CROATICA CHEMICA ACTA
CCACAA **78** (3) 393–396 (2005)

ISSN-0011-1643
CCA-3026
Original Scientific Paper

High Estimated Likelihood Ratio Might Be Insufficient in a DNA-lead Process of Identification of War Victims*

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RECEIVED NOVEMBER 26, 2004; REVISED APRIL 18, 2005; ACCEPTED MAY 10, 2005

With the advance in typing tools and extraction procedures in recent years, DNA analysis has developed into an amazingly powerful method for forensic analysis. For a number of years, autosomal STR (Short Tandem Repeat) typing has been used as a tool in the process of identification of war victims in Croatia. Although DNA typing is very effective in detecting possible identities of exhumed skeletal remains, this approach bears some risk of false identification. The paper presents the case of a match between skeletal remains and the son and wife of a missing person in 13 STR loci. Even though these skeletal remains also matched in 13 loci the mother of the same missing person, additional genetic testing (Y-chromosome and mitochondrial DNA) unequivocally excluded the proposed identity. Although likelihood ratio is the best measure of the significance of a genetic match between exhumed skeletal remains and relatives of the missing person, the meaning of likelihood ratio is not as clear in database matching as in simple paternity cases and great care is needed to avoid wrong interpretation. To reduce the risk of possible false identifications, in addition to DNA evidence, other types of evidence (such as information about the time, place and other conditions of disappearance), as well as on anthropological and other »classical« forensic data are being used as a »control mechanism« in the DNA-lead process.

Keywords
• amplified fragment length polymorphism
• Y chromosome
• mtDNA
• DNA typing
• human identity
• paternity

INTRODUCTION

Owing to recent advances in typing methods and extraction procedures, DNA analysis has developed into an amazingly powerful tool for forensic analysis and is today routinely used in casework, paternity analysis and identification of victims of mass fatality events. Increase in the number of analyzed loci significantly increased the evidential value of STR typing and this system is now generally considered sufficient to determine iden-

* Dedicated to Professor Željko Kućan on the occasion of his 70th birthday. Presented at the Congress of the Croatian Society of Biochemistry and Molecular Biology, $HDBMB_{2004}$, Bjelolasica, Croatia, September 30 – October 2, 2004.

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tity or paternity with very high probability of inclusion or exclusion. The evidential value of a genetic match is usually expressed as the likelihood ratio (LR) that tends to be astronomically high and seem very convincing. Although genetically and statistically sound and widely accepted, these values do not have the same significance in all situations, and as recently reported, can sometimes be quite misleading. This is particularly the case when DNA typing is used as a tool to lead the process of identification in mass fatality events. The fact that a potential match was found by searching through thousands or hundreds of thousands of unrelated genotypes could decrease the evidential value of the calculated likelihood ratio. In addition, a factor that cannot be accurately included in these calculations is the effect of local inbreeding. Even though it is logical to assume that local inbreeding will occur in isolated or partly-isolated subpopulations, it is very hard to quantify the significance of this phenomenon, as it may not be visible when larger populations are examined.

In the process of identifying skeletal remains of war victims, an unexpectedly large number of matches was observed between skeletal remains and relatives of missing persons that were later shown not to be related with the particular skeletal remains. An extreme example of a genetic match between 17 independent STR alleles that was subsequently shown to be a random genetic match is reported.

EXPERIMENTAL

DNA Extraction

DNA was isolated from powdered samples of skeletal remains as previously described. Briefly, tooth or bone samples were decalcified for 48 h at 56 °C in 50 mmol dm^{-3} Tris-HCl buffer (pH = 8.0) containing 50 mmol dm^{-3} EDTA, 100 mmol dm^{-3} NaCl and 0.7 mg/ml Proteinase K. DNA was isolated by extraction with phenol-chloroform-isoamyl alcohol (25:24:1), followed by n-butanol extraction. Isolated DNA was concentrated and further purified by ultrafiltration on Centricon-100 concentrators (Millipore, Bedford, MA, USA). Blood samples from relatives of missing persons were collected on FTA cards (Whatman Bioscience, Cambridge, UK) and DNA was purified by Chelex extraction. Bone extraction was performed four times, and blood extraction two times in two independent laboratories (DNA Laboratory of the School of Medicine in Osijek and the Laboratory for Clinical and Forensic Genetics of the University Hospital in Split).

DNA Analysis

Autosomal DNA loci were amplified using the AmpFlSTR Identifiler Kit (Applied Biosystems, Foster City, CA USA) according to the manufacturer's instructions. Identical results were obtained by independent analyses in two different laboratories. The probability of parenthood was calculated as described earlier using local population data. Six Y chromosome DNA loci (DYS 393, DYS 19, DYS 389II, DYS 390, DYS 391, and DYS 385a/b) were analyzed using a Y-Plext 6 Kit from Reliagene (New Orleans, LA) according to the manufacturer's instructions. Mitochondrial DNA was analyzed using immobilized sequence-specific oligonucleotides as previously described.

