Available online at www.sciencedirect.com
ScienceDirect
Forensic Science International: Genetics 1 (2007) 175–179
FSI GENETICS
www.elsevier.com/locate/fsig

Application of novel ''mini-amplicon'' STR multiplexes to high volume casework on degraded skeletal remains

Thomas J. Parsons [a,*], Rene Huel [a], Jon Davoren [a,1], Cheryl Katzmarzyk [a], Ana Miloš [a], Arijana Selmanović [a], Lejla Smajlović [a], Michael D. Coble [b,2], Adnan Rizvić [a]

[a] International Commission on Missing Persons, 45A Alipašina, 71000 Sarajevo, Bosnia and Herzegovina
[b] National Institute of Standards and Technology, Gaithersburg, MD, USA

Received 30 January 2007; accepted 3 February 2007

Abstract

The International Commission on Missing Persons (ICMP) conducts high throughput STR profiling on degraded skeletal remains, primarily recovered from mass graves relating to conflicts from 1992 to 1999 in the former Yugoslavia. To date, over 11,000 individuals have been identified through comparison of bone profiles to a large database of profiles from family members of the missing. To increase success rates in STR recovery, three short amplicon STR multiplexes (a 7-plex, a 6-plex, and a 5-plex) have been devised and implemented. These target loci from large commercial multiplexes, with an average decrease in amplicon size of 144 bp. The ICMP ''miniplexes'' have proven to provide substantially greater recovery of DNA data from a certain subset of difficult samples. However, the circumstances under which miniplexes provide additional data are restricted, and their advantages do not outweigh those of large commercial multiplexes for a majority of cases. The miniplexes, however, also have a very powerful use in DNA testing to support large scale reassociation of commingled, partial skeletons recovered from secondary mass graves.

Keywords: Human identification; Mass graves; DNA typing; Mini-STR

1. Introduction

The International Commission on Missing Persons (ICMP) was established in 1996 with a primary mission to assist in resolving the fate of ~40,000 individuals missing from the conflicts in the former Yugoslavia during 1992–1995, and in 1999 (Kosovo). To identify missing persons on such a vast scale, the ICMP has developed a DNA-led process of matching DNA profiles from skeletal remains to DNA profiles from family members of the missing. Operating in high throughput mode since 2001, this model has proven to be extremely effective. An integrated system of public education efforts, sponsorship of family organizations, and mobile blood collection teams has resulted in the collection of over 82,000 family reference blood samples representing over 27,000 missing persons. These blood samples have been typed for autosomal STR profiles (using Promega PowerPlex 16) and entered into a database. Likewise, DNA typing from skeletal remains, primarily recovered from mass graves, occurs in high-throughput mode, in the last few years averaging over 100 extractions per working day. To date, DNA profiles have been successfully obtained from over 20,000 bone samples, representing ~14,500 individuals. Matching software developed by the ICMP, combined with extended kinship analysis through DNA-View [1], has resulted in bone:family DNA match reports for more than 11,400 individuals. These results are communicated to government authorities with whom the ICMP has established effective protocols, and result in official identifications.

One particular challenge posed in this process is the fact that many of the large mass graves that are investigated are ''secondary'' mass graves. Individuals were murdered and then buried in a primary grave. In an attempt to hide the atrocity, the primary graves were later exhumed with heavy machinery and the remains trucked to a series of secondary graves. This gave rise to extensive fragmentation and commingling of the bodies,

* Corresponding author.
E-mail address: thomas.parsons@ic-mp.org (T.J. Parsons).
[1] Current address: The Bode Technology Group, Lorton, VA, USA.
[2] Current address: Armed Forces DNA Identification Laboratory (AFDIL), Rockville, MD, USA.

