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Digging up the recent Spanish memory: genetic identification of human remains from mass graves of the Spanish Civil War and posterior dictatorship

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ABSTRACT

The Spanish Civil War (1936–1939) and posterior dictatorship (until 1970s) stands as one of the major conflicts in the recent history of Spain. It led to nearly two hundred thousand men and women executed or murdered extra-judicially or after dubious legal procedures. Nowadays, most of them remain unidentified or even buried in irretraceable mass graves across Spain. Here, we present the genetic identification of human remains found in 26 mass graves located in Northern Spain. A total of 252 post-mortem remains were analyzed and compared to 186 relatives, allowing the identification of 87 victims. Overall, a significant success of DNA profiling was reached, since informative profiles (≥12 STRs and/or mitochondrial DNA profile) were obtained in 85.71% of the remains. This high performance in DNA profiling from challenging samples demonstrated the efficacy of DNA extraction and amplification methods used herein, given that only around 14.29% of the samples did not provide an informative genetic profile for the analysis performed, probably due to the presence of degraded and/or limited DNA in these remains. However, this study shows a partial identification success rate, which is clearly a consequence of the lack of both appropriate family members for genetic comparisons and accurate information about the victims' location. Hence, further perseverance in the exhumation of other intact graves as well as in the search of more alleged relatives is crucial in order to facilitate and increase the number of genetic identifications.

1. Introduction

DNA analysis has become a key tool for identifying victims from massacres, particularly in cases in which conventional forensic methods (i.e. radiology, anthropology, or odontology) are not enough discriminative.

DNA isolated from skeletal samples offers the possibility of determining the identity of unknown post-mortem remains by comparative genetic analyses with their alleged biological relatives. In the past, mitochondrial DNA (mtDNA) analysis was the first choice -and sometimes the only alternative-for successful DNA profiling from skeletal remains, due to its high copy number per cell, resistance to degradation, and high mutation rate [1,2]. Because of its maternal inheritance pattern, this analysis was restricted to cases where maternal relatives were available. Fortunately, nowadays the development of more efficient DNA extraction methods, as well as more sensitive marker panels, enables to obtain nuclear DNA profiles from degraded remains [3,4]. Indeed, the study of autosomal short tandem repeats (STRs) is currently the preferred technology for identification purposes because of its simplicity, sensitivity, and high discrimination power [5]. The implementation of STRs of the Y-chromosome (Y-STRs), which reveals the paternal lineage, becomes particularly important for cases in which only male relatives are available for genetic comparisons [6]. Additionally, the study of X-chromosome STRs (X-STRs) has turned into a very useful complement of autosomal STRs in complex kinship analyses, due to its particular inheritance, which differs in male and female descendants [7]. On the other

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hand, the application of the analysis of reduced size amplicons, known as mini-STRs, has increased the success in typing challenging samples [8].

For the last decades, DNA analyses have played a major role in victim identification of natural disasters such as the Asian tsunami disaster in 2004 and Hurricane Katrina in 2005 [9]. Likewise, DNA profiling was fundamental in the identification of missing persons from terrorism attacks including the World Trade Center mass fatality in September 2001 [10] and Madrid in March 2004 [11]; dictatorship victims from Chile [12], Argentina [13] or Guatemala [14] in the 1970s and 1980s; or from war conflicts such as the Balkans in the 1990s [15,16] or more recent conflicts in Iraq or Libya; and even soldiers from the II World War [6].

Spain is the second country in the world, after Cambodia, in number of enforced disappearances whose remains in mass graves have not been recovered or identified [17]. During the Spanish Civil War (1936–1939) and the posterior dictatorship period (until 1970s), nearly 200,000 men and women were executed or murdered extra-judicially or after dubious legal procedures [18]. More than 300 mass graves and 5000 skeletons have been already exhumed across the Spanish territory [19] , and some of these victims have been identified by multidisciplinary studies [20–23]. However, this barely represents 15% of the estimated 2000 burial sites; consequently, there is still a vast reservoir of remains and thousands of families who keep searching for the fate or whereabouts of their relative ones who disappeared during that inauspicious period.

Here we present the results of our experience of the last six-years (2010–2015) in genetic identification of human remains recovered from Spanish Civil War mass graves in the north of Spain.

