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ORIGINAL ARTICLE

Improved Y-STR typing for disaster victim identification, missing persons investigations, and historical human skeletal remains

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Abstract

Bones are a valuable source of DNA in forensic, anthropological, and archaeological investigations. There are a number of scenarios in which the only samples available for testing are highly degraded and/or skeletonized. Often it is necessary to perform more than one type of marker analysis on such samples in order to compile sufficient data for identification. Lineage markers, such as Y-STRs and mitochondrial DNA (mtDNA), represent important systems to complement autosomal DNA markers and anthropological metadata in making associations between unidentified remains and living relatives or for characterization of the remains for historical and archaeological studies. In this comparative study, Y-STR typing with both Yfiler™ and Yfiler™ Plus (Thermo Fisher Scientific, Waltham, MA, USA) was performed on a variety of human skeletal remains, including samples from the American Civil War (1861–1865), the late nineteenth century gold rush era in Deadwood, SD, USA (1874–1877), the Seven Years' War (1756–1763), a seventeenth-century archaeological site in Raspenava, Bohemia (Czech Republic), and World War II (1939–1945). The skeletal remains used for this study were recovered from a wide range of environmental conditions and were extracted using several common methods. Regardless of the DNA extraction method used and the age/condition of the remains, 22 out of 24 bone samples yielded a greater number of alleles using the Yfiler™ Plus kit compared to the Yfiler™ kit using the same quantity of input DNA. There was no discernable correlation with the degradation index values for these samples. Overall, the efficacy of the Yfiler™ Plus assay was demonstrated on degraded DNA from skeletal remains. Yfiler™ Plus increases the discriminatory power over the previous generation multiplex due to the larger set of Y-STR markers available for analysis and buffer modifications with the newer version kit. Increased haplotype resolution is provided to infer or refute putative genetic relationships.

Keywords Skeletal remains · Y-STR typing · Degraded DNA · Yfiler™ Plus · Lineage testing · Historical remains
Rapidly mutating Y-STRs

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Introduction

There are many scenarios encountered in forensic casework in which bone may be the only viable sample type for DNA testing, including fires, terrorist attacks, natural disasters, war conflicts, airline crashes, homicide, and mass graves from oppressive regimes [1–16]. In addition, skeletal remains often are the only samples available in historical and archaeological cases [17–22]. Skeletal remains are among the most challenging sample types for DNA testing due to prolonged exposure to a variety of environmental insults, including the effects of soil acidity. Humic and fulvic acids in soil damage DNA and, if co-purified, inhibit PCR amplification. Since DNA recovered from bone often is degraded and in low quantities, autosomal STR typing sometimes fails or results in partial profiles which may not be sufficient for rendering an

identification. In cases involving unidentified males, Y-chromosome lineage markers can serve to supplement autosomal STR results and anthropological metadata to increase statistical confidence in identification efforts.

Although the accuracy of identification of skeletal remains increases as the number of relatives typed increases, in some cases the number of reference samples available can be quite limited [23, 24]. One approach to improving the power of identification is to type additional markers [23–29]. Lineage-based Y-chromosome markers can provide additional data to support or refute putative familial relationships. In some cases, lineage markers may be the only informative markers for making associations between unidentified remains and living relatives [23, 30–32].

Two early generation kits, PowerPlex® Y (Promega Corporation, Madison, WI, USA) and Yfiler™ (Thermo Fisher Scientific, Waltham, MA, USA), contain reagents to simultaneously amplify 12 Y-STRs and 17 Y-STRs, respectively. Both of these kits contain the core Y-STR loci advocated by the Scientific Working Group on DNA Analysis Methods (SWGDAM) and the European minimal haplotype [33]. A considerable amount of effort has since been dedicated to identifying additional Y-STR loci that may increase the discriminatory power of Y-haplotype data, as well as be more effective for amplification of challenged samples [34–39]. Both PowerPlex® Y23 (Promega) and Yfiler™ Plus (Thermo Fisher Scientific) are enhanced Y-STR multiplex kits that may increase the typing capability of Y-STRs in bone samples.

