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Research Paper

Evaluating the efficacy of three Y-STRs commercial kits in degraded skeletal remains

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ABSTRACT

Y chromosome short tandem repeats (Y-STRs) typing is a useful tool in scenarios such as mass graves analysis or disaster victim identification and has become a routine analysis in many laboratories. Not many comparisons have been performed with the currently available commercial kits, much less with degraded skeletal remains. This research aims to evaluate the performance of three commercial Y-STR kits: Yfiler™ Plus, PowerPlex® Y23, and Investigator® Argus Y-28 in 63 degraded skeletal remains from mass graves. PowerPlex® Y23 yields more reportable markers and twice the RFU on average, while Yfiler™ Plus and Investigator® Argus Y-28 exhibited a similar behaviour. Additionally, Argus Y-28, which has not been tested with this kind of samples in literature before, showed a good performance. Finally, a predictive model was attempted to be developed from quantification and autosomal STR data. However, no acceptable model could be obtained. Nevertheless, good Y-STR typing results may be expected if at least 50 pg DNA input is used or 13 autosomal markers were previously obtained.

1. Introduction

Y chromosome short tandem repeats (Y-STRs) typing has been demonstrated to be of great importance in various applications, including human identification by paternal lineage, paternity testing, differentiation of male relatives, geographic origin inference, and molecular anthropology studies [1]. It has also been used in sexual assault cases [2] and historical and archaeological case studies [3,4]. The forensic use of Y-STRs has been validated by the International Society for Forensic Genetics (ISFG) [5,6], and it has become a routine technique in forensic laboratories [7]. Furthermore with the advent of massive parallel sequencing platforms, it is now possible to analyze up to 100 Y-STR loci, as well as Y-chromosomal single nucleotide polymorphisms (Y-SNPs) [8]. The utility of Y-STRs has been demonstrated in disaster victim identification and mass graves scenarios, especially when only distant relatives sharing paternal lineage are available for comparison, thus enhancing the identification success rate when combined with autosomal STRs [9,10].

This approach has been particularly valuable for our laboratory, which focuses on identifying the victims of the Spanish Civil War (1936–1939) and the postwar period buried in Andalusia, as part of the agreement between the Andalusian Regional Government and our University for the identification of the victims found in mass graves. To date, we have received 3303 individual remains, and 2085 buccal swabs from the relatives of the victims. The DNA extracted from these skeletal remains is highly degraded, a phenomenon well-documented in scientific literature and attributed to environmental conditions such as temperature [11], humidity, and pH [12]. As a result, the DNA is subject to molecular damage and fragmentation [13] leading to the generation of partial autosomal STR profiles. However, the analysis of Y-STRs has proven valuable in strengthening the results of our identifications, even allowing for the differentiation of the degree of consanguinity through the use of rapid mutation markers (RM-Y-STRs), as previously noted in literature [14].

Commercial Y-STR typing kits have evolved similarly to autosomal STR kits, with an increase in the number of loci due to advances in capillary electrophoresis. The first commercial kits were launched during the early 2000 s and included 12–16 loci [15]. In recent years, advancements in dyes incorporation and primer design have allowed these kits to expand, resulting in the currently available Yfiler™ Plus

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(Thermofisher, Waltham, MA, USA), PowerPlex® Y23 System (Promega, Madison, WI, USA), and Investigator® Argus Y-28 QS (QIAGEN, Hilden, Germany). The differences among these three kits are shown in Table 1.

Not many comparisons have been performed among the different commercial kits, with these few studies primarily focusing on sexual assault casework samples [16], or NIST DNA profiling standard reference material [15]. However, both Yfiler™ Plus [17,18] and PowerPlex® Y23 [19] have been successfully tested on degraded skeletal remains. In contrast Investigator® Argus Y-28 QS has not yet been evaluated with this type of samples. Furthermore, no systematic study has attempted to predict Y-STR typing success from autosomal STR typing profiles or quantification data.

