10.2| 9.3,9.3 | 14,14   | 18,20   | 14,14   | 16   | 17     | 9.3 | 9.3 |
| 10              | 30.2,30.2| 10.2,10.2| 9.3,9.3 | 14,14   | 18,20   | 14,14   | 16   | 17     | 9.3 | 9.3 |
| 11              | 30.2,30.2| 10.2,10.2| 9.3,9.3 | 14,14   | 18,20   | 14,14   | 16   | 17     | 9.3 | 9.3 |
| 12              | 30.2,30.2| 10.2,10.2| 9.3,9.3 | 14,14   | 18,20   | 14,14   | 16   | 17     | 9.3 | 9.3 |
| 13              | 30.2,30.2| 10.2,10.2| 9.3,9.3 | 14,14   | 18,20   | 14,14   | 16   | 17     | 9.3 | 9.3 |

* Consensus profile
† NA – not contained in the kit used.
‡ X – no results obtained.
§ Missing one allele at heterozygous locus.
II Wrong allele.

mtDNA typing

Four laboratories submitted mtDNA typing results for Sample 1, and 6 laboratories submitted results for Sample 2 (Table 6). Laboratories used different protocols for mtDNA amplification. Laboratories 1, 3, and 19 used primers as described by Eichmann et al. (22), Laboratory 4 used primers that generated amplicon sizes of 385 bp (HVR1) and 240 bp (HVR2). Laboratory 6 used primers that generated amplicon sizes of 220 bp (HVR1) and 242 bp (HVR2) (23,24). Laboratory 11 used primers that generated amplicon sizes of 461 bp (HVR1) and 445 bp (HVR2), Laboratory 13 used primers that generated amplicon sizes of 449 bp (HVR1) and 391 bp (HVR2), and Laboratory 15 used primers that generated amplicon sizes of 249 and 228 bp (HVR1), and 203 and 301 bp (HVR2).
TABLE 3B. Results of autosomal short tandem repeat (STR) typing of Sample 2 by the laboratories participating in Collaborative Exercise

| Laboratory | NGM STRs | Minifiler STRs | Additional loci |
|------------|----------|---------------|-----------------|
|            | D8S      | D21           | D18             | D10 | D22 | D2S | D1S | D7S | CSF | D13 | D16S | D25S | D18 |
| D8S        | 208      | 1179          | 11358TH01       | S11 | S15 | S138P43 | VWA | S51 | AME | FGA | S1248S1045 | 441 | 1656 | S391 |
| D21        | 1179     | S15           | 11358TH01       | S11 | S15 | S138P43 | VWA | S51 | AME | FGA | S1248S1045 | 441 | 1656 | S391 |
| D18        | 11358    | 1358TH01      | 1179            | D21 | S15 | S138P43 | VWA | S51 | AME | FGA | S1248S1045 | 441 | 1656 | S391 |
| D10        | 1179     | 1358TH01      | 1179            | 11358 | S15 | S138P43 | VWA | S51 | AME | FGA | S1248S1045 | 441 | 1656 | S391 |
| D2S        | 11358    | 1358TH01      | 1179            | 11358 | S15 | S138P43 | VWA | S51 | AME | FGA | S1248S1045 | 441 | 1656 | S391 |
| D1S        | 11358    | 1358TH01      | 1179            | 11358 | S15 | S138P43 | VWA | S51 | AME | FGA | S1248S1045 | 441 | 1656 | S391 |
| D7S        | 11358    | 1358TH01      | 1179            | 11358 | S15 | S138P43 | VWA | S51 | AME | FGA | S1248S1045 | 441 | 1656 | S391 |
| CSF        | 11358    | 1358TH01      | 1179            | 11358 | S15 | S138P43 | VWA | S51 | AME | FGA | S1248S1045 | 441 | 1656 | S391 |
| D13        | 11358    | 1358TH01      | 1179            | 11358 | S15 | S138P43 | VWA | S51 | AME | FGA | S1248S1045 | 441 | 1656 | S391 |
| D16S       | 11358    | 1358TH01      | 1179            | 11358 | S15 | S138P43 | VWA | S51 | AME | FGA | S1248S1045 | 441 | 1656 | S391 |
| D25S       | 11358    | 1358TH01      | 1179            | 11358 | S15 | S138P43 | VWA | S51 | AME | FGA | S1248S1045 | 441 | 1656 | S391 |
| D18        | 11358    | 1358TH01      | 1179            | 11358 | S15 | S138P43 | VWA | S51 | AME | FGA | S1248S1045 | 441 | 1656 | S391 |
| D10        | 11358    | 1358TH01      | 1179            | 11358 | S15 | S138P43 | VWA | S51 | AME | FGA | S1248S1045 | 441 | 1656 | S391 |