The Yfiler™ Plus kit includes the 17 Y-STR markers from the original Yfiler™ kit plus 10 additional highly polymorphic markers (DYS449, DYS460, DYS481, DYS518, DYS533, DYS570, DYS576, DYS627, and DYF387S1a/b). Seven of the ten additional loci included in the Yfiler™ Plus kit are rapidly mutating Y-STRs (DYS449, DYS518, DYS570, DYS576, DYS627, and DYF387S1a/b) [40]. Several validation and population genetic studies have been performed using the Yfiler™ Plus kit [41–46], but none evaluating its ability to type DNA extracted from skeletal remains. Therefore, the study herein evaluated the efficacy of the Yfiler™ Plus kit for typing degraded DNA from human skeletal remains from the American Civil War (1861–1865) [17], the Black Hills Gold Rush in Deadwood, SD, USA (1874–1877) [18], the Seven Years' War (1756–1763) [47], a seventeenth-century archaeological site in Raspenava, Bohemia (Czech Republic), and World War II (1939–1945) [10]. Comparisons were made to the previous generation Yfiler™ kit on equal amounts of DNA from the same skeletal remains samples. Various performance criteria for each kit were assessed, including total number of alleles detected, total signal (RFU) per locus, total average signal across all loci, inhibitor tolerance, and success with amplification of larger Y-STR loci, the latter of which often drop out of the profile in challenged samples.

Materials and methods

Protocols for minimizing contamination during handling and processing of the skeletal remains used in this study were the same contamination controls standardly recommended for archaeological and ancient DNA specimens, including (a) use of protective suits, gloves, and masks; (b) bleach decontamination and UV-irradiation of work benches and associated equipment; (c) physical removal and/or chemical destruction of contaminant/exogenous DNA on external bone surfaces; (d) extraction of bone samples in a designated low-copy area; (e) PCR amplification in a location that is physically separated from the extraction area; (f) use of appropriate negative controls, reagent blanks, and positive controls; and (g) replicate testing [48–53].

Human skeletal remains

Historical bone samples included the 120-year-old skeletal remains (right femur, both tibiae) of an exhumed American Civil War soldier [17]; 140-year-old human skeletal remains from the Black Hills gold rush era, discovered by a construction crew in Deadwood, SD, USA [18]; a femur of a soldier from a mass grave in Liberec, Northern Bohemia, from the Battle of Reichenberg in 1757 between the Austrian and Prussian armies (a battle of the Seven Years' War) [47]; seventeenth century skeletal remains from an archaeological site in Raspenava, Bohemia (Czech Republic); and skeletal remains (femora) of three Finnish World War II soldiers [10].

Bone processing and DNA extraction

The diaphysis of each bone was surface-sanded with a Dremel® 4000 Rotary Tool and sterile grinding stone (Robert Bosch Tool Corporation, Mount Prospect, IL, USA), followed by sectioning with a Stryker® autopsy saw (Mopec, Oak Park, MI, USA). DNA extractions with 0.5–1.0 g bone powder were performed in a designated low-copy number area of the laboratory using three different methods, as described in [17].

DNA quantification

The quantity of DNA in each extract was determined using the Quantifiler® Trio DNA Quantification Kit (Thermo Fisher Scientific) and an ABI 7500 Real-Time PCR System. The assay was carried out in a 20-μl total reaction volume (18 μl Quantifiler® Trio master mix and 2 μl DNA extract). Sample concentrations were determined by comparison to a standard curve. A degradation index (DI) was generated for each sample, and the quantification value for the small autosomal target was used to calculate total DNA input for downstream PCR applications (per manufacturer recommendations) [54].

Amplification of Y-STR loci

Amplifications with Yfiler™ Plus and Yfiler™ kits were carried out according to the manufacturer's recommendations [40, 55]. Thermal cycling was performed with an ABI GeneAmp® 9700 PCR System (Thermo Fisher Scientific), and the same input quantity of DNA was used for both kits.

