How High Should Paternity Index Be for Reliable Identification of War Victims by DNA Typing?

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Aim. To analyze statistically and logically the significance of genetic matches between skeletal remains and relatives of missing persons in the process of identification of war victims by DNA typing.

Methods. DNA was isolated from bone and blood samples and short tandem repeat (STR) loci were typed by using AmpFLSTR Profiler, Profiler Plus, and Identifiler kits. Novel mini-haplotype analysis that compares matches in all three-locus combinations of alleles was developed and used in the analysis of inbreeding in the group of 295 unrelated individuals.

Results. While comparing 98 skeletal remains exhumed in the process of identification of war victims in Croatia with over 3,000 genotypes of relatives of missing persons, we revealed 20 cases of 14-locus matches and 4 cases of 15-locus matches between unrelated people. We hypothesized that this unexpectedly high number of false matches might be a consequence of local inbreeding and supported this hypothesis with very low correlation between the probability of a genotype and the number of matching genotypes in the database ($R^2 = 0.36$). Further support for the hypothesis was obtained by the analysis of mini-haplotypes, which revealed up to 90% overrepresentation of some mini-haplotypes.

Conclusions. STR DNA typing is the “golden standard” of human identification, but evidential value of a genetic match can be easily misinterpreted. Therefore, careful use of statistical methods is essential for the proper evaluation of laboratory results. Whenever possible, multiple relatives should be analyzed and other evidence based on the information about time, place, and other conditions of disappearance, as well as anthropological and other “classical” forensic data should always be put together and compared before any final decision about the identity is made.

Key words: paternity; pedigree; polymorphism (genetics); tandem repeat sequences

Over a decade has passed since the 1991-1995 war in Croatia and more than a thousand families are still looking for the missing members of their families (1). Unfortunately, most missing persons have been exhumed from mass graves and their identity has been determined by DNA typing and other forensic methods. The value of DNA typing significantly increased in the last decade due to the increase in the number of analyzed short tandem repeat (STR) loci, and is considered today sufficient for the determination of identity and paternity (2,3). However, since the evidential value of a genetic match may vary considerably, elaborate statistical methods have been developed to quantify this significance (4).

In “normal” situations, we use DNA typing to confirm that two samples originate from the same individual or to dispute fatherhood. In the process of identification of war victims, on the other hand, we randomly compare genotypes of each unidentified body with genotypes of several thousands of potential relatives in our database (5,6). Large number of unrelated genotypes being compared in a search for a matching genotype significantly reduces the evidential value of a genetic match. We have recently reported significant risks of false inclusion associated with this approach when 9- or 12-locus STR systems are used (7), and have subsequently increased the discriminatory power of our analysis by switching to a system that analyzes 15 different loci. However, in some cases even this system has not been sufficient. We report here on several 15-locus matches between unrelated individuals and suggest a hypothesis that could explain these unexpected matches.

Material and Methods

DNA Typing

One hundred forty-six powdered samples of skeletal remains, tooth or bone, were decalcified for 48 h at 56 °C in 50
mmol/L Tris/HCl buffer pH 8.0 containing 50 mmol/L EDTA, 100 mmol/L NaCl, and 0.7 mg/ml Proteinase K (Invitrogen, Carlsbad, CA, USA). 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 973 relatives of missing persons were collected on FTA® cards (Whatman Bioscience, Cambridge, UK) and DNA was purified by Chelex extraction (8). Puriﬁed DNA was ampliﬁed with AmpFLSTR Proﬁler, AmpFLSTR Proﬁler Plus, and AmpFLSTR Identiﬁer kits (Applied Biosystems, Foster City, CA, USA) according to manufacturer’s recommendations.

