Dead migrants in the Mediterranean: genetic analysis of bone samples exposed to seawater

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ABSTRACT

In April 2015, a fishing boat that departed from Libya with about 1,000 migrants on board sank in the Mediterranean Sea. Most of the migrants were packed in the hull of the boat and drowned in the shipwreck. After fifteen months, the ship was recovered from the seabed and brought to a Sicilian naval area for forensic investigations. Skeletal remains belonging to more than 700 people were retrieved. A selected sample composed of 80 victims was considered in order to evaluate the possibility of achieving genetic profiles useful for a positive identification from these challenging specimens. The molecular features of the DNA recovered from a significant number of real casework samples exposed to seawater for long periods of time were described for the first time. Three different DNA extraction protocols and three different commercial kits were employed in order to generate genetic profiles based on the characterization of 21 autosomal STR loci. The combination of multiple DNA extractions and the cross-checking of multiple PCR amplifications with different kits allowed to obtain reliable genetic profiles characterized by at least 16 STR markers in more than 70% of the samples. The factors that could have affected the different quality of the genetic profiles were investigated and the bone preservation was examined through microscopic and macroscopic analyses. The approach presented in this study could be useful in the management of the genetic analysis of bone samples collected in other similar DVI scenarios. The genetic profiles recovered from the bone samples will be compared in kinship analysis to putative relatives of the victims collected in Africa in order to obtain positive identifications.

1. Introduction

On April 18th 2015 a medium sized fishing boat sank, just off Libyan waters, drowning hundreds of migrants packed on board. The victims were believed to be trapped in the cargo, 400 m beneath the sea. This disaster is considered the deadliest shipwreck in seaborne migration from Africa towards Europe [1]. The Italian government created a task force for the recovery and identification of the victims. In July 2016, the vessel was recovered and 528 decomposed bodies were retrieved along with many commingled remains (over 30,000) [2]. To date, the total number of victims is still unknown.

A sample set of the victims (80 bodies) was chosen in order to investigate the possibility of generating genetic profiles from bone samples in this DVI (Disaster Victim Identification) case.

The scenario presented in this study is very challenging as the bodies remained in sea water at a depth of 400 m for different time frames: corpses thrown outside the boat, laying on the seabed, were recovered from 3 to 10 months after the shipwreck while the largest part of the victims, trapped in the inner part of the boat, were retrieved after 15 months once the boat was dredged up from the sea. The boat emptying operations lasted about two weeks and were

Abbreviations: DVI, Disaster Victim Identification; MPI, Missing Person Identification; LR, Likelihood Ratio

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focused on the preservation of the maximum integrity of the bodies; once recovered, they were frozen at − 10 °C until post-mortem examinations which were completed within three months after the recovery of the boat.

While many are the papers dealing with the genetic analysis of bone samples in different DVI or MPI (Missing Person Identification) scenarios [3–5], there are very few studies reporting the genetic results obtained in situations similar to the ones outlined in the present research, most of which are case reports or technical notes [6–9]. Mameli et al. [10] reported a situation similar to the one presented in this paper where a degraded, partial DNA profile was obtained from a mandibular bone exposed to seawater for three months. A pilot study [11] was performed on waterlogged pig bones in order to understand the effect of water on DNA recovery, and the most efficient DNA extraction method. Recently, the Armed Forces DNA Identification Laboratory analyzed bone samples soaked in seawater and fuel oil and then buried in tropical environment belonging to US soldiers who died inside the USS Oklahoma during the Pearl Harbor attack [12]. Finally, human remains (among which a femur section and a metacarpal bone) belonging to a male individual were retrieved from a marine decomposition context and the following DNA profiling by STR-CE and MPS led to the identification of the victim [13].