2. Materials and methods

A total of 252 human remains recovered from 26 graves of the Spanish Civil War and posterior dictatorship, located in the north of Spain, were analyzed. More specifically, the remains of the following graves were studied (Fig. 1): La Tejera (Alava) ($n = 1$), Intxorta (Gipuzkoa) ($n = 3$), Ziardamendi (Gipuzkoa) ($n = 4$), Etxaguen (Alava) ($n = 15$), and Bóveda (Alava) ($n = 1$) in the Basque Country; Ezkaba ($n = 3$), Añezcar ($n = 1$), Berriosuso ($n = 1$), Aibar ($n = 3$), Corella ($n = 2$), Tudela ($n = 1$), Urzante ($n = 21$), Sima del Raso de Urbasa ($n = 10$), Peralta ($n = 1$), and Elia ($n = 3$) in Navarre; Santa Eulalia de Gállego ($n = 13$), Movera ($n = 4$), and Vera de Moncayo ($n = 10$) in Aragon; La Pedraja (Burgos) ($n = 103$), Loma de Montija (Burgos) ($n = 24$), Espinosa de los Monteros (Burgos) ($n = 9$), Sorriba (Burgos) ($n = 4$), Villamediana (Palencia) ($n = 3$), Ágreda (Soria) ($n = 3$), and Barcones (Soria) ($n = 6$) in Castile and Leon, and Vilarmaior ($n = 3$) in Galicia. Prior to genetic analysis, anthropological studies were carried out in order to individualize the skeletal remains and obtained basic biological data on sex, age-at-death, stature, ante mortem fractures, or possible cause of death, among other data.

In order to avoid contamination, recommendations suggested for work with ancient DNA (aDNA) were followed [24,25]. These include physically isolated work area laboratories for skeletal remains and reference samples, quantitation studies, and contamination monitoring in all steps using blank samples and negative controls.

For genetic analysis, long bones (i.e. femur, tibia or humerus) or teeth were preferably sampled. A 5 to 10 cm fragment was taken from each bone, and in the case of teeth, the complete piece was used. Prior to DNA extraction, bone surface was cleaned by physical

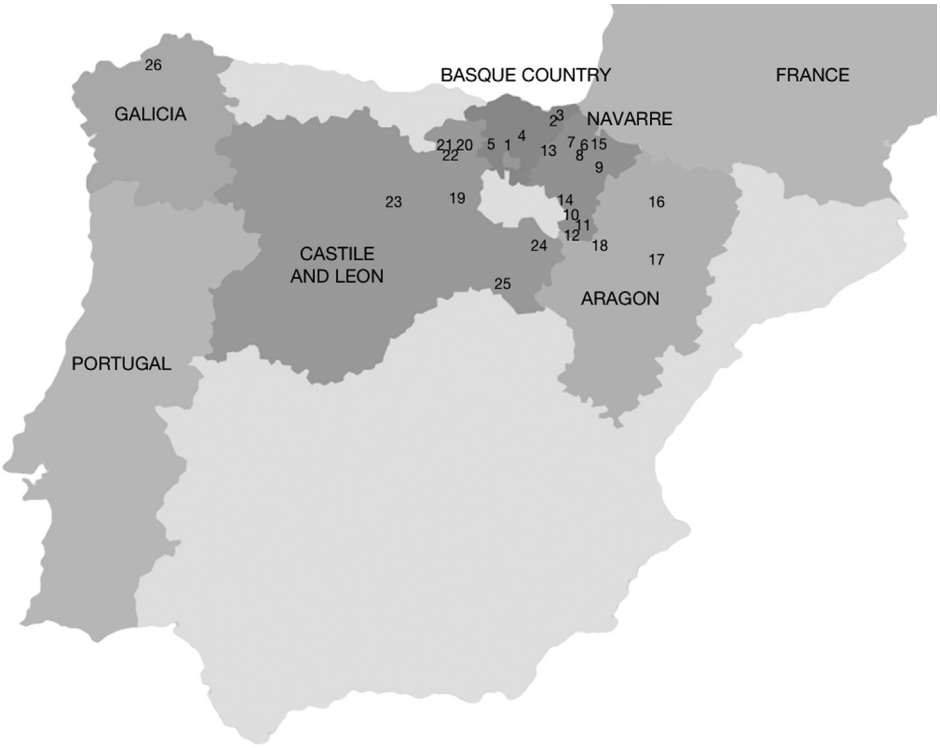

Fig. 1. Map of the mass graves under study from Spanish Civil War and posterior dictatorship. Basque Country: (1) La Tejera (Alava); (2) Intxorta (Gipuzkoa); (3) Ziardamendi (Gipuzkoa); (4) Etxaguen (Alava); (5) Bóveda (Alava). Navarre: (6) Ezkaba; (7) Añezcar; (8) Berriosuso; (9) Aibar; (10) Corella; (11) Tudela; (12) Urzante; (13) Sima del Raso de Urbasa; (14) Peralta; (15) Elia. Aragon: (16) Santa Eulalia de Gállego; (17) Movera; (18) Vera de Moncayo. Castile and Leon: (19) La Pedraja (Burgos); (20) Loma de Montija (Burgos); (21) Espinosa de los Monteros (Burgos); (22) Sorriba (Burgos); (23) Villamediana (Palencia); (24) Ágreda (Soria); (25) Barcones (Soria). Galicia: (26) Vilarmaior.

