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Population Substructure Can Significantly Affect Reliability of a DNA-led Process of Identification of Mass Fatality Victims

ABSTRACT: Aiming to evaluate the effects of population substructure on the reliability of a DNA correspondence in the process of human identification, we used the model of "in silico" constructed populations with and without substructure. Effects of population substructure were evaluated at the level of locus heterozygosity, Hardy–Weinberg equilibrium and mini-haplotype distribution. Inbreeding in a subpopulation of 100 individuals through 10 generations did not significantly alter the level of heterozygosity and Hardy–Weinberg equilibrium. However, analysis of mini-haplotype distribution revealed a significant homogenization in separated subpopulations. Average observed mini-haplotype frequency (f_o) increased to threefold from expected values (f_e), and the number of mini-haplotypes with f_o/f_e above 10 increased over sixfold, suggesting that the effects of population substructure on calculated likelihood ratios (LR) might be larger than previously estimated. In most criminal cases, this would not represent a problem, whereas for identifications in large-scale mass fatality events, population substructure might considerably increase the risk of false identification.

KEYWORDS: forensic science, human identification, DNA typing, STR loci, population substructure, inbreeding, mini-haplotypes

Nowadays, DNA analysis is routinely used in casework, paternity analysis, and the identification of victims of mass fatality events (1–3), and the widely applied fifteen STR loci system is generally considered sufficient to determine the identity or paternity with a very high probability of inclusion or exclusion (4–6).

DNA evidence is often interpreted using the genetic model known as the product rule, and evidential value of a genetic match in biological relationship testing is usually expressed as likelihood ratio, which tends to be astronomically high and seem very convincing. Product rule assumes both within and between loci independence, although it has been argued that population subdivision inevitably invalidates this assumption (7–10). Nevertheless, product rule approach is widely accepted (11,12). Recently, we reported that likelihood ratio can be quite misleading in some situations when DNA typing is used in the process of identification in mass fatality events (13–15). The fact that a potential correspondence between two DNA profiles was found by searching through thousands (or hundreds of thousands) of unrelated genotypes could decrease the evidential value of calculated likelihood ratio (due to undefined and highly variable prior probabilities). Another factor that cannot be accurately included in the calculation is the effect of population substructure. The effects of inbreeding due to population subdivision have long been recognized in the forensic literature, and several methods have been proposed to account for them when estimating correspondence probabilities (16–19). However, main indication of inbreeding is the deviation from Hardy–Weinberg proportions of homozygous and heterozygous loci, and even in well-segregated population, this is frequently not the case (20). Analysis of clearly separated recent migrant populations concluded that the effects of inclusion of co-ancestry coefficients F_{ST} and inbreeding coefficients F_{IS} in the formula for calculation of likelihood ratios are relatively small (21). Recent simulation of population substructure concluded that departure from Hardy–Weinberg and linkage equilibrium in subpopulations appear to have rather small effect (22).

During our work on the process of identification of war victims in Croatia, we observed a number of correspondences between genotypes that were associated with very high calculated LR, but were later proven to be unrelated (13,15). We have hypothesized that these false correspondences are a consequence of local inbreeding or hidden consanguinity, and developed the method of analyzing 3-loci haplotypes (mini-haplotypes), as a tool to verify the existence of within-population similarities (14,23). The foundation of this approach is the fact that the probability that an individual will share a combination of alleles on several loci with a relative is far larger than the probability that he/she will share this combination with an unrelated person.

We have demonstrated significant deviations from expected frequencies of many mini-haplotypes in the Croatian population (14), but its undefined stratification (the same is true for any other "real" population) did not allow us to estimate reliably its potential impact. Aiming to demonstrate the effects of inbreeding on the frequency of mini-haplotypes in subpopulations, we analyzed the effect of population stratification on the frequency of individual alleles and mini-haplotypes in populations generated "in silico."

Materials and Methods

In silico *mating experiment*

We generated genotypes of 1000 virtual "individuals" on 15 STR loci (D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, D19S433, vWA, TPOX, D18S51, D5S818 and FGA), using allele frequencies that exist in "real" Croatian population. In one experiment, the population of 1000

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"individuals" was mated through 10 generations. Two "children" were derived from one parental pair in the first generation, and the offspring from that generation was then used as population of parents for the second generation, and so long to the 10th generation. 1000 genotypes of 10th generation were used for further analysis, as a population without substructure.