DNA recovery from bones submerged in saltwater is a complicated issue because the bone structure could be modified by environmental or biological processes. For example, the bone dissolution process could have been enhanced by salt ions absorption which causes an increase in the porosity of the structure and the breakdown of the collagen component from the hydroxyapatite matrix; this situation could accelerate DNA degradation because the nucleic acid is no longer protected or stabilised by the inorganic matrix [14]. In addition, marine microboring (weld-type tunneling) originated by microscopic organisms can contribute to the alteration of the bone structure, partially dissolving the mineral portion [15]. An overview of the environmental and biological factors affecting the DNA stability in skeletal remains, and a review of the conventionally used DNA extraction and genotyping methods is reported in [16–19].

In the present paper, the genetic analysis of a significant number of real casework samples exposed to seawater for long periods of time is described. Different extraction protocols were evaluated in order to hopefully achieve informative genetic profiles. Statistical analyses were performed in order to investigate which environmental factor could have affected the quality of the resulting genetic profiles. This paper wished to focus specifically on the issues related to the difficulty of DNA extraction in such an unusual but “controlled” scenario. All activities were authorized by the judicial authority and all that is reported in this paper is part of the endeavor to identify these victims.

2. Materials and methods

2.1. Molecular characterisation of the victims' bone samples (PM - Post Mortem)

A selected sample composed of 80 victims was chosen on the basis of the finding of “documents” reporting personal data suggesting a possible identity of the victim (such as ID, driving licences, birth certificates, passports or UNHCR - United Nations High Commissioner for Refugees – cards) which were found by a clothing inspection during the post-mortem examinations. The documents showed that the individuals were from African countries such as Mali, Sudan, Ethiopia, and Eritrea. Bodies exhibited various decomposition rates, ranging from early/advanced stage of decomposition to partial/complete skeletonization, with the early stages restricted mainly to the cadavers recovered in the first months from the seabed (see [20] for more details).

Complete, ten centimetres long cross sections of 58 femoral and 21 tibial diaphyses were selected for this study, together with a clavicle. These bone samples were collected during autopsies, then frozen and finally transferred to the LABANOF (Forensic Anthropology and Odontology Lab) of the University of Milan for the final storage. The post-mortem interval for the samples considered in this study varied from 3 (the first bodies found on the seabed) to 18 months (the last bodies examined three months after the recovery and emptying of the boat) and was calculated as the difference between the day of the autopsies and the day of the shipwreck. Twenty-five bones (15 tibiae and 10 femurs) were sampled from the bodies found outside the boat recovered from 3 to 10 months after the shipwreck while the remaining fifty-five ones (7 tibiae, 47 femurs and 1 clavicle) were sampled within the set of autopsies performed during the three-months-period following the recovery of the boat.

2.1.1. DNA extraction

Three different DNA extraction methods were applied sequentially, not to test the best performing approach but to provide, by combining the results of multiple extractions, reliable genetic profiles useful for individual identification of the victims.

The surface of the bone samples was cleaned with a blade and briefly decontaminated with bleach (2%) before collecting bone powder by drilling the middle third of the bone diaphyses cross sections [21]. Three different DNA extraction kits were employed:1) Promega Bone DNA Extraction Kit followed by automated extraction with DNA IQ casework PRO kit for Maxwell 16 (Promega, USA), starting from 100 mg of bone powder; 2) Prepfiler BTA forensic DNA extraction kit (Thermo Fisher, USA), starting from 100 mg of bone sample; finally, the most challenging samples were treated increasing the amount of bone powder to 500 mg, which was then submitted to a 3-days pre-decalcification step with EDTA 0.5 M before DNA extraction with 3) QIAmp DNA Blood Maxi kit Qiagen (Qiagen, Germany) with minor modifications. The bone samples were extracted in a thermomix (Mixer HC, StarLab, Italy) at 56 °C o.n., setting up an orbital mixing at 900 rpm. All samples were recovered in 50 µl of each corresponding elution buffer.

2.1.2. DNA quantification

Two-microliter aliquots from each sample were quantified using the Quantifiler Duo DNA Quantification kit (Thermo Fisher, USA) following the manufacturer's recommendations, on a 7500 Real Time PCR System (Thermo Fisher). Calibration was performed in duplicate. Negative control samples were always included.