This study stands out in the field of forensic genetics as a pioneering exploration into the comparative analysis of the currently available Y chromosome Short Tandem Repeats (Y-STRs) commercial kits, focusing on the analysis of degraded skeletal remains from mass graves. With a comprehensive assessment of three leading commercial Y-STR kits (Yfiler™ Plus, PowerPlex® Y23, and Investigator® Argus Y-28 QS), it not only unveils the performance differentials among them but also introduces Investigator® Argus Y-28 as a previously untested yet robust contender for this challenging scenario. Moreover, the endeavour to create a predictive model by a correlation matrix and multiple regression from quantification and autosomal STR data, though ultimately inconclusive, showcases a novel approach to improve Y-STR typing outcomes and to economize forensic laboratories resources, providing valuable insights into the ever-evolving field of forensic DNA analysis.

2. Material and methods

All procedures were carried out in a low copy number (LCN) DNA facility, following international standards for ancient DNA work [20,21]. Contamination prevention measures included working in a clean room equipped with room UV light and HEPA filtered air under positive pressure. Surfaces were cleaned with bleach and DNAZap™, we also used sterile labware, negative extraction control, and staff genotyped to ensure minimal contamination. Quantification data (low DNA concentration in samples), degradation index (higher small DNA target detection), and 'ski-slope' profiles (indicating fragmentation of the DNA) were used as indicators of potential contamination.

2.1. Samples preparation

A total of 63 degraded skeletal samples (see Table 2) from mass graves in Andalusia (Southern Spain) were analysed. These samples were buried in mass graves at depths of 3–4 m, located in the Western region of Andalusia, for a period of 70–80 years. The climate in this region is characterized by extreme temperatures, with an average of 28 °C and maximum temperatures reaching 45 °C in summer, and minimum temperatures of 13 °C in winter. Rainfall is scarce with an average of 500 mm per year [22]. The soil in the area is acidic [23].

Following anthropological studies, the samples were sanded using a Dremel® rotatory tool, and the bones were cut into small pieces. Fragments and teeth were then pulverized using a TissueLyser II (QIAGEN, Hilden, Germany).

Table 2
Analyzed skeletal remain samples.

Skeletal remain	Tooth	Femur	Tibia	Ulna	Total
N	30	21	10	2	63

2.2. DNA extraction

DNA was extracted from the samples using an in-house protocol based on the phenol/chloroform/isoamyl alcohol organic extraction method [24]: 5 mL of extraction buffer (EDTA 0.5 M, SDS 10 %, proteinase K 10 mg/mL and DTT 1 M) was added to 1.0 g bone or tooth powder and incubated at 56 °C overnight, the lysate was mixed with phenol/chloroform/isoamyl alcohol (25:24:1) and the supernatant was concentrated in an Amicon Ultra-4 Centrifugal Filter Unit 30 kDa (Merck KGaA). Extracts were then purified with MinElute PCR Purification Kit (QIAGEN).

2.3. DNA quantification

The purified DNA extracts were quantified using the Quantifiler™ Trio on a QuantStudio™ 5 instrument (ThermoFisher), following the manufacturer's recommendations [25]. In general terms, the DNA concentration of the small target of the majority of the analysed samples ranged from 0.001 to 0.030 ng/μl, while the degradation index ranged from 2 to 25 (more than 40 in some of the samples).

2.4. DNA amplification

For DNA amplification, the samples were processed using the GlobalFiler™ kit following the manufacturer's recommendations [26]. A 15 μL DNA extract input was used. After confirming the sex of each sample with the amelogenin marker, every DNA extract was amplified using three different Y-STR amplification commercial kits: Yfiler™ Plus [27] (ThermoFisher), PowerPlex® Y23 [28] (Promega), and Investigator® Argus Y-28 QS [29] (QIAGEN). The amplifications for each kit were performed according to the manufacturer's specifications, and the same quantity of DNA extract input (10 μL DNA extract) was used in all three kits to ensure homogeneity. Following amplification, the fragments were analysed by capillary electrophoresis in an ABI 3500 instrument (ThermoFisher), following manufacturer's instructions.