DNA separation, detection, and analysis

Amplified products were size-separated and detected on an ABI 3500xl Genetic Analyzer (Life Technologies) using 1 μl PCR product, 9.6 μl Hi-Di™ formamide, and 0.4 μl GeneScan™ 600 LIZ™ Size Standard v2.0 (Thermo Fisher Scientific). An allelic ladder was included at least once per injection on the 96-well plate. Samples were denatured at 95 °C for 5 min and then immediately cooled on ice for 5 min. Electrophoresis was performed on a 36-cm capillary array with POP-4™ polymer (Thermo Fisher Scientific) using standard injection parameters (1.2 kV, 24 s). STR data were sized and typed with GeneMapper® ID-X Software Version 1.4 (Thermo Fisher Scientific) and analyzed using a common threshold of 75 RFU for comparison of kit performance.

Results and discussion

DNA was not detected in any of the extraction reagent blanks or amplification negative controls. Positive controls (control DNA 007, human male) yielded the correct type in all Yfiler™ and Yfiler™ Plus reactions. A female analyst conducted all testing for each set of remains, including surface cleaning, bone grinding, DNA extractions, quantification, PCR amplifications, and genotyping. Male individuals involved in the exhumation or recovery of the remains and male laboratory personnel were excluded as contributors, supporting that the Y-STR haplotypes obtained were endogenous to the decedents. A common threshold of 75 RFU was used in this study as a basis to compare performance between the kits. However, internal validation studies should be performed to formally establish analytical thresholds prior to implementation of the Yfiler™ or Yfiler™ Plus assay into casework.

Total number of alleles detected: Yfiler™ Plus versus Yfiler™

Figure 1 summarizes the total number of alleles detected for each bone sample after amplification with the Yfiler™ Plus and Yfiler™ kits. Although 1 ng of input DNA is recommended for both Y-STR multiplexes, this target quantity was not available for the samples used in this study, a limitation commonly encountered with degraded skeletal remains. For this sample set, the range of input DNA was 0.100–0.827 ng. The total amount of input DNA, degradation index (DI), and total number of alleles detected for each bone sample is reported in Supplementary Table 1. Allele designations for the loci detected and in common between Yfiler™ and Yfiler™ Plus were concordant for all bone samples.