2.1.3. DNA amplification

Three different amplification kits were used with the aim to generate genetic profiles based on the characterisation of 21 autosomal STR loci. All the DNA samples were amplified with the PowerPlex ESX 17 Fast System (Promega, USA) and AmpFlSTR Identifiler Plus (Thermo Fisher, USA) kits in a 15 µl final PCR volume. Poor quality profiles were amplified with the Powerplex ESI 17 Fast System as well. DNA amplification was performed by adding 250 pg of template DNA to the PCR reaction or the maximum input DNA volume for each kit, for those samples showing low DNA amounts (max input DNA volume: 10.5 µl). The number of PCR cycles were according to the manufacturers' recommendations. Negative extraction and amplification controls, as well as the 2800 M human DNA control (positive DNA control), were always included. Our strategy for a reliable genetic typing, especially in case of challenging samples, was to use all the information recovered by cross-checking the genotypes obtained from multiple amplifications of the DNA samples, using the same kit or multiple kit configurations.

2.1.4. DNA data analysis

DNA electrophoretic separation was performed on an ABI-PRISM 310 sequencer (AppliedBiosystems, USA) and the genotypes were analysed with the software GeneMapper ver. 3.2.1. The analytical and stochastic thresholds were respectively 50 and 350 rfu.

2.1.5. DNA profile quality score

In order to describe the quality of the genetic substrate extracted from the bones, a DNA degradation/fragmentation index was calculated for each sample, based on the allelic peak heights of the Promega ESX profiles. When evidence profiles are visualised, a regression coefficient is generated by the software Euroformix [22,23], calculating the average fragment lengths versus the summed peak heights at each observed locus. Peak heights are then converted into natural logarithm and the regression equation provides the degradation slope which varies from 0 (completely degraded DNA) to 1 (high molecular weight DNA). Ten positive control DNA profiles (2800 M cell line) were checked in order to calculate a reference degradation slope for a high molecular weight DNA profile.

2.1.6. Bone tissue preservation

In order to evaluate if the different quality of the profiles could be related to bone tissue preservation, each sample was analysed both macroscopically and microscopically.

Since no scoring system has been defined in literature to categorise samples coming from aquatic environment, macroscopic bone appearance was evaluated recording the presence/absence of organic sheen, fat leaching, adipocere and soft tissue and describing bone tissue as in [24].

Microscopic investigations were performed from a complete bone cross section carried out in the middle diaphysis of each sample following the calcified protocol as described in [25]. Bone tissue preservation was classified according to the Oxford Histological Index (OHI) as reported by [26]. In particular, six stages were described (from 0 to 5), considering the quantity of well-preserved bone tissue and the possibility to identify bone components such as osteons, lamellae and osteocyte lacunae. Bone sections showing less than 5% of the tissue preserved and no appreciable bone structures were scored as “0”, while bone sections with well-preserved bone tissue and well recognizable bone structures were classified as “5”.

2.1.7. Statistical analysis

The statistical data analysis was conducted using Microsoft Excel and R Studio, Version 1.2.1335 (RStudio© 2009–2019, RStudio, Inc.). ANOVA analysis was performed. Statistical significance was assessed when the p-value computed for the observed data under the given null hypothesis of the test was found lower than the significance level chosen ($p < \alpha = 0.05$).

3. Results and discussion

3.1. Molecular features of the bone samples

3.1.1. DNA quantification results

The DNA was extracted from all the 80 bone samples using the Promega Bone DNA Extraction Kit. Each sample was then quantified using the Quantifiler Duo DNA quantification kit, which provided human DNA amounts (autosomal probe) below the Limit of Quantification (LOQ=23 pg/µl) in 77% of the extracts with 19% of the total samples negative for the presence of measurable amounts of human DNA (undetermined) and only a single sample showing a remarkable high amount of genetic substrate (725 pg/µl) (see Table 1).