2.5. Data analysis

The raw data was analysed using GeneMapper™ IDX v1.4. Statistical analyses, including mean, standard deviation, coefficient of quartile variation [30], Shapiro-Wilk p, One-Way ANOVA (Welch's), One-Way ANOVA (Fisher's), homogeneity of variances test (Levene's), effect size measurement (eta-squared, η^2) Games-Howell Post-hoc test, correlation matrix and multinomial regression, were performed using jamovi 2.2.5 [31].

3. Results and discussion

The genetic profile results of each sample and each kit are presented in Fig. 1; which includes the number of alleles with peak height higher than the analytical threshold (>AT) (set at 100 RFU after internal

Table 1
Y-STRs commercial kits comparison.

Kit	Loci	RMs*	Dyes	Cycles	Recommended DNA input (ng)	Maximum DNA input (μL)
Yfiler™ Plus	25	7	6	30	0.5	10
PowerPlex® Y23	22	2	5	30	0.5	17.5
Investigator® Argus Y-28 QS	26	6	6	30	0.5	15

* Rapidly Mutating Y-STR markers.

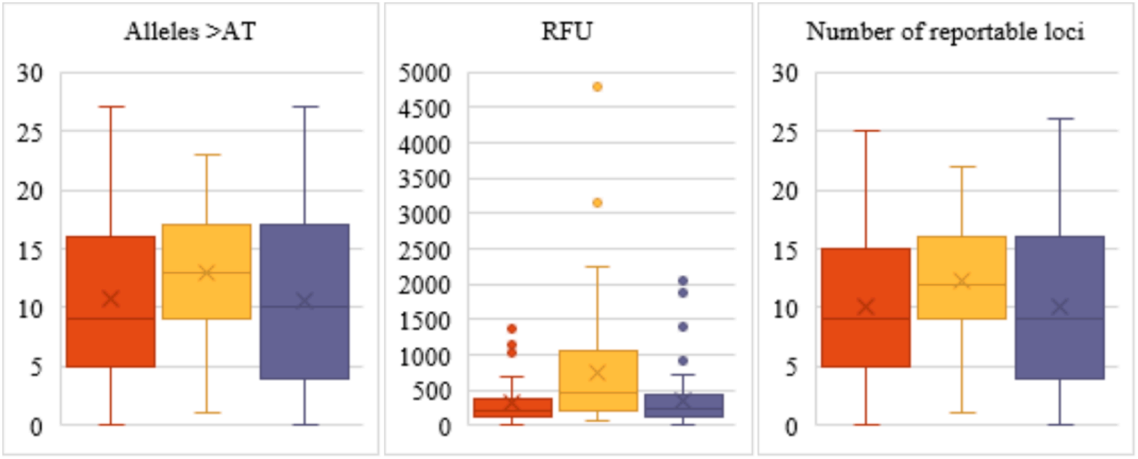

Fig. 1. Boxplots of alleles that exceeded the analytical threshold (>AT), relative fluorescence units (RFU), and reportable loci for each Y-STR commercial kit.

validation), average relative fluorescence units (RFU), average peak height ratio (PHR), and the number of reportable loci (defined as alleles that surpass the 100 RFU analytical threshold or, in the case of biallelic markers with only one amplified allele, 370 RFU stochastic threshold).

PowerPlex® Y23 was the commercial kit that detected the highest number of alleles on average (13) followed by Yfiler™ Plus and Argus Y-28 (11). However, the average peak height ratio in biallelic markers was approximately the same for all three kits (0.7). PowerPlex® Y23 achieved around twice the average RFU (750), which ultimately resulted in this kit having the highest number of reportable markers on average (12). Furthermore, it exhibited the least variation in the number of detected alleles and reportable loci (see Fig. 1), indicating that this kit achieves greater precision. It should be noted that the same volume of DNA input (10 μl) was used for the normalization of the experiment, however, PowerPlex® Y23 allows 17.5 μl of DNA input, so even more positive results may be expected if the maximum DNA input volume is used.