1872-4973/$ – see front matter © 2007 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.fsigen.2007.02.003

and parts of a single individual could be deposited in multiple different secondary graves. The ICMP uses an integrated forensic science approach to this extremely complex identification challenge, involving: state-of-the-art forensic excavation techniques with spatial mapping to recover discrete intact bodies or body parts, to the extent possible; systematized anthropological examination to generate reassociation hypotheses, or in some cases to definitively reassociate skeletal elements; and, strategic sampling of remains for DNA typing to associate remains through testing of anthropology-based hypotheses, or by blind matching. DNA sampling on this scale requires a strong emphasis on cost optimization. Often in mass disaster situations, both reassociation of body parts and primary identification follow the same technical approach [e.g., 2]. However, in the work of the ICMP, where individual identification is achieved almost exclusively through kinship analysis (requiring a large number of loci), it may be most efficient to approach these tasks using different testing approaches. For reassociation, based on direct matches between skeletal elements, a smaller number of loci can provide suitably high statistical strength.

The skeletal remains tested by the ICMP are generally between 7 and 15 years postmortem, and have been recovered singly or in mass graves from a wide range of depositional environments. These include burial in soil (the most common), scattered surface collections, or recovered from water, in wells or caves, etc. The DNA preservation in these degraded samples is, of course, highly variable, and depends substantially on the type of bone available for testing. Overall, the success of DNA profiling from optimal samples (dense femoral sections, or intact teeth) is high, with a ~90% success rate (with success defined as the recovery of 12 or more loci from the 16-plex kit). For less optimal samples (e.g. less dense bones such as vault bones, cranial bones, vertebrae, innominates, etc.) the success rate is substantially lower, however.

In recent years, there has been much attention and promise regarding the use of reduced-amplicon length STR typing for increasing the success of allele recovery from degraded DNA samples [3–7]. Obviously, given the scale and nature of the DNA testing at the ICMP, the prospect of higher recovery of alleles from degraded DNA is attractive. Based on published primer sequences for shorter amplicons for STR loci found in commercial kits [3], the ICMP has devised and tested three novel multiplexes (a 7-plex, a 6-plex, and a 5-plex). These have been applied to casework samples in order to increase the recovery of amplicons from more highly degraded samples, as well as to serve as less expensive typing systems for the purposes of reassociation. This paper will present our experiences with mini-amplicon kits for degraded skeletal remains casework, and discuss considerations on the use and limitations of short amplicons for increasing success.

2. Materials and methods

2.1. DNA extraction from bone and teeth

The superficial several millimetres of a bone sample is removed by sanding with a rotary sanding stone bit. The remaining bone sample, or a single intact tooth, is decontaminated in a sterile hood by washing with distilled water, inversion for 30 s in 10% bleach, and inversion for 30 s in 96% ethanol (twice). Following the chemical cleaning, samples are dried at 50 °C for 2 h and then ground into a fine powder using a Waring® blender. DNA extraction is based on the QIAamp DNA Blood Maxi kit (Qiagen), with some modifications. Two to six grams of bone powder, or the entire tooth powder, are incubated in 15 ml (tooth, 5.5 ml) of ATL extraction buffer (Qiagen) with 10 mg (tooth, 3 mg) of proteinase K and incubated for 18 h at 56 °C in a shaking water bath. This is followed by addition of 14 ml (tooth, 5 ml) of buffer AL (Qiagen), inversion for 30 s, and incubation at 70 °C for 1 h. The remaining bone material is removed by centrifugation at 1811 × *g* for 5 min and the supernatant transferred to another 50 ml tube. Twenty-two millilitres (tooth, 5 ml) of absolute ethanol is added and the samples are mixed by inversion for 15 s. The total volume is loaded to Qiagen blood maxi columns by 3 rounds (tooth, one round) of sample addition and centrifugation. The columns are then washed with 10 ml (tooth, 5 ml) of AW1 (Qiagen), centrifuged at 1811 × *g* for 5 min and the flow through discarded. A second wash with 10 ml (tooth, 5 ml) of AW2 (Qiagen) is followed by centrifugation at 1811 × *g* for 15 min and the flow through discarded. The DNA is eluted by the addition of 3 ml of AE buffer (Qiagen) preheated to 70 °C and incubated at 70 °C for 5 min, and then centrifuged at 1811 × *g* for 2 min. A second elution is performed by the addition of 3 ml of AE buffer (Qiagen), incubation at room temperature for 5 min, and then centrifugation at 1811 × *g* for 10 min. The 6 ml of eluted DNA is washed with water and concentrated to approximately 0.3 ml using 15 ml Centriplus YM-100 columns (Millipore) followed by Centricon YM-100 columns (Millipore).