3.1.2. Autosomal STR profiling

The DNAs were then amplified with the PowerPlex ESX 17 Fast System and AmpFlSTR Identifiler Plus kits, in order to hopefully get genetic profiles based on the characterization of 21 autosomal STR loci. This set is the recommended panel of markers for the following DNA screening approach through kinship analysis in DVI cases, according to [27–29]. In selected cases, the PowerPlex ESI 17 Fast System, amplifying the same STR loci as PowerPlex ESX but with primers pairs designed to achieve a complementary genotyping of the loci, was used [30]. The amplifications of the bone samples showed mostly partial genetic profiles with loss of the high molecular weight markers, and peak heights imbalance for the heterozygous genotypes. Twenty DNA samples provided genetic profiles with a number of autosomal STR loci ≥ 16 confirmed in different amplifications while the remaining DNAs showed degraded profiles with a variable number of STR markers below 16.

The bones belonging to these last sixty samples were then re-extracted using the Prepfiler BTA forensic DNA extraction kit and the DNAs were quantified as above. The quantification results showed an increased number of samples with human DNA amounts below the LOQ (50 out of 60, 83%) among which only 7% of the re-extracted samples (4 out of 60) were negative for the presence of amplifiable DNA (see Table 1). PCR amplification of the DNA extracts, according to the above-mentioned protocol, resulted in thirty-six samples showing 16 or more autosomal STRs, fourteen with a number of markers between 10 and 15 and ten samples showing a very limited number of STR loci (below 10).

Finally, in order to hopefully increase the amount of DNA for these ten challenging bone samples, five times more bone powder (500 mg) was collected and extracted with QIAmp DNA Blood Maxi kit columns following a 3-days pre-decalcification step with 0.5 M EDTA (pH 8). This protocol allowed to obtain DNA amounts below and above the LOQ in 80% and 20% of the samples, respectively. Only one sample delivered an almost complete STR profile (20 out of 21 autosomal STRs), three other samples showed genetic profiles characterised by 12–15 STRs, while the remaining specimens yielded no results or low quality profiles with a number of markers ≤ 8.

The workflow focusing on the analytical approach employed to achieve the genetic profiles from the 80 selected samples is shown in Supplementary Fig. 1.

The results of the DNA quantifications performed on the 80 bone samples, according to the three different DNA extraction protocols used in this study, are summarized in Table 1. The median of the values that provided measurable DNA amounts was very similar among the three different DNA extraction protocols, while the median recovery value per 100 mg of bone tissue was about five times lower for the Qiagen protocol compared to the other two methods, thus confirming the challenges in genotyping the last ten bone samples whose DNA was likely the most damaged.

Laboratory’s interpretation guidelines were set before reviewing the DNA typing results according to the Ge.F.I. recommendations for personal identification analysis by forensic laboratories [31], leading to a two-levels final database. In the first conservative level only replicated genotypes in multiple amplifications with the same or with different kits and high-quality profiles were stored. The second level contained, together with the reliable markers, additional low-quality genotypes from other STRs, which might be eventually considered in view of genetic comparison with the relatives of the victims; this approach is reported in [32] where it is suggested that even loci of insufficient quality should be evaluated for consistency between the profiles. It is obvious that these low-quality genotypes have to be evaluated with caution and that potential matches must be reviewed carefully by the experts even considering the possibility of re-extracting the bone sample to confirm or complete low-quality genotypes.

Table 1
Molecular DNA quantification results obtained using the *Quantifiler Duo DNA quantification kit (autosomal probe)*. For each of the three different DNA extraction protocols used in this study, the highest DNA amount obtained (maximum value), the median of the values which provided measurable DNA amounts (median of the values different from 0) and the corresponding median DNA amount normalised to 100 mg of bone samples are reported. In square brackets are the number of bone samples analysed for each DNA extraction protocol. The number of samples with DNA amounts above and below the LOQ and with no DNA (*undetermined*) together with the percentages are reported.