Both Yfiler™ Plus and Argus Y-28 demonstrated similar performance, achieving approximately the same number of reportable alleles, with similar average RFU values, leading to nearly the same number of reportable loci. This result suggests that Argus Y-28 may be a suitable option for DNA analysis from degraded skeletal remains.

Data were found to not be normally distributed (Shapiro-Wilk test p-value < 0.05), so non-parametric analyses were conducted. One-way ANOVA was used with the commercial kit as the grouping variable. To check for variance homogeneity, Levene's test was applied. For variables with non-homogeneous variance (>AT, RFU, and Loci), Welch's one-way ANOVA and Games-Howell post-hoc test were used, while for variables with homogeneous variance (PHR, %Reportable profile), Fisher's one-way ANOVA and Tukey post-hoc test were applied. The results of one-way ANOVA showed statistically significant differences only in RFU (p-value < 0.001, $F=7.65$, $df2 = 113.3$, $\eta2 = 0.111$, suggesting a medium effect size), and percentage of reportable profile (p-value < 0.001, $F=7.24$, $df2 = 186$, $\eta2 = 0.072$, indicating a medium effect size). Post-hoc tests revealed that PowerPlex® Y23 showed statistically significant difference in RFU compared to Yfiler™ Plus (p-value < 0.001, t-value $= 3.923$, df $= 77.8$), and Argus Y-28 (p-value $= 0.003$, t-value $= 3.37$, df $= 87.6$), as well as in percentage of reportable profile compared to Yfiler™ Plus (p-value $= 0.006$, t-value $= 3.097$, df $= 186$) and Argus Y-28 (p-value $= 0.002$, t-value $= 3.46$, df $= 186$). This is understandable considering the marked difference in the number of detected alleles (>AT) among the kits, and the percentage of reportable markers helps to normalize this parameter. It is worth noting that PowerPlex® Y23 has 5 dyes instead of 6, resulting in in 3–4 fewer markers. Yfiler™ Plus and Argus Y-28 showed no statistically significant differences between them in any of the analyzed parameters.

As mentioned earlier, there are not many systematic comparisons among these three kits in scientific literature. However, one study positions PowerPlex® Y23 as the most sensitive, particularly with low quantities of DNA [16], while another comparison conducted by QIAGEN found that Argus Y-28 recovered more alleles in the presence of inhibitors such as calcium or humic acid [32]. The usefulness of Argus Y-28 quality sensors is noteworthy as they allow for checking if a negative result is due to sample nature or the presence of inhibitors/purification failure, which is an important advantage when typing these types of samples.

In Fig. 2 the performance of each locus by commercial kit is displayed in a heat map. All the alleles were concordant between kits, and no drop-in was observed. This visualization also helps to explain the observed differences between PowerPlex® Y23 and the other kits as more markers of each channel are successfully amplified. This suggests that PowerPlex® Y23 may be less prone to DNA degradation. If a 250 bp threshold is set, PowerPlex® Y23 has the lowest number of markers in that amplicon size range. The inhibition problem can be ruled out since the extraction process includes a purification step, and any inhibitor would have been detected in the quantification step (IPC, considering its reported limitations with certain inhibitors such as calcium [33]) or by Argus Y-28 quality sensors. The difference may be attributed to primer design. Additionally, it is worth noting that PowerPlex® Y23 shows less allelic drop-out in biallelic markers than the other two kits. Regarding the amplification of rapidly mutating markers, it should be noted that their number vary among the three commercial kits as shown in Table 1. If we define the success rate as the ratio of successfully amplified RM loci to the total number of RM loci in the kit, then the results are as follows: Yfiler™ Plus has a success rate of 35 % and Investigator Argus Y-28 has a rate of 38 %, while Promega's Y23 achieves a rate of 90 %. However, this commercial kit contains only 2 RM Y-STRs, which may be insufficient for the analysis of closely related males.