removal using a rotary sanding tool (Dremel) and teeth were cleaned with 5% bleach followed by water rinse. All samples were UV-irradiated 30 min on each side, and grounded into a fine powder in a 6750 Freezer Mill (Spex SamplePrep, USA). DNA was isolated using 500 mg powder through a silica-based DNA extraction method (Qiagen, Hilden, Germany) (modified of Marshall et al.) [26] adapted to the Hi-Flow® DNA Purification Spin Columns (Generon, Berkshire, UK). A final concentration step to 30–40 μl was carried out using Amicon-30 columns (Millipore, MA, USA). Quantification of the DNA extracted and determination of inhibition (IPC: Internal Positive Control) was performed by real-time quantitative PCR (qPCR) using Quantifiler™ Human DNA Quantification kit (Quantifiler, AB/LT/TFS: Applied Biosystems™, Life Technologies, ThermoFisher Scientific, Waltham, MA, USA). According to quantification results, decalcification based on incubation with EDTA 0.5 M pH8 overnight and posterior re-extraction was applied in samples where inhibition and/or poor DNA yield were obtained.

DNA from buccal swabs of 186 relatives was obtained under informed consent, following the ethical standards of the Helsinki Declaration. DNA was extracted using Gentra Puregene System (Qiagen) and quantified by Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, USA) in a DTX880 Multimode Detector (Beckman Coulter, Fullerton, USA) or NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

Different DNA systems were used for DNA amplification. For autosomal STRs, the following commercial systems were selected: AmpFlSTR® NGM™ PCR Amplification kit (NGM, AB/LT/TFS), AmpFlSTR® NGM SElect™ PCR Amplification kit (NGMSE, AB/LT/TFS), and MiniFiler™ PCR Amplification Kit (Minifiler, AB/LT/TFS). In the case of family reference samples, I-DNASE21 system was used [27]. For Y-STRs amplification, AmpFlSTR Yfiler PCR Amplification Kit (YFiler, AB/LT/TFS) or PowerPlex® Y23 System (PPY23, Promega Corporation, WI, USA) kits were used, whereas X-STRs were analyzed with the decaplex of the GHEP-ISFG [28]. All PCR amplification kits were used according to manufacturers' recommendations. MtDNA hypervariable segments I and II (HVS-I and HVS-II) in the skeletal remains were studied by sequencing six overlapping fragments ranging from 277 to 317 bp (data not published). For reference samples, mtDNA control region was studied as described in Cardoso et al. [29]. In one case, subtyping of mtDNA haplogroup H by SNaPshot minisequencing [30,31] was performed in order to increase the statistical certainty. The posterior electrophoresis of the PCR products was conducted on an ABI Prism 3130 Genetic Analyzer (AB/LT/TFS). Finally, GeneMapper® Software v.4.0 (AB/LT/TFS) was used for allele designation, using 50 relative fluorescent units (RFU) as threshold for allele calling in heterozygotes and 75 RFU for homozygotes. As for mtDNA, sequences were edited and compared to the reference sequence rCRS [32] using ChromasPro v.1.5 and SeqScape Software v 2.5 (AB/LT/TFS). Replicate analyses were routinely performed in order to guarantee the authenticity of the DNA profiles.

Genetic profiles obtained from the mass graves victims and their alleged relatives were compared, and likelihood ratio (LR) and posterior probabilities (PP) were calculated for autosomal STRs, using the statistical software Familias v.1.97 [33] or GFF v.2.55 [34], based on a Spanish allele frequency database (2014 GHEP-ISFG collaborative exercise). MtDNA and Y-STR haplotype frequency was assessed through EMPOP (EDNAP Mitochondrial DNA Population Database) [35] and YHRD (Y-STR Haplotype Reference Database) [36] databases, respectively. X-STR statistics were calculated based on an allele frequency database for the Spanish population [37].

3. Results

A total of 252 post-mortem remains from 26 mass graves in the north of Spain were genetically studied with identification purposes. Most samples analyzed were teeth (47.13%) or femur (45.59%), followed by tibia and humerus (2.68% and 2.30%, respectively) and other bones such as coxal, calcaneus, talus or temporal bone.