	Promega Bone DNA extraction Kit [80]	Prepfiler BTA forensic DNA extraction kit [60]	QIAmp DNA Blood Maxi kit [10]
Maximum value	725 pg/µl	56 pg/µl	35 pg/µl
Median of the values ≠ 0	11 pg/µl	12 pg/µl	13 pg/µl
Median DNA amount/100 mg bone tissue	550 pg	600 pg	130 pg
N. of samples with DNA amount ≥LOQ	18 (23 %)	10 (17 %)	2 (20 %)
N. of samples with DNA amount < LOQ	62 (77 %)	50 (83 %)	8 (80 %)
N. of samples with no DNA	15 (19 %)	4 (7 %)	2 (20 %)

PCR artefacts such as allele drop-outs or allele drop-ins were sometimes observed, but these ambiguities were easily resolved by cross-checking the multiple amplifications with the same kit or with different kits. With regard to the drop-outs, it is to mention that in five samples the height of the surviving amplified allele of the heterozygous genotype was clearly above the stochastic threshold, that is the threshold above which if a single allele is seen the analyst should be confident in assigning a homozygous genotype; in two cases, the height of the surviving allele was even from three to four times that threshold (see Supplementary Fig. S2). This finding warns against the assignment of homozygous genotypes after a single amplification of a sample, even if a good quality profile is obtained.

At the end of the task, the strategy of combining different profiles obtained from multiple amplifications using the same kit or multiple kit configurations resulted in more than 70% of the samples giving genetic profiles characterized by at least 16 STR markers, which is the number of markers already used for preliminary kinship calculations in a similar DVI case [27,28]. In Fig. 1 is reported the number of high quality, replicated STR markers (first level database) defining the genetic profiles of each of the 80 bone samples, according to the DNA extraction protocol employed.

The number of amplifications to which the samples were subjected ranged from 2 to 8, depending on how difficult the genetic typing of that specific bone sample was, with a mean value of 4 PCRs per sample.

3.2. Taphonomic features of the bone samples

In order to investigate which factor could have affected the different quality of the obtained genetic profiles, bone preservation was examined through microscopic and macroscopic analyses.

The analyses on all the 80 samples revealed a well-preserved bone tissue showing a still greasy external bone surface, with soft tissue and bone marrow in the medullary cavity still present. Similarly, microscopic investigations highlighted a good preservation of the bone microstructure, where bone components such as osteons and lamellae were well-recognizable. Only three of the total number of samples, recovered outside the vessel, showed pattern of marine scavengers both macroscopically and microscopically. Nevertheless, bone micro-structure was well preserved, with no signs of destructive foci and with an appearance similar to fresh bone samples (see Fig. 2).

Likewise, the following molecular characterization of these three samples provided good quality genetic profiles each one defined by at least 16 autosomal STR loci.

3.3. Factors affecting DNA quality

Other factors could have had a significant effect on the quality of the genetic profiles. Among them, the post-mortem interval, varying from 3 (the first bodies found on the seabed) to 18 months (the last bodies examined three months after the recovery and the emptying operations of the boat), was calculated as the difference between the day of the autopsies and the day of the shipwreck. The position of the bodies outside or inside the boat: among the 80 victims, 26 were found outside the boat, on the seabed, and were recovered in a time span ranging from 3 to 10 months after the shipwreck. Most of the bodies were contained in the hull of the ship, and were retrieved when the boat hold was emptied starting from the upper part, the peak compartments, where a limited number of victims were contained in these small spaces, then carrying on to the inner part, the cargo, where most of the victims were crammed. Out of the 80 victims, 26 and 28 were found in the peak compartments and in the cargo, respectively. The presence/absence of clothing on the skeletal remains could have either protected or exposed the bones to the micro or macro marine fauna attack. For this reason, the presence of trousers (or a shirt, for the clavicle) covering the bone portion selected for the sampling was recorded. Finally, the quality of the profiles could be related to the different types of long bones (femur and tibia) and to the different DNA extraction protocols adopted in this study.