2.2. Amplification primers

Primers are obtained from Applied Biosystems (Foster City, CA) labeled with either 6FAM, VIC, NED or PET. Primer sequences are from [3].

Primer mixes are made up in 25× aliquots, with the following primer concentrations (forward and reverse concentrations the same for each pair):

- *MP1*. Mini Amplicon Kit #1 (25× concentration): TH01 (6FAM), 3 μM; Amelogenin (6FAM), 3 μM; FGA (6FAM), 10 μM; D18251 (VIC), 10 μM; Penta D (NED), 35 μM; D2S1338 (PET), 10 μM; D21S11 (PET), 50 μM.

  Note. An earlier version of MP1 included D16S539 instead of Penta D. Primer concentrations for D16S539 were 5 μM, labeled with NED.
- *MP2*. Mini Amplicon Kit #2 (25× concentration): vWA (6FAM), 7.5 μM; CSF1P0 (VIC), 5 μM; D13S317 (NED), 5 μM; D7S820 (NED), 10 μM; D8S1179 (PET), 27 μM; D21S11 (PET), 15 μM.
- *MP3*. Mini Amplicon Kit #3 (25× concentration): FGA (6FAM), 10 μM; CSF1P0 (VIC), 5 μM; D21S11 (VIC), 20 μM; Penta D (NED), 25 μM; Penta E (PET), 40 μM.

2.3. PCR amplification

PCR reactions are carried out in 25 μl volumes, consisting of 1× Gold Star buffer (Promega), 0.5–1 unit AmpliTaq Gold® polymerase (ABI) and 1× mini amplicon kit primer mix. DNA template volumes are typically 10 μl.

Thermocyling conditions are as follows: 95 °C for 11 min; 96 °C for 1 min; 10 cycles of 94 °C 30 s, 55 °C 30 s, 72 °C 1 min; 22 cycles of 90 °C 30 s, 55 °C 30 s, 72 °C 1 min; 60 °C for 45 min.

2.4. Analysis of samples on ABI 3100 or 3100 Avant

Samples are run on either type of Genetic Analyzer using the G5 matrix set using matrix standards DS-33 (ABI). These include 6FAM (blue), VIC (green), NED (yellow), PET (red) and LIZ (orange). Samples are prepared using 18.5 μl of Hi-Di™ formamide, 0.5 μl of GeneScan™ 500 LIZ® size standard (both ABI) and 1 μl of PCR product. Samples are denatured for 5 min at 95 °C, then snap cooled on ice. Both the 3100 and 3100 Avant are equipped with 36 cm arrays, with separation of fragments using POP-4™ polymer.

3. Results and discussion

The short amplicon primer sets used here are as previously reported [3]. However, we have selected novel combinations of primer sets into three multiplexes, in an attempt to maximize utility for our purposes (Table 1). We selected one multiplex (MP1) for high stand-alone discrimination potential, as a principle tool for reassociation between skeletal elements. The average random match probability of MP1 is 1.9×10^8, which for direct matching is well sufficient for reaching ICMPs match reporting threshold (99.95% posterior probability). Multiplex 3 (MP3) was arranged to target mainly the loci of PP16 that have the largest amplicons, with the rationale that this would most often provide additional data to complement partial profiles obtained with PP16. The MP3 amplicons are an average of 212 bp shorter than the corresponding loci in PP16. Miniplex 2 is not as specialized in intent as the other multiplexes, but simply targets additional larger loci to fill in when needed with degraded samples. The multiplexes are designed to overlap in at least one locus to demonstrate confirmatory results between amplifications.