In another experiment, the same population of 1000 "parents" was separated into 10 subpopulations of 100 individuals that were randomly mated within its subpopulation. After 10 generations, 10 subpopulations, consisted of 100 children of 9th generation (groups CS–1 to CS–10) were merged to one, substructured population (CS-M).

This *in silico* experiment was repeated 10 times (starting from the same initial population of 1000 "parents") and all presented results are average values from these 10 experiments.

Analysis of three-locus haplotypes

Three-locus haplotypes of starting generation were generated using a home-developed computer program as previously reported (23). All possible combinations of alleles on all possible three-locus combinations of nine STR (D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539 and D2S1338) loci were used for further analysis. Even though it would be theoretically possible to make combinations for all fifteen loci, the number of generated mini-haplotypes would be too large to handle (more than 60 billion), so we limited our analysis to nine listed loci that can be combined in 60,631 mini-haplotypes with expected frequencies over 0.01% in Croatian population. An analyzed individual was considered to be "positive" for the specified mini-haplotype, if it had matching alleles on all three specified loci, either in homozygous or in heterozygous form. Expected frequency (f_e) of a given mini-haplotype "x, y, z" was calculated from the frequencies of individual alleles (p_x, p_y, p_z) in Croatian population using the following formula:

$$f_e = (1 - (1 - p_x)^2) \times (1 - (1 - p_y)^2) \times (1 - (1 - p_z)^2)$$

Observed frequency (f_o) of a mini-haplotype was determined by simply counting individuals with a specified mini-haplotype in a sample of 100 individuals from an analyzed population (e.g., a given genotype would count positive for D8S1179:12, D21S11:28, D7S820:9 mini-haplotype if it had allele 12 on D8S1179 locus, allele 28 on D21S11 locus and allele 9 on D7S820 locus, either in homozygous, or in heterozygous form).

Descriptive population statistics

Hardy–Weinberg equilibrium expected (He) and observed (Ho) heterozygosity were analyzed using Genetic Data Analysis (GDA) software (Lewis and Zaykin 2001. Genetic Data Analysis: Computer program for the analysis of allelic data. http://hydrodictyon.eeb.uconn.edu/people/plewis/software.php) (24). Inbreeding coefficients (f) were calculated using the same software by the following formula:

$$f = \frac{\mathrm{He} - \mathrm{Ho}}{\mathrm{He}} \quad (24)$$

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Results

Aiming to study the effects of population substructure on the reliability of the process of identification of missing people we generated a population of 1000 virtual "individuals" (represented as STR genotypes). As described in Materials and methods, this population was mated for 10 generations either as a single population, or divided into 10 separated subpopulations that were again merged after 10 generations. This *in silico* mating experiment produced two populations of 10th generation "children"; one with and one without population substructure.

Expected heterozygosity and observed heterozygosity were determined for all populations (Table 1), and, apparently, 10 generations of inbreeding in completely isolated populations of 100 individuals were not enough to generate significant deviations in the inbreeding coefficient. However, bottleneck effect and homogenization were observed as deviations from Hardy–Weinberg equilibrium for a number of loci (Table 2). Nevertheless, since different alleles were lost in different subpopulations, this disequilibrium was blurred when 10 subpopulations were merged into one population, indicating that even very clear stratification in the population cannot be detected in this way.

Mini-haplotype analysis is a novel method we developed for the analysis of similarities between individual genotypes (23). Since it analyses combination of alleles, and not individual alleles, it is much more sensitive than other methods and we have already demonstrated that it can be efficiently used for detection of aberrations in population substructure (14,23). In this study, we applied mini-haplotype analysis to examine the effects of population stratification in defined, *in silico* generated, populations.