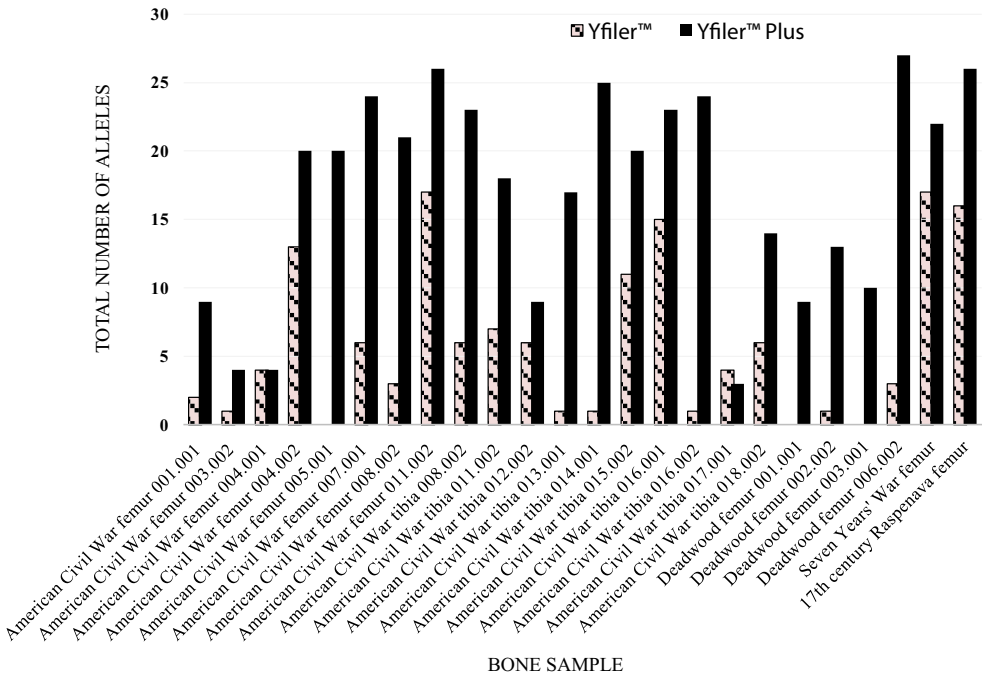

Fig. 1 Comparison of total number of Y-STR alleles recovered using the Yfiler™ and Yfiler™ Plus PCR amplification kits with the same quantity of input DNA (ng) from human skeletal remains from the American Civil War (1861–1865), the Black Hills Gold Rush era (1874–1877, Deadwood, SD, USA), the Seven Years' War (1756–1763), and a seventeenth century archaeological site in Raspenava, Czech Republic. Total input DNA and degradation indices (DIs) for each bone sample are reported in Supplementary Table 1

Table 1 Number of bone samples with amplification success at the largest Y-STR loci in common between the Yfiler™ and Yfiler™ Plus kits (total $n = 24$). Amplicon sizes provided by Lisa Calandro and Julio Mulero, Thermo Fisher Scientific (personal communication)

	Amplicon size		Number of bone samples with amplification success (n=24)	
Y-STR locus	Yfiler™	Yfiler™ Plus	Yfiler™	Yfiler™ Plus
DYS438	212–237 bp	212–237 bp	7	12
DYS635	246–270 bp	198–222 bp	11	18
DYS389II	254–294 bp	251–291 bp	5	13
DYS385a/b	243–315 bp	229–301 bp	6	15
DYS448	282–324 bp	282–324 bp	10	20
DYS392	294–327 bp	274–307 bp	4	11

Out of 18 bone cuttings tested (eight femur, ten tibia) from an American Civil War soldier (with a range of 0.100–0.440 ng input DNA), more alleles were detected for all samples using Yfiler™ Plus except for one femur sample (004.001) and one tibia sample (017.001). For the femur sample, the same number of alleles was detected after amplification with both Yfiler™ and Yfiler™ Plus. The minimal data obtained (i.e., four alleles) likely is a consequence of DNA degradation, as indicated by the degradation index (DI 8.39) for this sample, which was the highest among the cuttings from this set of remains. The latter sample (tibia 017.001) had a much lower DI (3.32), but minimal data also were observed, and slightly more alleles were detected using Yfiler™ compared to Yfiler™ Plus (i.e., four alleles and three alleles, respectively). Although the input quantities varied slightly among samples analyzed, there was not a strong correlation observed between DI values and typing success for these samples and the other remains tested in this study.

Four femur cuttings from the skeleton of a pioneer from the Black Hills Gold Rush era (1874–1877) were tested, all of which yielded a higher number of alleles with Yfiler™ Plus. The sample with the lowest DI (6.99) and greatest amount of input DNA (0.626 ng) yielded the most alleles. Moreover, there was a notable difference in typing success with this sample (as well as the others from this set of remains), resulting in a complete 27-locus Yfiler™ Plus Y-STR haplotype compared to recovery of only three alleles using the Yfiler™ kit.

The femur of a soldier from a battle of the Seven Years' War (1756–1763) yielded 17 and 22 Y-STR alleles with Yfiler™ and Yfiler™ Plus, respectively, even with less than half the recommended input DNA of 1 ng. For another set of remains from the Czech Republic, recovered from a seventeenth-century archaeological site in Raspenava, Bohemia, Yfiler™ Plus and Yfiler™ both performed well (16 alleles and 26 alleles, respectively). Detailed locus-by-locus data for each kit for each bone sample from the American Civil War, late nineteenth-century Deadwood, the Seven Years' War, and the seventeenth century archaeological site in Raspenava, Bohemia are reported in Supplementary Table 2.