Obtained genotypes of relatives of missing persons were included into the Croatian database of relatives of missing persons, which is being compiled by joint efforts of DNA laboratories in Zagreb, Split, and Osijek. We calculated the probability of parenthood (paternity index, Pi, and maternity index, Mi) using formulas for calculation of the system index with a parent missing (4) and local population data (9). Expected (He) and observed (Ho) heterozygosity were calculated with Genetic Data Analysis (GDA) software (10). Inbreeding coefﬁcients (I) were calculated by using the same software according to the following formula (10):

\[ I = \frac{H_e - H_o}{H_e} \]

Analysis of Three-locus Haplotypes

We developed a simple computer program for this study, which enabled us to generate all possible combinations of alleles on all possible three-locus combinations of STR loci covered by AmpFLSTR Proﬁler Plus kit (D3S1358, vWA, FGA, D8S1179, D21S11, D18S51, D5S818, D13S317, and D7S820). To determine the observed frequencies of all minihaplotypes (three-locus haplotypes), the program compared each individual genotype with all possible minihaplotypes. The genotype was considered “positive” for the speciﬁed minihaplotype if matching alleles were found at all three speciﬁed loci, either homozigous or heterozygous or heterozygous form.

The expected frequency (F) of a given minihaplotype “x,y,z” was calculated by using the following formula and local population data:

\[ F = 2p_x \times 2p_y \times 2p_z \]

Results

The genotype of each skeletal sample was compared with genotypes of all potential relatives in the database, which currently contains over 3,000 genotypes. A homemade algorithm in Microsoft Excel was used to identify putative parents and children. To be included in the group of possible parents or children, the compared genotypes needed to share at least one allele on each analyzed locus. These preliminary matches were then either conﬁrmed or eliminated by comparison with further relatives of the same missing person. Unfortunately, for some missing persons not only that no additional relatives were available, but sometimes there was no other forensic evidence. Thus, comparisons of two genotypes were the only available evidence. As of March 2003, we have determined 15-locus genotypes of 98 skeletal remains and compared them with all genotypes in the database. During the process, we have identiﬁed 20 cases of a 14-locus match and four cases of a 15-locus match between unrelated individuals (Table 1). Although the paternity index (Pi) for two of these matches was quite high (Pi ≥10,000), they were all eliminated as potential parents/children by comparisons with other relatives or forensic data (data not shown).

When averaged for all loci, expected frequency of a 15-locus genotype covered by the AmpFLSTR Identifer kit (D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D25S138, D19S433, vWA, TPOX, D18S51, D5S818, and FGA) is one in 2×1016 (as reported by the manufacturer). Single parent analysis is much less discriminatory since it relays on only half of the genotype, and the exact calculation of the false-match probability is not straightforward. Presciuttini and colleagues (11) recently showed that the probability (p) of two unrelated individuals sharing a single allele on a particular locus can be estimated from the heterozygosity (H) of that locus according to the following formula: p = 2H – 2H2. Using heterozygosity values for the Croatian population, we calculated that the cumulative probability for a false 15-locus match was approximately one in 6.7×1010. Comparing 98 genotypes of skeletal remains with over 3,000 possible relatives (approximately 300,000 random comparisons), we observed four false 15-locus matches, which was nearly two orders of magnitude more than estimated by statistical calculations.

<table>
<thead>
<tr>
<th>Locus</th>
<th>SR-1</th>
<th>RMP-A*</th>
<th>SR-2</th>
<th>RMP-B*</th>
<th>SR-3</th>
<th>RMP-C*</th>
<th>SR-4</th>
<th>RMP-D*</th>
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<td>6,6</td>
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*Each of the “relatives of missing persons” was excluded as a possible relative of the particular skeletal remains by analyzing other family members, or using other forensic evidence.

†PI – Paternity index, MI – Maternity index (4).
While performing manual analysis of all these potential parents/children, we noticed that the number of observed potential matches varied significantly between different skeletal remains. Some genotypes matched less than five genotypes, whereas others matched over a hundred. However, the majority of genotypes in our database of relatives of missing persons still consist of 9- or 12-STR loci, and these matches were 9- or 12-locus matches. Initially we assumed that the observed number of matches were in correlation with the probability of the individual genotype. To confirm the assumption that a genotype containing more frequent alleles would have more matches, we calculated the probabilities of each individual genotype and correlated it with the number of full matches. We were very surprised to find that the correlation between the frequency of a genotype and the percentage of relatives who could be possible parents/children of this genotype was only 0.36 (Fig. 1).