TABLE 1—*Heterozygosity in studied populations.*

Population	He	Ho	f
Parents	0.794 (0.003)	0.783 (0.009)	0.014 (0.011)
Children	0.791 (0.005)	0.793 (0.011)	−0.003 (0.011)
CS	0.776 (0.008)	0.788 (0.013)	−0.015 (0.013)
CS-M	0.793 (0.004)	0.789 (0.011)	0.006 (0.013)

Expected heterozygosity (He), observed heterozygosity (Ho) and inbreeding coefficients (f) were determined as described in Materials and methods for 10 subpopulations of 10th generation children (CS), merged substructured population created by combining individual subpopulations (CS-M) and 10th generation of children in population without defined substructure (Children).

TABLE 2—*Probabilities of Hardy–Weinberg disequilibrium in studied populations.*

	Population			
Locus	Parents	Children	CS	CS-M
D3S1358	9	5	28	6
VWA	6	9	22	6
FGA	16	7	33	7
TH01	3	4	21	9
TPOX	7	9	18	6
CSF1PO	3	11	24	9
D5S818	5	2	27	3
D13S317	5	8	17	11
D7S820	8	3	16	9
D8S1179	9	7	24	9
D21S11	7	13	27	8
D18S51	14	5	39	7
D16S539	6	3	19	7
D2S1338	21	9	28	11
D19S433	8	7	20	7

Exact tests for linkage and Hardy–Weinberg disequilibrium were performed as described in Materials and methods on 10 subpopulations of 10th generation children (CS1-CS10), 10th generation of children in merged population with substructure (CS-M) and 10th generation of children in population without defined substructure (C).

TABLE 3—*Mini-haplotypes in substructured and homogenous populations.*

	Population			
	Parents	Children	CS	CS-M
Number of mini-haplotypes	16052 (147)	16152 (478)	13431 (374)	16002 (491)
Number of mini-haplotypes over 2%	5510 (61)	5625 (109)	5719 (103)	5599 (143)
Average frequency (f_o)	5.89 (0.03)	5.95 (0.09)	6.31 (0.14)	5.95 (0.15)
Average expected frequency (f_e)	5.11 (0.04)	5.06 (0.07)	4.61 (0.08)	5.06 (0.09)
Average f_o/f_e	1.61 (0.05)	1.61 (0.07)	2.98 (0.19)	1.62 (0.06)
Haplotypes with $f_o/f_e > 10$	39 (7)	38 (13)	276 (42)	38 (12)

Population of 1000 of individual genotypes (''Parents'') was generated and randomly mated as a homogenous population through 10 generations to yield ''Children.'' The same population was divided into 10 subpopulations of 100 individuals that evolved separately for 10 generations to produce 10 subpopulations of children (CS). These 10 subpopulations were merged into one merged substructured population (CS-M). Presented values are average values (with standard deviations in parenthesis) of 10 independent experiments.

On the basis of the frequency of individual alleles on D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539 and D2S1338 loci in Croatian population, we have identified 60,631 different mini-haplotypes that are expected to exist in Croatian population with frequency larger than 0.01%. Out of these 60,000 theoretically possible mini-haplotypes, in each group of randomly selected 100 individuals from a parental population, we found approximately 16,000 different mini-haplotypes (Table 3). Mating in a small population will result in the elimination of some alleles and, consequently, lead to the reduction in the number of existing mini-haplotypes in the population. After 10 generations of mating within a population of 100 individuals we observed an average decrease of 12.1% (±4.9%) in the number of haplotypes. However, as each of the subpopulations lost different alleles, when mixed together, this was not visible and, as it is clearly shown in Table 3, even though there was a substantial reduction in the number of different haplotypes in individual subpopulations, a random sample of 100 individuals, from a population created by merging 10 separated subpopulations had nearly the same number of different haplotypes as did the random sample of 100 individuals from a population of the same size, which evolved as a single population.