The molecular quality of the DNA extracted from the 80 bone samples was estimated from the ESX genetic profiles obtained from each sample, according to their degradation/fragmentation patterns. In a high molecular weight DNA, the peak heights for each locus are expected to be very similar along the entire molecular weight range of the markers, while in a degraded DNA, a downward trend of allele peak heights relative to increasing fragment size is observed; this degradation slope can be modeled by a regression coefficient calculated by the software Euroformix [22,23]. This number (i.e., the degradation slope) should ideally approximate 1 in a high molecular

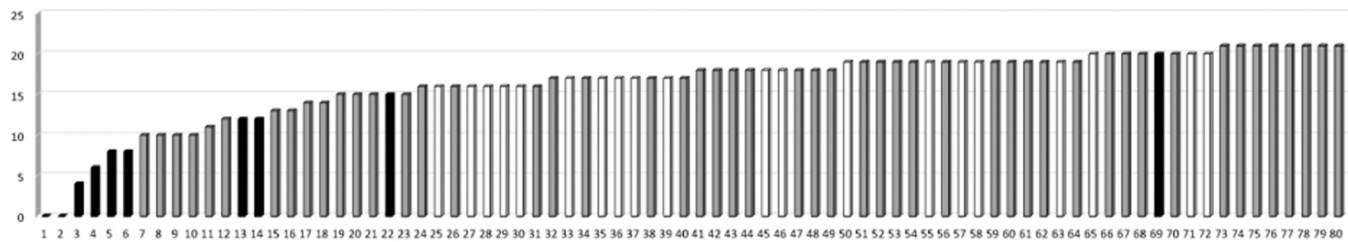

Fig. 1. Number of STR markers defining the genetic profiles of each of the 80 bone samples, according to the DNA extraction protocol employed. White bars: Promega Bone DNA Extraction Kit; grey bars: Prepfiler BTA forensic DNA extraction kit; black bars: QIAmp DNA Blood Maxi kit.