Table 1
Loci of ICMP miniplexes

Locus	Commercial kit size range	Size change ICMP mini	ICMP miniplex
Amel.	106–112	+20	1
vWA	123–171	−35	2
HUMTHO1	156–195	−105	1
D13S317	176–208	−88	2
D8S1179	203–247	−117	2
D7S820	215–247	−79	2
D21S11	203–259	−50	1, 2, 3
D16S539	264–304	−183	(1)
D2S1338	289–341	−199	1
D18S51	290–366	−177	1
CSF1PO	321–357	−232	2, 3
FGA	322–444	−197	1, 3
PentaD	376–449	−282	(1), 3
PentaE	379–474	−299	3

Amplicon sizes are compared to those of Promega PowerPlex 16, with the exception of D2S1338 which is not represented in PP16 (the commercial size is listed from ABI IdentiFiler). Two versions of MP1 have been used, the earlier version included D16S539 instead of Penta D; the latter is currently in use.

Initial trials were run varying a number of parameters prior to adoption of final conditions for further validation and final implementation. These include trials with four buffer/enzyme combinations (ABI TaqGold buffer, ABI AmpflSTR buffer, Takara Ex Taq buffer and Promega Gold Star buffer; Taq Gold or Takara Ex Taq), and a variety of $MgCl_2$ concentrations (not shown). The most suitable general combination appeared to be TaqGold enzyme with Gold Star buffer. Sensitivity studies were performed indicating sensitivity on non-degraded DNA comparable to the PP16 kit. Concordance studies were performed for all three miniplexes with both PP16 and AmpfliSTR SE-Filer on 300 unrelated individuals from the former Yugoslavia. Two differences were observed amongst the compared systems: in one case for D18S51, PP16 and MP1 showed a 14, 15, while SE-Filer amplified only the 15 allele, apparently reflecting a primer binding site mismatch; in the other case, for D8S1179 SE-Filer and MP3 showed a 13, 14 while PP16 apparently had a primer binding site mismatch that permitted only the slightest amplification of the 14 allele. Discrepancies with PP16 are of particular concern for our application, as all the family reference blood samples have been typed with this system. Overall the degree of concordance was quite high, and the single PP16 discrepancy is viewed as acceptable because the ICMP family match searching algorithms are permissive to single locus mismatches, to account for within pedigree mutations or other anomalies.

The success of typing of MP1 on degraded samples permitted it to become an inexpensive tool for bone:bone reassociation. To date more than 3000 MP1 profiles have been generated from bones processed for reassociation purposes. In these cases, DNA typing serves to confirm or refute hypotheses generated by physical anthropology specialists, as well as to provide a database where blind matches can also be made. This is particularly important since secondary mass graves are characterized by extensive commingling of body parts. Fig. 1 shows a skeleton that was recovered as eight sets of anthropologically associated/articulated partial remains, recovered from two secondary mass graves separated by 30 km. Typing with MP1 permitted definitive association amongst these partial sets that otherwise would have been impossible given the scale and context of the recovery. Obviously, reassociating skeletons in this manner is expensive, and raises the question of how far one should go in identifying different components of disassociated remains. This difficult issue, however, is reduced in severity by a highly informed anthropological sampling strategy, and by the significant reduction in cost that has been achieved in using the smaller, non-commercial MP1.