The power of combining two or more kits if a partial result is obtained should also be considered, as previously stated [34]. For example, if only the first two markers of each channel are successfully amplified (approximately a 250 bp threshold), using one kit as the primary Y-STR kit and complementing it with another kit as a complementary approach could confirm many markers and potentially add new ones to a pooled profile [35], thereby increasing the statistical strength of the result. For instance, if Yfiler™ Plus is used as the main Y-STR kit and is complemented by PowerPlex® Y23, and 10 markers are successfully amplified with the former, 5 markers will be confirmed, and 3 more markers may be added to the eventual consensus profile.

Another objective of this study was to assess if it is possible to predict an eventual Y-STR typing success based on quantification or autosomal STR amplification parameters. Three quantification parameters

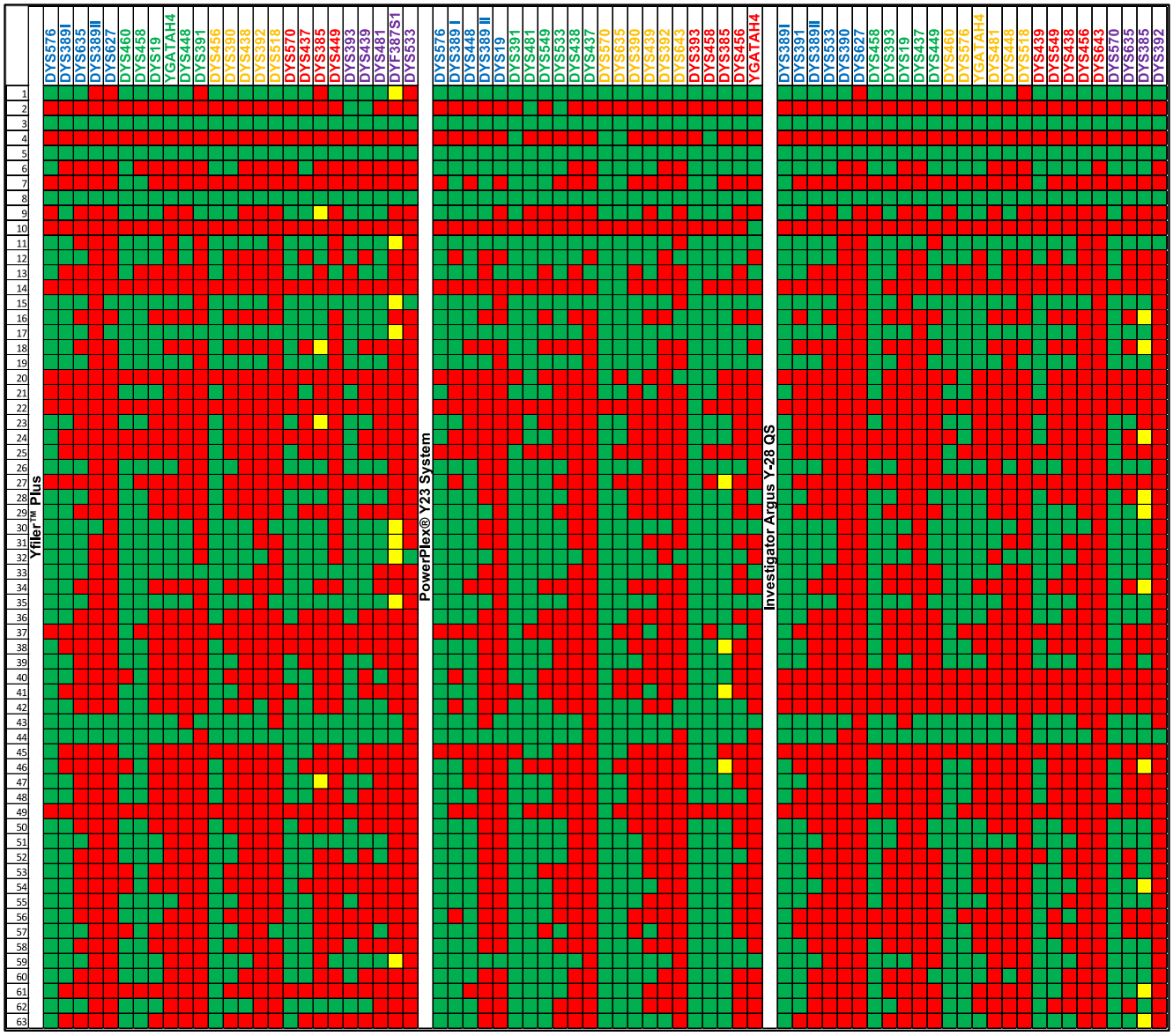

Fig. 2. Heat map of the three tested commercial kit by sample (each square of y-axis) and by marker. Green squares represent successfully amplified markers, red squares indicate failed markers, and yellow squares represent biallelic marker in which allelic drop-out could not be ruled out. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(detected small human DNA target, large human DNA target, and human male target) and four autosomal STR profile parameters (number of detected alleles considering a 50 RFU threshold, number of alleles considering a stochastic threshold of 365 RFU – both thresholds obtained after internal validation –, average RFU of the sample, and the number of autosomal STR reportable loci achieved by each sample) were considered as predictors. The variable to be predicted was the number of successfully amplified markers by each commercial kit. Table 3 shows the quantification clusters and Table 4 displays the DNA profiles. A threshold of 10 loci was established in order to consider a Y-STRs profile reportable, following the ISFG recommendations for disaster victim identification [36].