RESULTS

DNA was extracted from a bone sample originating from skeletal remains found in a mass grave in Vukovar, Croatia. STR genotype was successfully determined on 14 STR loci covered by AmpFLSTR Identifiler Kit (D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D19S433, vWA, TPOX, D18S51, D5S818, and FGA). D2S1338 locus did not amplify in any of the four separate extractions and purifications performed (Table I).

By comparing the obtained genotype with genotypes of relatives of missing persons in the database, a potentially significant match with the son and spouse of a missing person (subsequently referred to as missing person A) was identified. There was a match between skeletal remains and the child and wife of the missing person A in 13 loci (LR = 390 090). However, there was a mismatch in D3S1358 locus (Table I). Using the mutation rate (0.0013) and average probability of exclusion (0.53) for D3S1358 locus reported in the American Association of Blood Banks Parentage Testing 2002 Annual Report Summary, paternity index (PI) for this mutation was calculated to be 0.0023 (observed rate of mutation divided by average probability of exclusion), which decreased the LR to 897.2.

TABLE I. Autosomal STR genotypes of the skeletal remains "NN" and relatives (mother, son and wife) of the missing person "A"[(a)]

STR Locus	Spouse	Son	NN	Mother
D8S1179	13; 13	13; 14	14; 14	12; 14
D21S11	30; 31.2	29; 30	29; 31.2	29; 30
D7S820	10; 12	8; 12	8; 8	8; 10
CSF1PO	10; 12	12; 12	9; 12	9; 10
D3S1358	**16; 17**	**16; 16**	**15; 18**	16; 18
TH01	9.3; 9.3	7; 9.3	6; 7	7; 9.3
D13S317	8; 11	8; 9	**9; 11**	**10; 12**
D16S539	9; 13	11; 13	11; 13	11; 11
D2S1338	16; 19	17; 19	–	17; 17
D19S433	14; 15	14; 15	13; 15	14; 15
vWA	14; 18	14; 16	15; 16	16; 18
TPOX	8; 11	9; 11	9; 11	8; 11
D18S51	13; 14	14; 14	14; 18	18; 20
D5S818	11; 12	11; 12	11; 12	11; 12
FGA	21; 22	21; 22	21; 21	19; 21
Amelogenin	X; X	X;Y	X; Y	X; X

[(a)] Loci that were not in agreement with the proposed identity are bolded.

TABLE II. Y-chromosome STR genotypes of the skeletal remains "NN" and the son of the missing person "A"[a]

Locus	Son	NN
DYS 393	14	14
DYS 19	**15**	**14**
DYS 389II	**32**	**31**
DYS 390	25	25
DYS 391	**10**	**9**
DYS 385a/b	**12; 13**	**17; 19**

[a] Loci that led to exclusion of proposed identity are bolded.

TABLE III. Mitochondrial DNA genotypes of the skeletal remains "NN" and the mother of the missing person "A"[a]

Locus	Mother	NN
HVI A	**1**	**2**
HVI C	1	1
HVI D	**1**	**0**
HVI E	1	1
HVI 16093	1	1
HVII A	2	2
HVII B	**5**	**1**
HVII C	**2**	**0**
HVII D	1	1
HVII 189	**1**	**0**

[a] Loci that led to exclusion of proposed identity are bolded.

Mother of the missing person A also gave a blood sample for the identification process, and by comparing these two genotypes it was found that they matched in 13 of the 14 analyzed loci (Table 1). The likelihood ratio for the matching 13 loci was 4 980, and when a single mutation in D13S317 locus was included (calculated analogously to mutation in D3S1358 locus), LR for this mother/son relationship was 13.9. Since grandmother and grandson cannot be considered independent, it was not possible to multiply these two likelihood ratios, but the fact that these remains matched with the son and a wife in 13 STR loci and in addition with the mother of the same missing person also in 13 loci (4 independent alleles and 9 alleles that were shared between grandmother and grandson) was quite a strong evidence in favor of this identity.

To reach the final conclusion, additional analyses of Y-chromosome STR and mitochondrial loci were performed as described in the Experimental. Four of the six analyzed STR loci (Table II) and five of the ten analyzed mitochondrial loci (Table III) did not match and the hypothesis that the skeletal remains originated from the missing person A was excluded.

DISCUSSION

A random match between STR genotypes of exhumed skeletal remains and the wife and a son of a missing person in 13 STR loci is reported. In addition the same skeletal remains matched in 4 independent alleles (*i.e.*, different alleles from those matching with the son) with the mother of the same missing person. However, in each of the comparisons (mother with son, and father with wife and a child) there was a single locus that did not match, which raised significant doubt about the correctness of this hypothesis. By performing additional genetic analyses, it was proven that this match was a random event. Such a close match might indicate that these skeletal remains originated from a close relative, but this missing person has no close relatives that are still missing.