**Note:**
- Loci duplicated in different kits.
- Consensus profile.
- Not contained in the kit used.
- Missing data.
- False homozygous locus.
oratories for Sample 1, 6 laboratories for Sample 2), but the number of different mtDNA profiles reported for Sample 1 suggests that mtDNA typing in challenging and degraded bone samples is not a robust and reliable methodology for some laboratories and more investigation is needed. One possible explanation for the variable mtDNA sequence results may be that the primers used by the participating laboratories generate amplicon sizes that are larger than the fragmented DNA in Sample 1 and, thus, may select for low level contaminating exogenous DNA.

### TABLE 4A. Evaluation of autosomal short tandem repeat (STR) typing of Sample 1 by the laboratories participating in Collaborative Exercise*

| Laboratory code | Number of loci typed (depending on the kit used) | Number of loci with concordant results | Missing one allele at heterozygous locus | Number of loci with wrong results | Number of loci with no results | Percentage of success |
|-----------------|-----------------------------------------------|---------------------------------------|----------------------------------------|---------------------------------|-------------------------------|-----------------------|
| 2               | 19                                            | 5                                     | 2                                      | 1                              | 11                            | 36.8                  |
| 3               | 26                                            | 8                                     | 7                                      | 1                              | 10                            | 57.7                  |
| 4               | 15                                            | 2                                     | 1                                      | 2                              | 10                            | 20.0                  |
| 5               | 23                                            | 19                                    | 4                                      | 0                              | 0                             | 100.0                 |
| 8               | 17                                            | 11                                    | 2                                      | 0                              | 4                             | 76.6                  |
| 9               | 20                                            | 2                                     | 4                                      | 2                              | 12                            | 30.0                  |
| 10              | 19                                            | 1                                     | 0                                      | 0                              | 18                            | 5.3                   |
| 12              | 20                                            | 13                                    | 0                                      | 0                              | 7                             | 35.0                  |
| 13              | 18                                            | 12                                    | 0                                      | 4                              | 2                             | 66.7                  |
| 14              | 21                                            | 12                                    | 4                                      | 1                              | 4                             | 76.2                  |
| 15              | 22                                            | 1                                     | 0                                      | 0                              | 21                            | 4.5                   |
| 16              | 18                                            | 2                                     | 1                                      | 0                              | 15                            | 16.7                  |
| 17              | 9                                             | 9                                     | 0                                      | 0                              | 0                             | 100.0                 |
| 18              | 9                                             | 9                                     | 0                                      | 0                              | 0                             | 100.0                 |

*Loci duplicated in different kits are counted twice, including AMELOGENIN. Laboratories 3 and 13 encountered a problem with pull-up peaks in STR loci vWA (laboratory 3) and D21S11, D18S51, and vWA (laboratory 13). A locus was considered correct if concordant with organizer results (or consensus profile) or if one of the two alleles at a heterozygous locus was detected. The calculations of success rate (%) are based on a total of the loci used by the laboratory and readers should take into consideration that the number of STRs ranged from 9 to 26.