DNA was successfully quantified in 67.83% of the remains, taking into account the minimum detection limit of Quantifiler (≥0.023 ng/μl). Additionally, 24.03% of the samples yielded positive values below the detection threshold (Table 1), thereby raising success in DNA extraction up to 91.86%. Overall, teeth and dense cortical bones (tibia and femur) showed the highest yielding results, in agreement with previous studies [38,39], although some of these samples also failed at times in terms of DNA recovery, probably due to the high degradation of the DNA. Negative DNA results were also obtained in the few calcaneus and coxal samples analyzed. Interestingly, the only analyzed sample of talus displayed a high DNA recovery, reasserting a previous study that pointed to this bone as a high yielding sample [40].

Additionally, the presence of PCR inhibitors was detected in 11.76% of the samples (IPC >30 or undetermined in qPCR). Certain type of samples showed a full inhibition rate (i.e. calcaneus and coxal), whereas the rest did not show inhibition or this was considerably low compared to the high number of samples analyzed (teeth and femur, <12%).

Amplification was performed in all samples even when no DNA or inhibition was detected, as these results in the quantification reaction do not always affect the STRs analysis [41]. However, the quality and quantity of the DNA in the samples, shown by the qPCR results, were key factors to decide between the analyses based on STRs or mini-STRs, as the last markers are more efficient for degraded samples [42].

Table 1
Type of skeletal remains analyzed ranking by sampling frequency. The proportion of samples according to the amount of DNA extracted (mean and range of DNA in ng/μl) and inhibition (IPC detected <30 or IPC ≥30 or undetermined) are also detailed.

Type of skeletal remains	Percentage of type of samples	Amount of extracted DNA (ng/μl) (% samples)				Inhibition (IPC value) (% samples)		
		Mean amount	>0.5	≥0.023 ≤ 0.5	>0 < 0.023[a]	0	IPC < 30	IPC ≥ 30 or undetermined
Teeth	47.13	0.22	10.74	60.33	26.45	2.48	90.91	9.09
Femur	45.59	0.16	6.78	61.86	22.88	8.47	88.18	11.82
Tibia	2.68	0.46	28.57	42.86	–	28.57	83.33	16.67
Humerus	2.30	0.02	–	33.33	33.33	33.33	100	–
Coxal	1.15	0.00	–	–	–	100	–	100
Calcaneus	0.38	0.00	–	–	–	100	–	100
Talus	0.38	0.60	100	–	–	–	100	–
Temporal bone	0.38	0.01	–	–	100	–	100	–
Total	–	0.19	9.30	58.53	24.03	8.14	88.24	11.76

[a] Below the detection limit of Quantifiler™ Human DNA Quantification kit (0.023 ng/μl).

Different genetic markers -autosomal STRs or mini-STRs, Y-STRs, X-STRs and/or mtDNA-were studied in the remains mainly depending on the family relationships which were being examined and the gender of the victims.

Autosomal STRs were the preferred markers for identification (86.90% of the remains) through three different commercial kits, NGMSE (77.38%) or NGM (5.16%) for conventional STRs, and Minifiler (1.98%) for mini-STRs (Fig. 2). In some remains (2.38%), Minifiler was used as a complement to NGMSE to recover the longer NGMSE loci that may fail to amplify in highly degraded samples. In addition, Y-STRs were studied in 84.52% of the samples to assess paternal relationships, being the YFiler the most used kit (77.38%) compared to PPY23 (3.17%), or the combination of both (3.97%). The X-STRs decaplex of GHEP-ISFG was used in 6.35% of the remains, in order to study complex kinships or increase the discrimination of the autosomal results. Finally, mtDNA analysis (HVS-I, HVS-II and/or H subhaplogroup SNPs) was performed to investigate maternal line cases or highly degraded samples, in approximately 27% of the remains. MtDNA analysis was mainly performed by means of the combined study of HVS-I and HVS-II (21.03%). In some samples, the study was restricted to HVS-I (3.97%) or HVS-II (1.98%). Additionally, H subhaplogroup SNPs were studied in one case where the analysis of control region fragments was not sufficiently discriminative.