Over 29,000 individual minihaplotypes were identified, but only 6,310 minihaplotypes that occurred with frequency of over 2% were used in further analysis. The most frequent minihaplotype was D8S1179:13; D5S818:12; D13S317:11, found in 27.7% of all analyzed individuals. The average frequency of a minihaplotype was 4.8%. The frequency of some minihaplotypes was notably different from the expected frequency. A selection of minihaplotypes with particularly large deviations from expected values is listed in Table 3.

Discussion

In the process of identifying skeletal remains we observed unexpectedly high number of matches between skeletal remains and relatives of missing persons in our database, who were later shown not to be related. DNA typing, and in particular analysis of STR loci is a very powerful tool for human identification, but the significance of a genetic match can vary significantly and careful use of statistical formulas is essential for proper determination of their evidential value (12). Although genetically and statistically sound and widely accepted, calculations that we perform today (4) produce numbers that might not be fully applicable in all situations (13,14). One of the factors not included in these calculations is the effect of local inbreeding. Although it is logical to assume that something like that may have occurred in isolated or partly-isolated subpopulations, it is very difficult to quantify the significance of this phenomenon as it...
may not be visible where larger populations are examined (15). Significant local differences in allele frequencies were reported to exist in some regions of the world (16), but our attempts to find any significant difference in the frequency of individual alleles in our local subpopulations were not successful (data not shown).

Using a minihaplotype approach developed for this study, we demonstrated that some combinations of minihaplotypes were significantly overrepresented in the studied population. Since the presence of the same minihaplotype in two individuals would qualify them for a parent/child relationship, the significance of the genetic match would be overestimated when calculated on the basis of individual allele frequencies. The increased frequency of specific combinations of alleles, or minihaplotypes, could explain the unexpectedly high number of false 14- and 15-locus matches observed during the process of identification of war-victims in Croatia. The deviations that we have shown are already large enough to raise concern, but it is also quite reasonable to assume that these minihaplotypes would be even more overrepresented in some local communities, which would make the risk of assigning wrong identity even higher. The magnitude of this problem correlates with the size of the database, and through personal communication with International Commission on Missing Persons (ICMP; ref. 17) we have learned that similar effects have been observed in the process of identifying war victims in Bosnia and Herzegovina.

To provide additional support for our hypothesis that the probability of a match between two genotypes is not determined exclusively by the frequency of individual alleles, we analyzed the correlation between the probability of an individual genotype and the number of potential parents/children in the database. Current calculations of genetic match evidential value are based on general assumption that sharing a rare allele increases the probability of two individuals being related (4). However, our results clearly demonstrated that the correlation between the probability of a genotype and the number of potential parents/children in the database was very low. It is generally accepted that the comparison of genotypes of siblings or close relatives yields much higher probability of a match than that of unrelated people (18). In our study population, we found very low inbreeding coefficient, although some isolated populations are considered to be highly inbred. Perhaps the analysis of minihaplotypes in well-defined subpopulations will identify specific highly overrepresented allele combinations, which should be carefully screened before calculating the probability of a genetic match.

In conclusion, although STR DNA typing has become the “gold standard” of human identification, evidential value of a genetic match can easily be misinterpreted and careful use of statistical methods is essential for proper evaluation of laboratory results. This is especially the case in a situation where a number of people are missing and each individual genotype is being compared with hundreds or thousands of potential relatives. Using a novel minihaplotype approach, we showed that local inbreeding effects exist in Croatian population and cannot be identified by classical methods. We also found that in some cases even a relatively high PI of over 10,000 might not be sufficiently high for the correct determination of identity. Whenever possible, multiple relatives should be analyzed, and other evidence based on the information about time, place, and other conditions of disappearance, as well as anthropological and other “classical” forensic data should always be put together and compared before any final conclusion about the identity of the skeletal remains is made.

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