To evaluate the potential of the short amplicon multiplexes to improve recovery rates from difficult samples, we compared

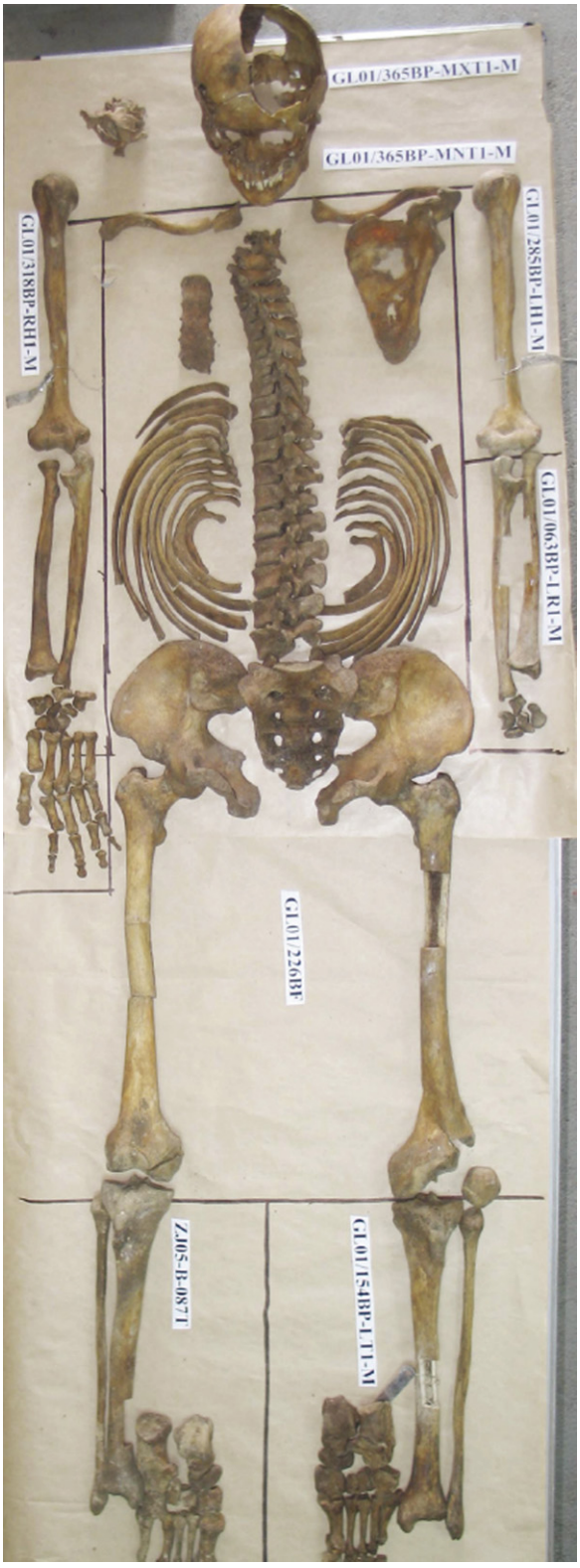

Fig. 1. An example of a bone:bone re-association composed of eight sets of anthropologically associated/articulated partial remains recovered from two secondary mass graves. Each set of remains, represented within the black lines, were sampled. PowerPlex 16 profiles of the torso and right lower leg were matched with family reference samples resulting in a personal identity for the victim. Miniplex typing of the other sets of remains provided matches to the PowerPlex 16 profiles, allowing for a virtually complete skeleton to be reassociated and returned to the family.

success rates on five bone samples that were recovered from a grave where lime ($Ca(OH)_2$) had been applied. The lime appeared to have deleterious effects on DNA preservation, and success was quite limited with PP16. Success rates for PP16 versus the miniplexes are shown in Fig. 2(a–c), as assessed by the percentage of alleles that were reportable according to ICMP calling thresholds for homozygotes or heterozygotes. With the exception of amelogenin, where typing was 100% successful for all multiplexes, the mini-amplicon multiplexes produced better results for all loci. Not surprisingly, this is most pronounced for MP3, which targets the largest of the PP16 loci.