If quantification data is considered, approximately 50 % of samples will achieve a partial Y-STRs profile with, at least, 0.005 ng/μl as DNA input, while almost all results are unsuccessful if less concentration is used, indicating a sensitivity threshold of 0.05 ng (total DNA amount in 10 μl input). This success percentage increases to 80 % if the PowerPlex® Y23 kit is used, supporting previous comparison that suggest this kit is more sensitive [16]. Using the number of successfully amplified markers by Globalfiler™ as an indicator of Y-STR typing success (at least 10 Y-STR loci), acceptable results can be obtained for samples that achieved at least 11–15 autosomal STR markers. This interval is reduced to 6–10 in the case of using PowerPlex® Y23.

A correlation matrix was created with all these variables, and they all showed significant correlations (p-value < 0.001. As expected, a positive correlation was found between the quantity of DNA and the number of loci successfully amplified. However, no correlation was observed between the degradation index and the number of reportable loci in any kit, with the exception of the Yfiler™ Plus kit, p-value $= 0.046$), No Pearson's r higher than 0.8 was obtained. Despite trying multinomial logistic regression with all variables and subsequently removing and adding them based on the improvement or worsening of the coefficient of determination (R^2) no acceptable model could be obtained. The best R^2 values achieved were 0.565 (Yfiler™ Plus loci prediction), 0.597 (PowerPlex® Y23), and 0.641 (Argus Y-28). In conclusion, no acceptable regression model was attainable.

Methodologically, the same DNA input was used for each Y-STR commercial kit, ensuring the same DNA quantity was amplified for all.

Table 3
Small, large, and male human DNA quantification clusters and the percentage of profiles of each group that achieve a minimum number of 10 reportable loci, for each Y-STRs commercial kit.