To assess success rate in DNA typing, informative profiles were defined as those with a minimum of twelve or more loci [43] for autosomal STRs, twelve or more Y-STRs, or a mtDNA profile. Overall, a significant success of DNA profiling was reached, since informative profiles were obtained in approximately 85.71% of the remains (216 out of 252 remains). Consequently, the number of samples without informative profiles for the analysis performed was relatively low (14.29%). A more detailed analysis of the success typing in the remains for STRs is provided in Table 2. Autosomal STR typing presented a success rate of 80.00% in terms of informative profiles. This rate increased to 87.56% when considering all profiles with seven or more reportable loci. The success results obtained with Minifiler were substantially lower to the other systems as it was mainly applied to challenging samples and, consequently, partial or null results were more probable to occur. All profiles obtained were unique, thus reinforcing authenticity of the results. Informative profiles for Y-STRs were obtained in 60.99% of the analyses and another 18.39% of the analyses provided partial profiles of 7–11 loci. A higher number of informative profiles were achieved with PPY23 compared to YFiler (72.22 and 60.00%, respectively); even though the comparison might be biased due to the fact that PPY23 was less frequently used. Nevertheless, it is worth mentioning that more than 17 Y-STRs were observed in 72.22% of the cases where PPY23 was applied (data not shown). This result differs with the 28.78% of cases in which Y-Filer analysis resulted in 17 Y-STRs (complete profile). Therefore, this result may be indicating the adequacy of PPY23 kit for challenging DNA sample analysis in order to increase the number of STRs successfully analyzed [44]. In addition, X-STRs analysis provided a success rate of 87.50% in profiles with more than seven reportable markers, and 37.50% of complete profiles (10 X-STRs). The mtDNA analysis showed great efficiency (Table 3), given that a complete or partial (>200 bp) mtDNA profile was obtained for all the samples analyzed.

For genetic comparison, a total of 186 family members were available (Table 4), being the parent/child cases the most frequent

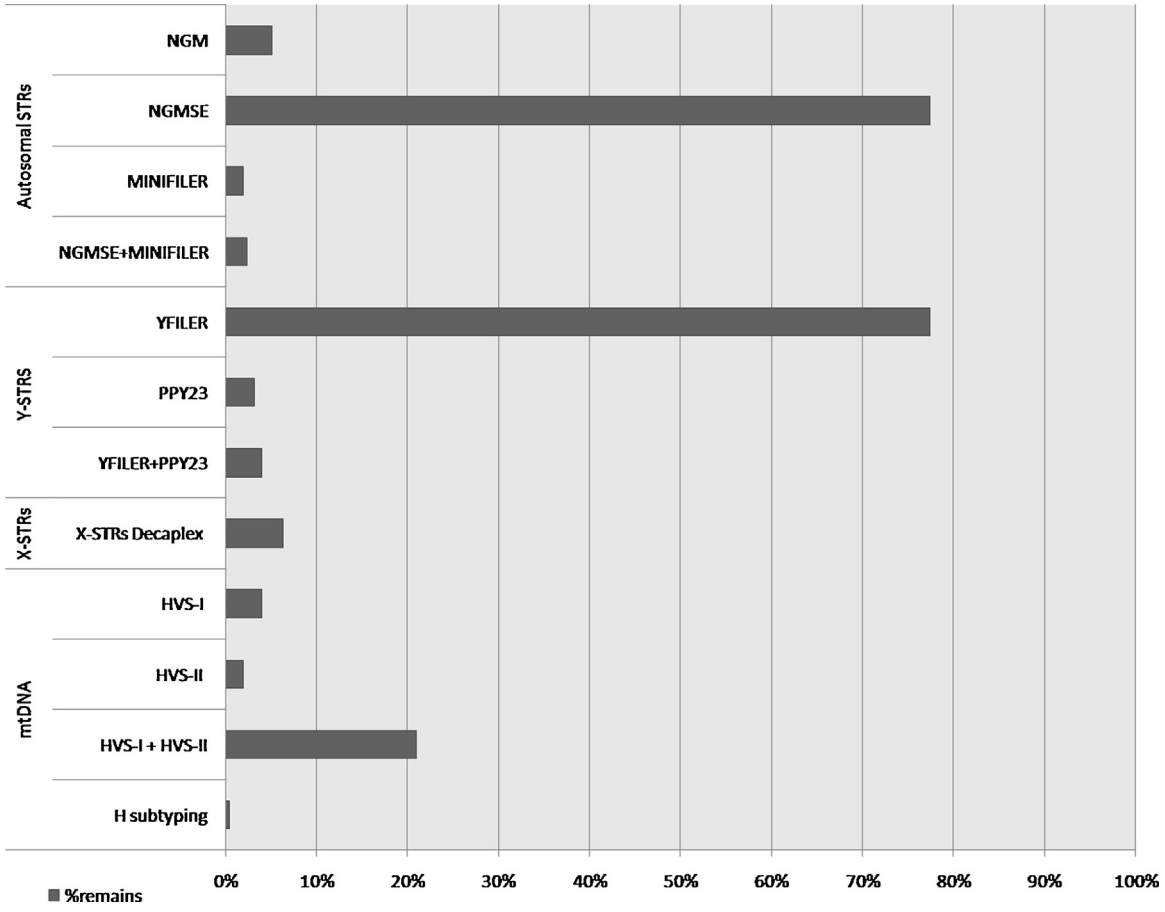

Fig. 2. Proportion of remains analyzed by the different genetic markers (autosomal STRs, Y-STRs, X-STRs, and mtDNA).