Since the amount of sample from the skeletal remains of three Finnish World War II soldiers was limited, these bone samples were typed only with Yfiler™ Plus due to the increased recovery of Y-STR genetic data observed with this kit. With a DI as high as 13.15 and with less than 1 ng input DNA for each sample (0.381, 0.664, and 827 ng), all three World War II bone samples yielded a greater number of alleles than would have been possible with the previous generation Yfiler™ kit (19, 21, and 27 alleles, respectively, compared to a maximum of 17 Y-STRs with Yfiler™) (Supplementary Table 1). One sample (femur 2011-287-1163) yielded a complete 17-locus haplotype for the common loci amplified by both Yfiler™ and Yfiler™ Plus, as well as a full 27-locus Yfiler™ Plus profile. For the other two World War II bone samples (femur 2010-224-1548 and femur 2011-104-310), only 14- and 13-allele haplotypes were obtained (respectively) for the 17 common loci between the kits. Although these samples were not tested with the earlier generation Yfiler™ assay, the results using Yfiler™ Plus exceed the maximum possible haplotype with Yfiler™. The additional data recovered for these samples using Yfiler™ Plus (ten alleles, five alleles, and seven alleles, respectively) increases the discriminatory power for identification of these soldiers' remains. Detailed locus-by-locus Yfiler™ Plus data for each World War II bone sample are reported in Supplementary Table 3.

Performance with larger Y-STR loci

Although a multiplex with a greater number of loci increases discriminatory power, another important consideration for degraded samples is amplicon size and potential inhibition. Skeletal remains often contain DNA of limited quantity and compromised quality, both of which contribute to reduction or loss of signal at larger loci. In these types of samples, incomplete genetic profiles due to allele and/or locus drop-out are well documented [17, 18, 56–60]. Therefore, due to the degraded nature and/or quality of the bone samples used in this study, the performance of the seven largest loci that are common between the two kits (DYS385a/b, DYS389II, DYS392, DYS438, DYS448, DYS635) was compared (Table 1). Out of

Table 2 Comparison of the performance of Yfiler™ Plus versus Yfiler™ on the same set of bone samples using the same quantity of input DNA: (A) total number of alleles detected using the Yfiler™ kit only; (B) total number of alleles observed using Yfiler™ Plus that were common and concordant with Yfiler™ amplification results; (C) number of additional alleles observed for the 17 loci in common between the two kits (i.e., alleles observed using Yfiler™ Plus but not observed in Yfiler™ only results); and (D) number of additional alleles observed for loci included only in the Yfiler™ Plus kit ($n = 10$ loci)

Bone sample	A Total number of alleles observed (Yfiler™ only)	B Number of common alleles observed (Yfiler™ Plus)	C Number of additional Yfiler™ alleles observed using Yfiler™ Plus	D Number of additional alleles observed (Yfiler™ Plus loci only, $n = 10$)
American Civil War femur 001.001	2	2	3	4
American Civil War femur 003.002	1	0	2	2
American Civil War femur 004.001	4	0	1	3
American Civil War femur 004.002	13	12	2	6
American Civil War femur 005.001	0	0	14	6
American Civil War femur 007.001	6	5	11	8
American Civil War femur 008.002	3	2	12	7
American Civil War femur 011.002	17	17	0	9
American Civil War tibia 008.002	6	6	10	7
American Civil War tibia 011.002	7	6	7	5
American Civil War tibia 012.002	6	3	3	3
Aemrican Civil War tibia 013.001	1	1	10	6
American Civil War tibia 014.001	1	1	16	8
American Civil War tibia 015.002	11	9	5	6
American Civil War tibia 016.001	15	15	2	6
American Civil War tibia 016.002	1	1	16	7
American Civil War tibia 017.001	4	0	1	2
American Civil War tibia 018.002	6	5	4	5
Deadwood femur 001.001	0	0	7	2
Deadwood femur 002.002	1	1	8	4
Deadwood femur 003.001	0	0	6	4
Deadwood femur 006.002	3	3	14	10
Seven Years' War femur	17	16	0	6
Seventeenth century Raspenava femur	16	16	0	10

24 bone samples tested, higher success rates for the larger common loci were achieved with the Yfiler™ Plus kit compared to the Yfiler™ kit. These data suggest that the Yfiler™ Plus kit can overcome inhibition of the downstream assay better than the Yfiler™ kit.