### TABLE 4B. Evaluation of autosomal short tandem repeat (STR) typing of Sample 2 by the laboratories participating in Collaborative Exercise*

| Laboratory code | Number of loci typed | Number of loci with correct results | Missing allele in heterozygous locus | Number of loci with wrong results | Number of loci with no results | Percentage of success |
|-----------------|----------------------|------------------------------------|-------------------------------------|----------------------------------|-------------------------------|-----------------------|
| 2               | 30                   | 30                                 | 0                                   | 0                                | 0                             | 100                   |
| 3               | 17                   | 17                                 | 0                                   | 0                                | 0                             | 100                   |
| 4               | 17                   | 17                                 | 0                                   | 0                                | 0                             | 100                   |
| 5               | 23                   | 23                                 | 0                                   | 0                                | 0                             | 100                   |
| 7               | 27                   | 27                                 | 0                                   | 0                                | 0                             | 100                   |
| 8               | 17                   | 17                                 | 0                                   | 0                                | 0                             | 100                   |
| 9               | 22                   | 15                                 | 2                                   | 0                                | 5                             | 77.3                  |
| 10              | 18                   | 18                                 | 0                                   | 0                                | 0                             | 100                   |
| 12              | 22                   | 21                                 | 1                                   | 0                                | 0                             | 100                   |
| 13              | 17                   | 17                                 | 0                                   | 0                                | 0                             | 100                   |
| 14              | 22                   | 22                                 | 0                                   | 0                                | 0                             | 100                   |
| 15              | 24                   | 24                                 | 0                                   | 0                                | 0                             | 100                   |
| 16              | 21                   | 21                                 | 0                                   | 0                                | 0                             | 100                   |
| 17              | 22                   | 22                                 | 0                                   | 0                                | 0                             | 100                   |

*Laboratories 2-8, 10, and 13-17 provided complete profile results with no discordance. Laboratory 9 did not obtain results for the loci D21S11, D2S1338, and FGA and failed to identify 1 of the alleles at the vWA and D18S51 loci using Identifiler Plus kit (Life Technologies, USA) but obtained correct results for the D251338 and D18S51 loci using the MiniFiler kit (Life Technologies, USA). Laboratory 12 failed to identify 1 of the alleles in the D165539 locus using the Identifiler kit (Life Technologies, USA), but obtained correct results using the MiniFiler kit (Life Technologies, USA). A locus was considered a success if concordant with organizer results (or consensus profile) or if one of the two alleles at a heterozygous locus was detected.
The concept of CE

The selection of a femur for the samples was based on the greater amount of material that could be obtained for distribution among laboratories. Recent findings might suggest that a femur may not be the best choice (32), but the amount of available specimen must be considered when preparing a sufficient quantity of operationally identical samples for all participating laboratories. The concept of future CEs on bone samples could clarify the typing results from the extraction-borne variations by sending the participating laboratories DNA extracted from aged bone samples. This approach may help to better identify the root cause(s) of particular DNA typing limitations, e.g., the extraction method. The next CE could also address the cleaning and grinding phase and the removal of humic acid inhibitors and modern DNA contaminants (18,33-41). Another improvement of the CE concept would be the inclusion of massive parallel sequencing (42,43) during the verification of the sample by the organizing laboratory.

Conclusion and methodology recommendations

The analysis of highly degraded and difficult bone samples, such as archeological specimens, may not yield reliable results in all laboratories. Contamination may be a concern that should be investigated further. Perhaps development of a quality-controlled commercial kit could reduce some forms of contamination. Those laboratories with inconsistent results may consider that findings should not be reported unless they are confirmed by a second independent laboratory (44). Future collaborative exercises could include male skeletal remains and Y-chromosomal STR typing to further investigate performance among laboratories.