The results above conform to expectations for increased recovery rates with short amplicons when applied to degraded samples. However, our experience indicates that there are many

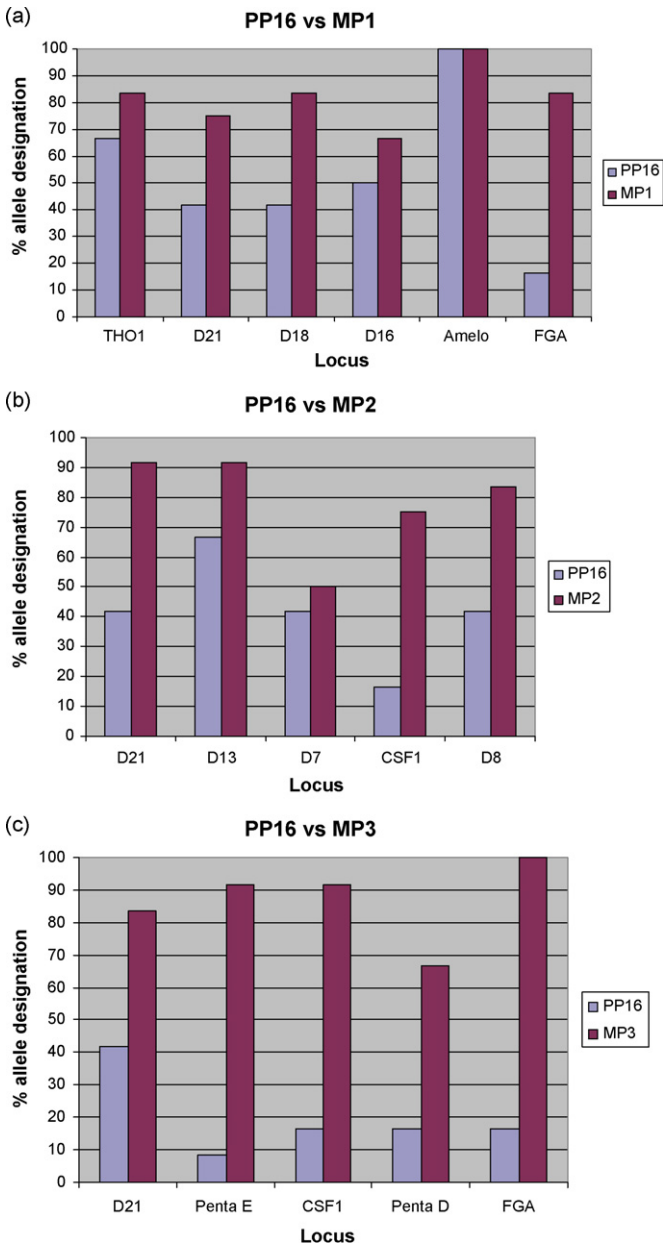

Fig. 2. Proportion of reportable alleles recovered from either PP16 or the ICMP short-amplicon multiplexes obtained from a series of five highly challenging bone samples. (a) Results for MP1, (b) results for MP2 and (c) results for MP3.

factors that affect the utility of these ''mini-plexes.'' First, there are limitations to the number of loci that can be included in multiplexes when minimizing amplicon size is a priority: multiple loci cannot be easily be separated by size within the same florescent dye layer. A 16-plex where the six largest amplicons loci drop out still provides more genetic information than a short amplicon 6-plex with 100% amplification success. Secondly, DNA length fragmentation is only one parameter that causes difficulties in successful amplification of degraded skeletal remains. Taq polymerase inhibition is a pervasive factor in this type of work that can affect PCR success independently of amplicon size—although it is also true that shorter targets can amplify more efficiently and might be more resistant to partial inhibition. Another is high ''sequence complexity'' caused by the presence of sometimes very large quantities of non-human DNA (bacterial, fungal, etc.) that mimics polymerase inhibition by greatly decreasing PCR efficiency—and occasionally gives rise to artifactual amplicons. Again, the higher intrinsic efficiency of shorter amplicons and multiplexes involving fewer loci may provide advantages in this regard as well.