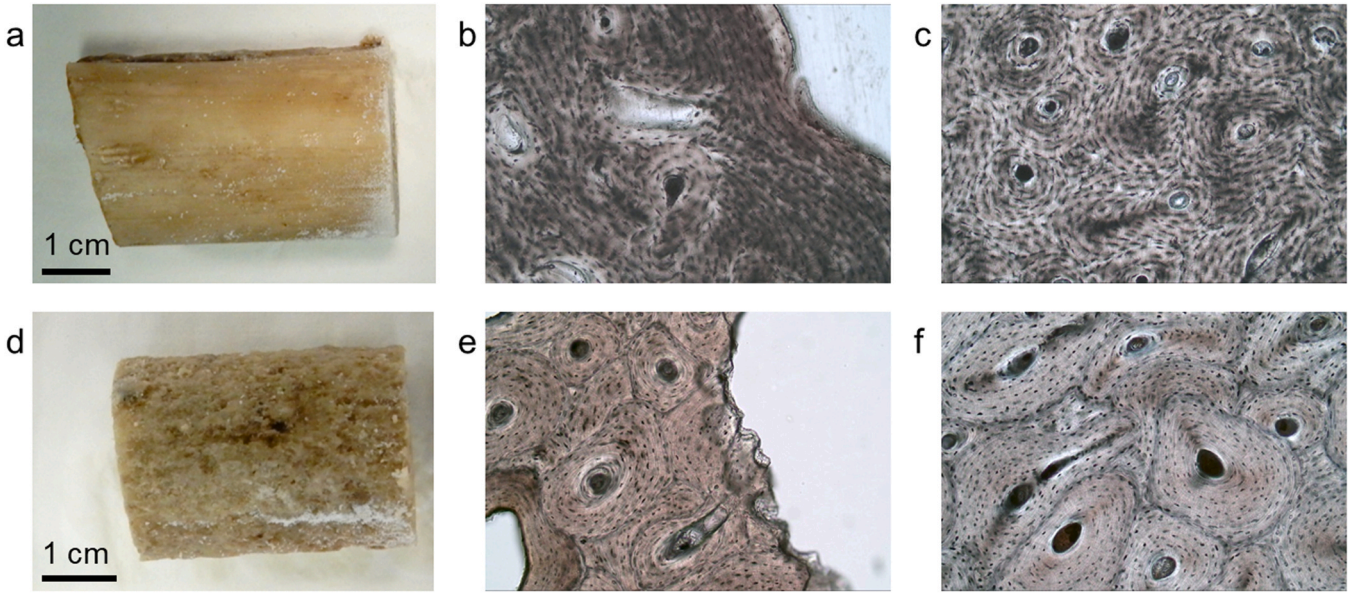

Fig. 2. Macroscopic (a, d) and microscopic (b-c, e-f) appearance of two representative bone samples: a-c: femur with an intact external surface; d-f: femur with signs of marine scavengers' activity. Despite the macrofaunal activity, the two samples were characterised by well-preserved bone tissue with no signs of destructive foci and appearance similar to that of fresh bones. Figs. b and e show the periosteal surface (microscopic pictures: 100x).

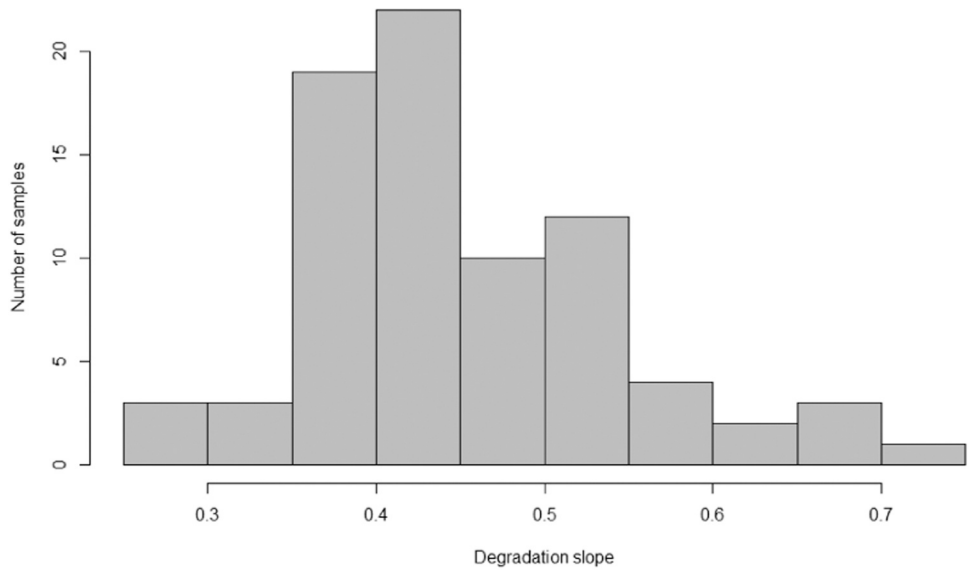

Fig. 3. Bar graph showing the frequency of the degradation slopes calculated by the software Euroformix for the 80 ESX profiles obtained from the bone samples.

weight DNA. This calculation was performed for 10 amplifications of the DNA control sample 2800 M to check this hypothesis. The results showed a mean value and confidence interval at the 95% level of probability ($\alpha = 0.05$) of 0.95 ± 0.03, thus describing a reference number for a good quality profile. The descriptive statistics of degradation slope values calculated for the 80 DNA samples are reported in Supplementary Table 1 while Fig. 3 illustrates the frequency of the degradation slope values calculated for the bone samples. The mean and median values of the degradation slope for the 80 samples were 0.45. However, the frequency data distribution appeared skewed and tailing due to the dispersion of the data towards the higher degradation slope values.

No correlation was found between the degradation slope and the post-mortem interval (R^2 = 0.003379). Strictly related to this result was the finding that no correlation could be found between the degradation slope and the sector of recovery of the bodies (see boxplot "A" in Supplementary Fig. S3). Also, no relation was highlighted between the different types of long bones and the degradation slope as confirmed by the results of one-way and two-way ANOVA tests of the data (see boxplot "B" in Supplementary Fig. S3). Furthermore, no relation existed between the three different DNA extraction methods employed in this study and the degradation slope values (see boxplot "C" in Supplementary Fig. S3).