| Laboratory code | X-STRs          |          |          |          |          |          |          |          |
|-----------------|-----------------|----------|----------|----------|----------|----------|----------|----------|
|                 | AME DXS7132     | DXS7423  | DXS8378  | DXS10074 | DXS10079 | DXS10101 | DXS10103 | DXS10134 |
| 1               | X,X             | 12,13    | 14,14    | 10,12    | 14,17    | 19,21    | 28,23,22 | 17,18    | 36,41,3  | 25,32    | 30,40,2  | 27,1,271 | 12,14    |
| 3               | X,X             | 12,13    | 14,14    | 10,12    | 14,17    | 19,21    | 28,23,22 | 17,18    | 36,41,3  | 25,32    | 30,40,2  | 27,1,271 | 12,14    |
| 7               | X,X             | 12,13    | 14,14    | 10,12    | 14,17    | 19,21    | 28,23,22 | 17,18    | 36,41,3  | 25,32    | 30,40,2  | 27,1,271 | 12,14    |

| Laboratory code | Reported haplotype* |
|-----------------|---------------------|
| Sample 1        | HVR1 (range of sequencing) | HVR2 (range of sequencing) |
| 1               | 16293A/G 16311C [15989-052] | 195C, 263G [001-293, 317-460] |
| 3               | 16293A/G 16311C/T 1632C/T [15989-052] | 195C 263G [16533-619] |
| 4               | 16163T 16126C 16294T 16301C [16050-16400] | 73G [072-240] |
| 11              | 16093C 16189C 16270T [16000-16461] | 073G 146C 150T 263G [034-479] |
| 15              | 16293G 16311C [15990-16239, 16163-16391] | 195C [48-251, 164-465] |
| Sample 2        | HVR1               | HVR2               |
| 1               | 16304C 16311C [15975-042] | 152C 263G [16524-635] |
| 3               | 16304C 16311C [15989-052] | 152C 263G [16533-619] |
| 6               | 16304C 16311C [16128-16348] | 152C 263G [45-287] |
| 11              | 16304C 16311C [16000-16461] | 152C 263G [034-479] |
| 13              | 16304C 16311C [15978-16427] | 152C 263G [9-399] |
| 15              | 16304C 16311C [15990-16239, 16163-16391] | 152C 263G [48-251, 164-465] |
| 19              | 16304C 16311C [15989-052] | 152C 263G [16533-619] |

*The haplotypes obtained for the bone samples from the participating laboratories. Laboratory 3 reported problems with the read of the Sample 2 HVR2 sequence. Laboratory 6 reported interpretation difficulties for Sample 1 and therefore did not report the sequence data. Sample 2 provided consistent mtDNA results among the submitting laboratories; homopolymer stretches were not included in the comparison as these subregions are often not used in forensic analyses and interpretation varies among laboratories (21). There were a number of inconsistencies among the laboratories regarding mtDNA results for Sample 1. No consensus approach could be achieved with the data from Sample 1.
The first recommendation we may make regarding the methodology is that DNA analysis of human skeletal remains should adhere to ethical and data protection issues. Furthermore, laboratories should establish procedures for efficient reduction of possible sources of contamination, such as separated bone extraction area, forensic grade consumables, and cleaning of the laboratory areas. Laboratories should use an extraction procedure providing the highest DNA yield and purity, eg, possibly silica-based extraction chemistry. Laboratories should determine the quantity of extracted DNA using a human mtDNA specific quantitative real-time PCR assay that also includes at least two internal positive controls to evaluate the presence of inhibitors and degradation. Laboratories should include an additional step of PCR inhibitor removal for samples with detected inhibition. Last but not least, laboratories should use short amplicons for both STR and mtDNA typing for analysis of very challenging samples.

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Declaration of authorship DV, BB, and MP designed the Collaborative Exercise and drafted the manuscript. DV, BB, and MP critically revised the manuscript, and all authors gave the final approval of the manuscript. DV, JDV, and AA performed DNA analysis and interpreted the data. DV, BB, and MP critically revised the manuscript, and all authors gave the final approval of the manuscript. The data and analysis were performed by the participants of the exercises. The tasks required by the participating laboratories (all remaining authors) included sample collection, DNA extraction, removal of inhibitors, DNA quantitation, fragment analysis, mtDNA sequencing, and interpretation of the results.

Competing interests All authors have completed the Unified Competing Interest form at www.icmje.org/coi_disclosure.pdf (available on request from the corresponding author) and declare: no support from any organization in the submitted work; no financial relationships with any organizations that might have an interest in the submitted work in the previous 3 years; no other relationships or activities that could appear to have influenced the submitted work.

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