Results for the remaining ten loci that are common between the two kits are reported in Supplementary Table 4. Because these loci are smaller, detection and typing success generally is greater in degraded samples. For these ten common loci, typable results were obtained for more bone samples using Yfiler™ Plus compared to Yfiler™ for every locus except DYS391 and DYS437. For the DYS391 locus, 14 bone samples yielded typable results with the Yfiler™ kit compared to only ten bone samples using the Yfiler™ Plus kit. This finding likely is due to a different primer set used during amplification with the Yfiler™ Plus multiplex, which results in a larger amplicon size for that marker (i.e., 353–377 bp compared to 152– 176 bp in Yfiler™). The opposite pattern was observed with the Y-GATA-H4 locus, in which the amplicon size in the Yfiler™ kit was much smaller than in the Yfiler™ Plus kit (122–142 bp compared to 227–247 bp, respectively), yet more bone samples yielded typable results with the Yfiler™ Plus kit. In the case of DYS437, results were obtained for an equal number of samples (19 out of 24 bones tested) with each kit.

Because the same DNA extracts and an equal amount of input DNA were used for amplification with each kit, the overall improved performance observed with Yfiler™ Plus compared to Yfiler™ on the same bone samples suggests that Yfiler™ Plus is more robust than its previous generation counterpart.

Sensitivity assessment: Yfiler™ Plus versus Yfiler™

In addition to increased discriminatory power and successful typing of larger Y-STR loci, an equal or improved level of sensitivity of detection is desirable for forensic casework. The low-signal (RFU) data encountered in analyses of bone

samples is an important consideration in assay selection. If addition of more loci to a multiplex reduces overall signal and performance, it may not be desirable for use with challenging casework samples. Even minimal reduction in signal across loci presents a risk of potentially losing valuable genetic data that could have otherwise been detected using a smaller, less discriminatory assay.

For the majority of bone samples tested (20 out of 24), Yfiler™ Plus performed the same or comparably (within one allele) on the common alleles observed from the Yfiler™ amplifications (Table 2, columns A–B). Allele designations were concordant between the two kits for all samples (Supplementary Table 2A). More importantly, for most of the samples (21 out of 24) undetected Yfiler™ alleles were recovered with Yfiler™ Plus amplifications (column C) as well as the ancillary benefit of detection of additional alleles with the expanded loci (column D). These observations further support that the Yfiler™ Plus kit is a more robust assay both in terms of sensitivity and in overcoming inhibition.

A general pattern observed was that samples which performed well with Yfiler™ also performed well with Yfiler™ Plus. However, some samples yielding poor results with Yfiler™ were substantially improved with Yfiler™ Plus. For example, although two of the Deadwood bone samples (femur 001.001, femur 003.001) yielded no typable data with Yfiler™, partial profiles of nine alleles and ten alleles, respectively, were obtained using Yfiler™ Plus. In another case, a complete 27-locus Y-STR haplotype was obtained using Yfiler™ Plus for a sample which yielded only three detectable alleles using Yfiler™ (Deadwood femur 006.002, Table 2, Supplementary Table 2). Furthermore, although complete 17-locus Yfiler™ profiles were obtained for two bones in this sample set (American Civil War femur 011.002, Seven Years' War femur), additional data was obtained when the samples were amplified using Yfiler™ Plus (nine additional alleles and five additional alleles, respectively), improving the discriminatory power.