Table 2
Compiled results of success in typing autosomal STRs, Y-STRs and X-STRs in the post-mortem remains. The different STR genetic panels available (with the number of STRs included) are indicated. The proportion of profiles (%) according to the number of STRs successfully typed is specified. The global efficiency of autosomal and Y-STR typing, expressed as the percentage of remains with results for each type of markers, is also indicated.

Type of markers	STR genetic panel (number of markers included)	Complete profiles	STRs successfully typed (% post-mortem remains)			
			≥12	7–11	1–6	0
Autosomal STRs	NGM (15)	76.92	84.62	7.69	–	7.69
	NGMSE (16)	65.17	84.08	6.97	5.97	2.99
	Minifiler (8)	9.09	–	18.18	72.73	9.09
	Global efficiency:	63.11	80.00	7.56	8.89	3.56
Y-STRS	YFiler (17)	28.78	60.00	18.54	14.15	7.32
	PPY23 (23)	27.78	72.22	16.67	–	11.11
	Global efficiency:	28.70	60.99	18.39	13.00	7.62
X-STRs	Decaplex (10)	37.50	–	87.50	–	12.50

Table 3
Compiled results of success in typing HVS-I, HVS-II or HVS-I+HVS-II in the post-mortem remains. The proportion of profiles according to the length of the sequence successfully analyzed is specified: informative profile (complete or partial sequence) or no results. The global efficiency for mtDNA typing is also indicated.

MtDNA typing (nucleotide positions)	% Post-mortem remains		
	Complete sequence	Partial sequence	No results
HVS-I (16024–16406)	100	–	–
HVS-II (73–340)	60.00	40.00	–
HVS-I + HVS-II	30.19	69.81	–
Global efficiency	52.07	47.93	–

(35.90%), followed by distant paternal (31.79%) or maternal lineages relationships (24.61%), siblings (6.66%) or others (1.03%). Most of the missing persons were male, only six were female.

The comparison performed between the skeletal remains and the alleged relatives showed that 87 of the 252 victims matched with reference relatives, which represents an identification success rate of 34.52%. Most of the found victims were male and only a total of three women were identified, all of them in the same grave (Sorriba, Burgos). In more detail, 18 victims were identified by comparison with their sons, with values for LR ranged between 2.8×10^2–6.3×10^8 in autosomal STRs, and 8.2×10^2–5.6×10^4 in Y-STRs, additionally a LR value of 2.5×10^3 of mtDNA analysis was also obtained in one mother-son case. The calculated values for combined LR of autosomal STRs and lineage markers (Y-STRs or mtDNA) were 5.7×10^6–1.6×10^{13}. Other 19 identifications were reached by comparison to daughters, with LR values for autosomal and X-STRs between 1.7×10^3–3.9×10^8 and 2.3×10^3–1.7×10^6, respectively. In these cases, combined LR values ranged between 5.6×10^7–6.4×10^{12}. Eight individuals were traced by comparison with their siblings. In the case of brothers, autosomal STRs ($LR = 1.7 \times 10^4$–4.2×10^{10}), Y-STRs ($LR = 8.9 \times 10^3$–4.1×10^4) or mtDNA ($LR = 6.6 \times 10^2$–2.7×10^4) were studied to establish the kinship. The combined LR values ranged between 1.5×10^8–1.7×10^{15}. For brother-sister cases, autosomal STRs ($LR = 3.8 \times 10^7$) or mtDNA ($LR = 1.0 \times 10^3$–1.5×10^3) were analyzed with combined LR values of 2.2×10^4–3.9×10^{10}. Moreover, 27 grandparents/uncles by paternal lineage and 15 by maternal lineage were traced through their grandchildren/nephews-nieces. The statistical analysis displayed high confidence results, particularly in the cases where close relatives were compared, that support the hypothesis that the remains are related to the family references rather than unrelated individuals.

The percentage of potential identifications was markedly lower compared to the great number of profiles obtained from the post-mortem remains. In 51.19% of the cases, the comparison between

Table 4
Detailed family relationships under study and their frequency are indicated, as well as the number of possible identifications and the corresponding LR values range, classified by relationships groups. Combined values are the combined result of LR obtained for autosomal and other markers (Y-STRs, X-STRs or mtDNA).