Quantifiler™ Trio Small DNA (ng/μL)	Yfiler™ Plus (≥10 loci)	PowerPlex® Y23 (≥10 loci)	Argus Y-28 (≥10 loci)
[0–0.005] (n = 14)	0 %	14 %	0 %
(0.005–0.05] (n = 38)	47 %	82 %	50 %
(0.05–0.1] (n = 7)	86 %	100 %	86 %
>0.1 (n = 4)	100 %	100 %	100 %
Quantifiler™ Trio Large DNA (ng/μL)	Yfiler™ Plus (≥10 loci)	PowerPlex® Y23 (≥10 loci)	Argus Y-28 (≥10 loci)
[0–0.005] (n = 45)	27 %	60 %	29 %
(0.005–0.05] (n = 15)	93 %	100 %	93 %
(0.05–0.1] (n = 2)	100 %	100 %	100 %
Quantifiler™ Trio Male DNA (ng/μL)	Yfiler™ Plus (≥10 loci)	PowerPlex® Y23 (≥10 loci)	Argus Y-28 (≥10 loci)
[0–0.005] (n = 13)	15 %	23 %	8 %
(0.005–0.05] (n = 40)	45 %	80 %	50 %
(0.05–0.1] (n = 6)	83 %	100 %	83 %
>0.1 (n = 3)	100 %	100 %	100 %

Table 4
Percentage of samples with reportable loci obtained by the Globalfiler™ autosomal STR kit and, within that group, the number samples (in percentage) that achieve at least 10 Y chromosome STRs in each commercial kit.

Globalfiler™ reportable loci	Yfiler™ Plus (≥10 loci)	PowerPlex® Y23 (≥10 loci)	Argus Y-28 (≥10 loci)
0–5 (n = 10)	10 %	10 %	10 %
6–10 (n = 16)	6 %	50 %	19 %
11–15 (n = 15)	60 %	100 %	53 %
16–21 (n = 22)	77 %	91 %	77 %

Additionally, each manufacturer's thermal cycling and injection parameters were applied to compare the commercial kit in their native conditions, as they have been validated for these specific parameters.

4. Conclusion

Y-STRs typing is a widely used approach in forensic genetics laboratories due to its advantages in paternal lineage and molecular anthropology applications. In contexts such as disaster victim identification or mass graves, it proves to be a useful tool for identifications, especially when combined with autosomal STR analysis or in cases where only distant family members are available for comparisons.

The research aimed to compare the efficiency of three Y-STRs commercial kits with degraded skeletal remains, evaluate the applicability of the newly developed Investigator® Argus Y-28 with these challenging samples, and establish possible indicators or predictors of Y-STR typing success.

The results suggest that Investigator® Argus Y-28 performs well with degraded skeletal remains, almost on par with the results the results achieved with Yfiler™ Plus. In comparison of the three kits, PowerPlex® Y23 showed the best performance, with twice the average RFU, and two more reportable loci on average. The comparison of successfully amplified markers suggests that PowerPlex® Y23 is less susceptible to DNA degradation, and more sensitive possibly due to its primers design. Finally, at least partial Y-STR profiles can be obtained with 0.05 ng DNA input or if 13 markers are successfully amplified with the Globalfiler™ kit. However, no satisfactory regression model could be obtained in the attempt to predict Y-STR typing success.

5. Finding source

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Ethical statement

Human bones used for this study were obtained as part of the agreement between the University of Granada and the Regional Government of Andalusia (Spain) for the genetic identification of the victims of the Spanish Civil War and postwar period (OTRI-6260). This research adhered to the legal requirements set forth by both institutions.

CRediT authorship contribution statement

Christian Haarkötter: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft. **María Isabel Medina-Lozano:** Validation, Investigation. **Diana C. Vinueza-Espinosa:** Software, Validation, Data curation, Formal analysis, Writing – review & editing. **María Saiz:** Software, Validation, Data curation, Formal analysis, Writing – review & editing. **Xiomara Gálvez:** Validation, Investigation. **Juan Carlos Álvarez:** Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration. **José Antonio Lorente:** Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scijus.2024.07.007.

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