In terms of signal intensity, there was a reduction in total average signal (RFU) per locus for some samples using Yfiler™ Plus, while signal was comparable between the two assays for other samples. For the Seven Years' War femur and the seventeenth century Raspenava archaeological femur, signal intensity decreased for almost every locus using Yfiler™ Plus compared to Yfiler™ (Supplementary Table 2B). However, the Yfiler™ Plus reactions generated additional genetic data for identification (six alleles and ten alleles, respectively; Supplementary Table 2A). For one of the American Civil War samples (femur 011.002), signal for all loci common between the two kits was comparable and alleles for five loci exclusive to the Yfiler™ Plus assay were detected (Supplementary Table 2). In another American Civil war bone sample (tibia 016.001), signal at 12 of the 17 common loci was increased using Yfiler™ Plus and was supplemented with detection of alleles at six of the ten additional loci included in the Yfiler™ Plus assay.

For the ten additional loci included in the Yfiler™ Plus multiplex, an average of six additional alleles was obtained across all bone samples (range 2–10 alleles, Table 2, column D). This data provides improved discriminatory power than could have been possible using the earlier generation Yfiler™ assay. The potential for increased data acquisition from the same quantity of DNA using Yfiler™ Plus warrants its consideration for use with challenged forensic samples types such as skeletal remains and bone fragments.

Value of rapidly mutating Y-STRs

Another important potential benefit of the Yfiler™ Plus kit relates to the assay's inclusion of rapidly mutating Y-STRs. Some studies have shown that rapidly mutating Y-STRs can increase the power of discrimination between unrelated males as well as between males of the same patrilineage [61–64]. In a study of 305 males from 127 separate familial pedigrees, the rapidly mutating Y-STR panel included in Yfiler™ Plus could distinguish between (1) 48.7% of fathers and sons, (2) 60% of brothers, and (3) 75% of male cousins. The discriminatory power of the previous generation Yfiler™ kit was considerably less with the same data set, at 7.7%, 8%, and 25%, respectively [62]. This increased ability to distinguish between related males could have considerable application for mass disaster scenarios in which multiple victims from the same family were traveling together, as well as for mass graves from past war conflicts (which may contain related male soldiers) and for mass graves containing multi-generational victims of oppressive regimes.

Although the rapidly mutating loci included in the Yfiler™ Plus kit could help improve the ability to discriminate between closely related male victims within a mass grave or male relatives killed in the same mass disaster, there is a potential additional complication that should be considered. The rapid mutation rate that provides increased discriminatory power between males of the same patrilineage also increases the possibility of detecting differences between male victims and reference samples of living relatives. This will make kinship testing with rapidly mutating Y-STRs more challenging. Mutations could result in false exclusions or the resultant likelihood ratio may be so diminished that the Y-STR data may not be informative. This must be taken into account when making an association or identification.

Conclusion

Y-STRs provide a valuable addition to other tools used (e.g., autosomal STRs, anthropological analyses) in identifying male skeletal remains. The Yfiler™ Plus multiplex of 27 Y-STRs offers greater power of discrimination than the previous generation 17-locus Yfiler™ kit as well as an overall increased robustness. A previous study with two models of inhibition, humic acid and

hematin, demonstrated that the Yfiler Plus™ kit was more robust to inhibition than the Yfiler™ kit [41]. Furthermore, the design of the Yfiler™ Plus assay, with inclusion of eleven mini Y-STR loci (< 220 bp), facilitates successful amplification of degraded templates [40, 41]. This study demonstrates that the improved polymerase and buffer systems in Yfiler™ Plus result in better performance on degraded DNA from human skeletal remains than the earlier generation assay. The skeletal remains used in this study were recovered from a variety of geographic locations, including both eastern and western regions of the United States, Russia, and the Czech Republic. The diverse environments to which the remains were exposed likely resulted in varying degrees of DNA damage as well as different combinations of lesions, each of which posed a unique challenge for Yfiler™ and Yfiler™ Plus chemistry. The results demonstrate the efficacy of Yfiler™ Plus on a diverse sample set of bones, which can be some of the most difficult samples for forensic analyses.

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