By this example it is pointed to the high risk of making false conclusions in the case of mass fatality events. Such situations frequently require isolation of DNA from skeletal remains, which is extremely difficult and due to a low amount of DNA, high level of DNA degradation, and the presence of contaminations, frequently associated with inability to amplify all analyzed loci. If in the presented case one of the mismatched loci originally analyzed (D3S1358) had not amplified, as was the case with D2S1338 locus, even with a single mutation included in the calculation, a very convincing false conclusion would be made. There would be a full 13-loci match between skeletal remains and the son and mother of the missing person A with LR = 390 090, and additional 13 loci match with the mother of the same missing person with LR = 13.9 (with mutation included in the calculation). In this case, the logical conclusion would be that there was a single mutation, and that the skeletal remains belonged to the missing person A, an identity that was excluded by analyzing Y-STR loci and mitochondrial DNA.

Here it is important to note that this problem is associated only with mass fatality events where reference samples of missing people do not exist and the identity is being determined by reverse paternity analysis. Contrary to other types of databases (*e.g.,* databases of convicted offenders) where all alleles provide useful information, in the case of reverse paternity it is known that one of the child's alleles on each loci originates from the mother, and only the other allele can be used to identify the missing father.

DNA typing, and particularly the analysis of STR loci, is a very powerful tool for human identification, but the existence of a match between two genotypes should not be directly converted into identity. This is especially the case when numerous people are missing, because the fact that the observed match is a consequence of random comparisons of thousands of genotypes in the database significantly reduces the evidential value of the match. On the other hand, dividing calculated likelihood ratios by the number of genotypes in the database is meaningless (especially as databases become larger and larger), because it unjustly reduces the significance of the »real« match and could even pose an insurmountable barrier in

cases where only a limited number of relatives of a specific missing person are available. In addition to obtaining as much DNA evidence as possible (Y-STR, mitochondrial DNA), identification of war victims in Croatia, heavily relies on other types of evidence (such as the information about time, place and other conditions of disappearance), as well as on anthropological and other »classical« forensic data as a »control mechanism« in the DNA-lead process. This approach appears to be quite effective in pointing to and correcting situations when DNA evidence (genetic matches) points to a wrong direction. If in the presented case only one of the mismatched loci had not amplified when originally analyzed, our cumulative likelihood ratio (with included the probability of a single mutation) would be over 4 million, what significantly exceeds currently used threshold of 10 000, and would most probably result in erroneous identification. This problem might turn out to be even more important for the identification of victims in more complex situations (like Bosnia and Herzegovina, or Iraq) where the number of missing people is much higher and will require a several orders of magnitude larger number of random comparisons of genotypes than in Croatia.

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SAŽETAK

Visoke vrijednosti omjera vjerojatnosti ne moraju biti dostatne u procesu identifikacije žrtava rata s pomoću analize DNA

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Zahvaljujući stalnom napretku u metodama izolacije i karakterizacije, analiza DNA razvila se u vrlo moćnu metodu forenzičke analize. Već niz godina analizu autosomnih STR regija primjenjuje se kao temeljnu metodu u procesu identifikacije žrtava rata u Hrvatskoj. Iako je analiza DNA vrlo učinkovita u pronalaženju mogućega identiteta ekshumiranih posmrtnih ostataka, ovakav pristup nosi i određeni rizik pogrešne identifikacije. Ovdje je opisan slučaj poklapanja posmrtnih ostataka sa sinom i suprugom nestale osobe u 13 STR lokusa. Iako su se isti posmrtni ostaci također poklapali i s majkom iste nestale osobe u 13 lokusa, dodatnim genetičkim ispitivanjima (Y kromosom i mitohondrijska DNA) nedvojbeno je isključena mogućnost da se radi o toj osobi. Omjer vjerojatnosti najbolji je pokazatelj značajnosti genetičkoga podudaranja skeletnih ostataka i rodbine nestalih osoba, no kad je to podudaranje pronađeno nasumičnim pretraživanjem baze podataka, značaj omjera vjerojatnosti nije tako jasan kao u jednostavnome utvrđivanju očinstva i potreban je velik oprez kako ne bi došlo do njegove pogrešne interpretacije. Radi smanjivanja rizika pogrešne identifikacije, osim na rezultate analize DNA, pri utvrđivanju identiteta velik značaj potrebno je dati i drugim tipovima dokaza (poput vremena, lokacije i uvjeta nestanka) te antropološkim i ostalim »klasičnim« forenzičkim dokazima, kao kontrolnome mehanizmu u procesu identifikacije predvođenom analizom DNA.