Relationship under study (relatives)	*N* (%)	Number of possible identifications	LR				
			Autosomal STRs	Y-STRs	X-STRs	mtDNA	Combined
Son	16.41	18	2.8×10^2–6.3×10^8	8.2×10^2–5.6×10^4	–	2.5×10^3	5.7×10^6–1.6×10^{13}[a]
Daughter	19.49	19	1.7×10^3–3.9×10^8	–	2.3×10^3–1.7×10^6	–	5.6×10^7–6.4×10^{12}
Brother	4.10	6	1.7×10^4–4.2×10^{10}	8.9×10^3–4.1×10^4	–	6.6×10^2–2.7×10^4	1.5×10^8–1.7×10^{15}[a]
Sister	2.56	2	3.8×10^7	–	–	1.0×10^3–1.5×10^3	2.2×10^4–3.9×10^{10}
Paternal grandson/nephew/ grandnephew	20.51	24	1.5×10^2–3.9×10^3	1.7×10^2–7.8×10^4			1.2×10^3–1.1×10^8
Paternal granddaughter/niece/ grandniece	11.28	3	1.3×10^3–3.3×10^4	–	–	–	
Maternal grandson/ nephew/ grandnephew	11.28	9	–	–	–	1.3×10^2–2.8×10^4	
Maternal granddaughter/niece/ grandniece	13.33	6	3.4×10^2–5.96×10^3	–	–	4.1×10^3–2.8×10^4	3.5×10^6
Second cousins	1.03	0	–	–	–	–	

[a] These combined LR range includes LR values obtained for autosomal and Y-STRs; or autosomal STRs and mtDNA.

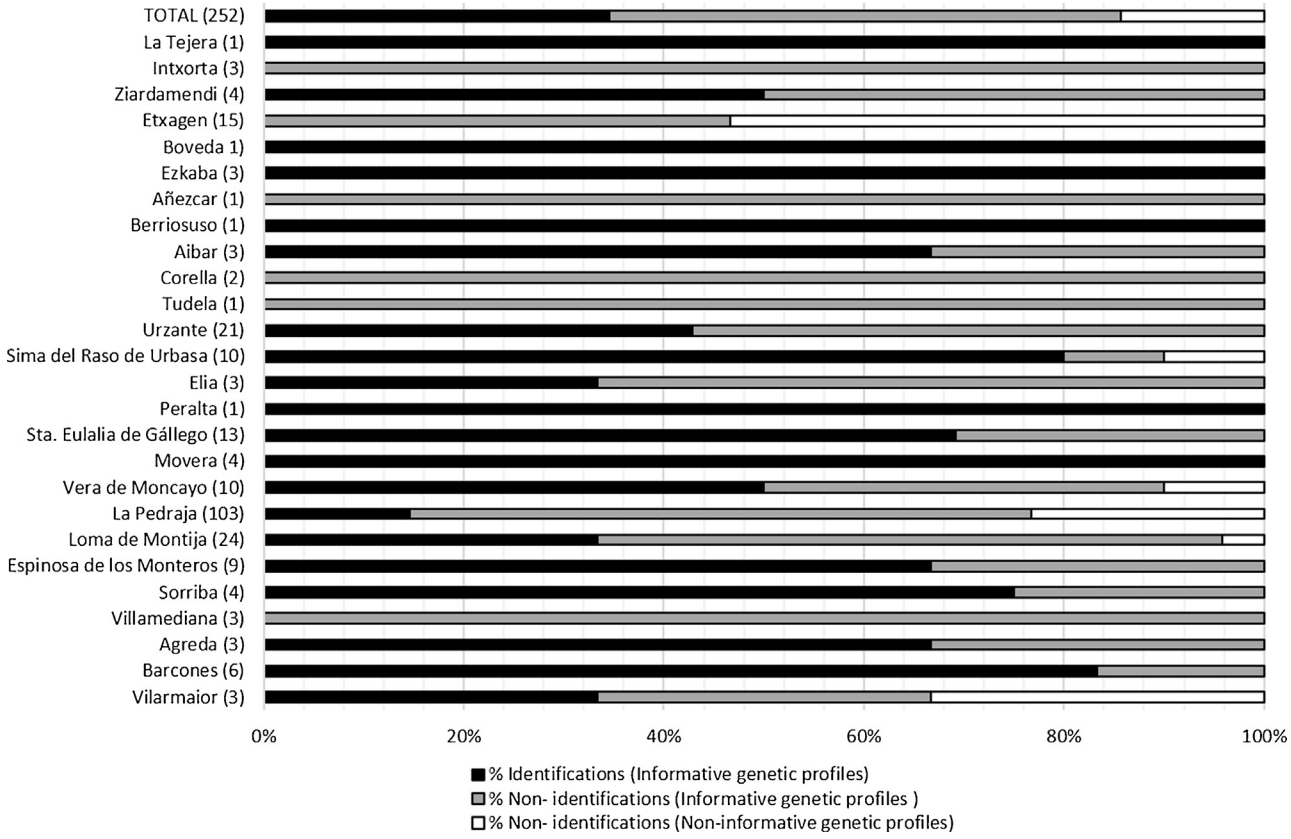

Fig. 3. Mass graves (number of remains) under study with the proportion (%) of identified remains (black bars), unidentified remains with informative profiles (grey bars) and unidentified remains without informative profiles (white bars). Average values for the studied remains are shown in the first row.