On balance, in our experience the circumstances under which the mini-plexes provide substantial additional capability in recovering genetic data are rather narrow. Our success rate for ''typical'' samples is high enough that the advantages of standard commercial kits rule the day. For the subset of more difficult cases, ~15–20% of our samples, we are able to recover usefully more data with the mini-plexes approximately 35% of the time. In other instances, amplicon size apparently is either not the key limitation (within the relevant differential provided by the mini-plexes), or DNA preservation is so poor that reliable profiles cannot be obtained from either approach. Improving success with these difficult cases may be best approached with a parallel emphasis on improvements in DNA extraction methods [8]. Nonetheless, reduced amplicon multiplexes are clearly an important tool in skeletal remains casework, and the low cost and high success rate permits them to be a major benefit to the challenging issue of complex reassociation of commingled cases.

Acknowledgements

The ICMP acknowledges the generous support provided by the governments of Canada, Denmark, Finland, France, Germany, Greece, the Holy See, Iceland, Ireland, Italy, the Netherlands, Norway, Sweden, Switzerland, the United Kingdom, and the United States, as well as the European Union and the C.S. Mott Foundation.

References

[1] Charles Brenner, http://dna-view.com/dnaview.htm.
[2] L.G. Biesecker, J. Bailey-Wilson, J. Ballantyne, H. Baum, F.R. Bieber, C. Brenner, B. Budowle, J.M. Butler, G.P. Carmody, P.M. Conneally, B. Duceman, A. Eisenberg, L. Forman, K.K. Kidd, B. LeClair, S. Niezgoda, T.J. Parsons, E. Pugh, R. Shaler, S.T. Sherry, A. Sozer, A. Walsh, DNA identification of victims from the 9/11 World Trade Center Attack: scientific challenges and policy implications, Science 310 (2005) 1122–1123.
[3] J.M. Butler, Y. Shen, B.R. McCord, The development of reduced size STR amplicons as tools for analysis of degraded DNA, J. Forensic Sci. 48 (2003) 1054–1064.
[4] P. Grubwieser, R. Mühlmann, B. Berger, H. Niederstätter, M. Pavlic, W. Parson, A new ''miniSTR-multiplex'' displaying reduced amplicon lengths for the analysis of degraded DNA, Int. J. Leg. Med. 120 (2006) 115–120.
[5] D.T. Chung, J. Drabek, K.L. Opel, J.M. Butler, B.R. McCord, A study on the effects of degradation and template concentration on the amplification efficiency of the STR miniplex primer sets, J. Forensic Sci. 49 (2004) 733–740.
[6] L.A. Dixon, A.E. Dobbins, H.K. Pulker, J.M. Butler, P.M. Vallone, M.D. Coble, W. Parson, B. Berger, P. Grubwieser, H.S. Mogensen, N. Morling, K. Nielsen, J.J. Sanchez, E. Petkovski, A. Carracedo, P. Sanchez-Diz, E. Ramos-Luis, M. Brion, J.A. Irwin, R.S. Just, O. Loreille, T.J. Parsons, D. Syndercombe-Court, H. Schmitter, B. Stradmann-Bellinghausen, K. Bender, P. Gill, Analysis of artificially degraded DNA using STRs and SNPs-results of a collaborative European (EDNAP) exercise, Forensic Sci. Int. 164 (2006) 33–44.
[7] M.D. Coble, J.M. Butler, Characterization of new miniSTR loci to aid analysis of degraded DNA, J. Forensic Sci. 50 (2005) 43–53.
[8] O.M. Loreille, T.M. Diegoli, J.A. Irwin, M.D. Coble, T.J. Parsons, High efficiency DNA extraction from bone by total demineralization, Forensic Sci. Int. Genetics 1 (2007) 191–195.