The presence of clothing on the bodies was also considered. From the inspection of the bodies, it was recorded that the victims were wearing from 1 to 3 pairs of trousers or shirts (probably to defend themselves from the cold), and just a few of them were found without them (see boxplot "D" in Supplementary Fig. S3). One would reasonably assume that the clothes protected the bone from external degradation agents. However, boxplot D in Supplementary Fig. S3 suggests that the highest degradation slopes were those measured in samples extracted from bare bones, but statistics tests evidenced no relation between the presence /absence of clothing and the

degradation slope of bone samples (ANOVA, p-value 0.193, Kruskal-Wallis test p-value 0.126).

An overview of the 80 bone samples tested with the description of the position of the bodies outside or inside the boat from which each specific sample was recovered, the associated DNA extraction protocol used, and the number of loci recovered is reported in Supplementary Table 2.

4. Concluding remarks

In the present study, a selected set of bone samples belonging to migrant victims exposed to seawater for 3–15 months was submitted to genetic and anthropological investigations. The dense cortical portion of the weight-bearing bones femur and tibia was collected during autopsies and selected for DNA extraction, together with a single clavicle. These skeletal remains were preferentially sampled for forensic testing due to their high success rate in DNA recovery [29,33]. In this DVI scenario, three different DNA extraction methods were applied sequentially, not to test the best performing approach but to provide, by combining the results of multiple extractions, reliable genetic profiles useful for individual identification of the victims. Multiple STR kits were chosen to maximize the possibility to achieve a powerful genetic information for kinship statistics. In fact, this approach can be useful when very degraded DNA is recovered from challenging samples; in those situations, the last generation multiplex STR kits, showing superior discrimination power, could deliver genotypes just for the lower molecular weight markers while the higher molecular weight amplicons could provide only low quality genotypes or could not be amplified at all. The combination of more STR kits in different primer configurations can overcome this issue, producing a complementary genotyping of the markers in independent amplifications.

No correlation was found by comparing the DNA molecular quality index (degradation slope) to the post-mortem interval, the position of the bodies inside or outside the boat, the bone element, the DNA extraction kits used and the presence/absence of clothing. High and low quality genetic profiles were obtained in all the categories, thus suggesting that other factors, different from the ones considered in this study, could act on the stability of the DNA molecule.

Studies focusing on the identification of other parameters explaining the different quality of the genetic profiles are in progress among which the microscopic analysis of decalcified bone sections and densitometric radiological investigations (BMD, bone mineral density) of the bone diaphyses in order to investigate the organic and inorganic components. It is even possible that chemical-physical factors acting at a sub-microscopic level (intra-bone pH and/or pressure variations, for example) could have originated the different degree of degradation recorded among samples exposed to similar environmental conditions.

The STR profiles from the migrant victims can be compared with large datasets of DNA profiles obtained from putative relatives collected in African countries, in order to get positive genetic identifications by kinship analysis.

CRediT authorship contribution statement

Emilie Bertolini: Investigation, Formal analysis. **Pierangela Grignani**: Investigation, Methodology, Formal analysis, Writing – review & editing. **Barbara Bertoglio**: Investigation, Methodology, Formal analysis, Writing – review & editing. **Giorgio Marrubini**: Formal analysis. **Debora Mazzarelli**: Methodology, Investigation. **Stanilla Lucheschi**: Methodology, Investigation. **Alessandro Bosetti**: Resources. **Paolo Fattorini**: Methodology, Formal analysis, Writing – review & editing. **Cristina Cattaneo**: Methodology, Formal analysis, Writing – review & editing. **Carlo Previderé**: Writing – original draft, Writing – review & editing, Formal analysis.

Conflict of interest

The authors have declared no conflict of interest and that the study was performed with the authorization of the Italian Government's Office of the Commissioner for Missing Persons (UCPS-Ufficio Commissario Straordinario per le Persone Scomparse) and of the judicial authority.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.forsciint.2022.111421.

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