the victim profile and the reference database did not show positive matches. It is remarkable that the percentage of identifications, as well as the DNA profiling success, widely differed in the studied graves (Fig. 3). More specifically, genetic studies allowed the positive identification of all the victims in six graves and at least 50% of matches were reached in other nine graves, whereas any identification were reported in five graves. In terms of profile success rate, informative profiles were obtained in all graves. Indeed, only seven of the analyzed graves showed a percentage of not successfully typed remains.

4. Discussion

During the last six years, we performed the genetic analysis of 252 post-mortem remains from graves of the Spanish Civil War (1936–1939) and posterior dictatorship (until 1970s), located in Northern Spain, with identification purposes.

Informative genetic profiles were obtained in 85.71% of the remains analyzed. Therefore, approximately 14.29% of the samples did not provide an informative genetic profile for the analyses performed. The probability of successfully obtaining genetic results from a skeletal remain is largely dependent on the amount of preserved DNA, the level of DNA damage, and the presence of amplification inhibitors [45]. In order to maximize DNA profiling success, the present study followed the current recommendations for DNA analysis from skeletal remains [46] whereby cortical bones, such as femur or tibia, or teeth are preferentially used. Indeed, these samples provided higher yielding results than others bone samples, such as coxal or calcaneus. Consequently, skeletal sample selection constituted an important factor in the success of DNA recovery and posterior profiling. The minor percentage of non- success obtained in the samples analyzed herein, may be due to the influence of exogenous factors such as the post-mortem interval or the grave location (climatic conditions, chemical or soil properties) [47–49]. These factors have been previously reported to cause DNA damage (degradation or structural modifications), and the subsequent absence of informative or complete multiloci STR profile [50]. Furthermore, the presence of endo- or exogenous inhibitors in some of the extracted samples may be also negatively affecting the DNA amplification. Accordingly, the different influence of the aforementioned factors could explain the variability in typing success among the samples and among the analyzed graves.

Nevertheless, the overall success in DNA profiling obtained denotes that the silica-based extraction method used in the present study is highly effective recovering DNA and removing or diminishing the inhibitors present in the samples. Furthermore, it also reflects the sensitivity of the multiplexes applied as they successfully allowed the DNA profiling of a large number of samples.

The identification of 87 victims was accomplished through the comparison with family relatives. However, this barely represents the 34.52% of the total remains recovered, and subsequently the 65.48% of the victims' samples could not be matched to living relatives. As previously stated, a minor percentage of the samples did not provide any genetic profile in the analyses performed or this was highly partial, which impeded a viable identification. Therefore, in 51.19% of the cases an informative profile was obtained, but no match was found with the family members compared. This circumstance is mainly explained by the difficulty to track down living relatives to identify the victims due to the long time elapsed since the end of the conflict, and the lack of information about the identity of the exhumed remains since in that period the victims were dispersed throughout Spain in order to avoid their identification by acquaintances. In the present study,

only 186 presumptive relatives were available for comparison with the 252 post-mortem remains, which approximately represents 74%. Furthermore, the adequacy of relatives for genetic comparisons was also an impediment. The availability of first-degree family members (i.e. children or siblings) represented less than 50% of the cases. Consequently, genetic comparison with more distant familial relationships (i.e. nephews, grandchildren) was frequently the unique alternative, with the subsequent decrease of the statistical certainty as allele sharing decreases. In this situation, the combination of genetic markers (autosomal, Y-chromosome, X-chromosome and/or mtDNA) was applied to obtain satisfactory kinship probabilities.

5. Conclusion

DNA analysis constitutes an inestimable tool in the process of victims' identification from mass graves of the Spanish Civil War and posterior dictatorship. The efficacy and sensibility of the DNA extraction and amplification methods used herein provided an important success in genetic profiling from victims remains despite the intrinsic difficulty of skeletal samples analysis. The identification of individuals has been partially limited by the lack of available and adequate family members used for genetic comparisons. Further perseverance in the exhumation of other intact graves as well as in the search of more alleged relatives is required in order to facilitate and increase the number of genetic identifications.

Conflict of interest

Authors declare no competing interest in the content of this manuscript.

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