To reduce the effects of random sampling, further analysis of mini-haplotypes was performed only on mini-haplotypes that were observed with frequency over 2%. Between 5,500 and 6,000 different mini-haplotypes were found to be present with frequency over 2% in all analyzed populations. In parental populations the average observed frequency (f_o) of mini-haplotypes was 5.89%, while the average expected frequency (f_e) of the same haplotypes was 5.11%. Each observed mini-haplotype was on average approximately 60% ($f_o/f_e = 1.61$) more represented than expected, and this difference was the reflection of random sampling (since samples of 100 individuals were analyzed, minimal observed frequency of each haplotype was 1%, while their expected frequencies were generally lower). However, after 10 generations of mating within closed subpopulations, average f_o/f_e ratio nearly doubled ($f_o/f_e = 2.98$). The effect of population homogenization was even more obvious when mini-haplotypes that were observed with frequency that was more than 10-times higher than their expected frequency were compared. In parental subpopulations, on average, there was approximately 40 such mini-haplotypes, what represent approximately 1% of mini-haplotypes (with frequency over 2%), and can be explained by statistical fluctuations. However, in 10th generation of children the number of such mini-haplotypes increased more than sixfold (Table 3). Nearly 300 genotypes with f_o/f_e over 10 in a population of 100 individuals mean that each member of the subpopulation on average had three such 10-fold overrepresented mini-haplotypes.

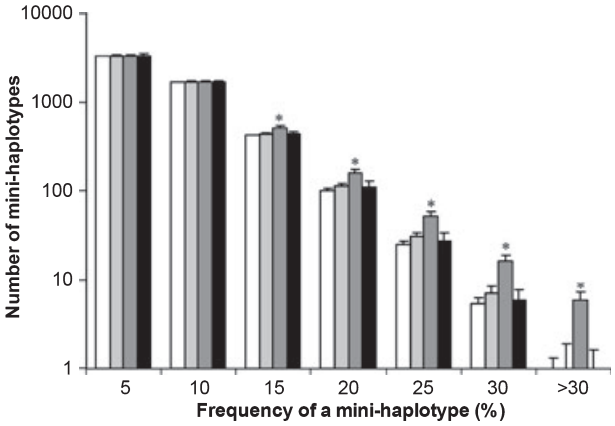

FIG. 1—*Distribution of frequencies of mini-haplotypes (only mini-haplotypes with frequency over 2% were evaluated) in (1) parental population of 1000 individuals (white bars); (2) children that evolved in a single population of 1000 (light gray bars); (3) children who evolved in subpopulations of 100 individuals (dark gray); and (4) population created by merging 10 individual subpopulations of children that evolved separately (black bars). Error bars are standard deviations from 10 experiments. Number of mini-haplotypes is presented on a logarithmic scale to make changes in the distribution of more frequent mini-haplotypes visible. Statistically significant differences ($p < 0.01$)* are marked with ''*''.

When 10 individual subpopulations of 100 people were merged into single population of 1,000, all these deviations became cryptic. Neither f_o/f_e ratio, nor frequency distribution analysis were able to detect any differences between our two populations (Table 3, Fig 1). Nevertheless, those (on average) three mini-haplotypes with $f_o/f_e > 10$ that were observed before merging of subpopulations were still present in each individual genotype, and were making members of each subpopulation much more alike to individuals from their own subpopulation, than to individuals from any other subpopulation.

Discussion

Using our two model populations, we have evaluated the effects of population substructure on the validity of current methods for evaluating evidential value of calculating likelihood ratios. For any two putative relationships among individuals, the likelihood ratio can be calculated in order to assess the relative support of the observed DNA profiles for one relationship compared with the other. For paternity and other relationship testing, likelihood ratios are usually calculated assuming independence of genes, but the

presence of population subdivision invalidates this assumption (25). Hence, within a subpopulation, DNA profiles with corresponding alleles are more common than expected by the independence assumption, even when two individuals are not directly related.

There is no doubt that some level of genetic homogenization in isolated population will occur, but methods to evaluate population substructure are generally not available. Inbreeding as a result of population subdivision is generally manifested in the increased proportion of homozygous individuals and deviations from Hardy–Weinberg equilibrium (17). However, this occurs in relatively late phases, and might not be visible in recently isolated subpopulations. For this experiment, sizes of populations and the number of generations were selected aiming to simulate situations that might have occurred in the "real" world during the past few centuries. Surprisingly, even in very small populations (100 individuals), 10 generations of inbreeding without any contribution to gene pool by migrant or mutation, were not enough to generate visible inbreeding. As any other subpopulation model, ours is an idealized one; it does not take in count mutation, nor migration; nor it can emulate full complexity of human mating habits, but all these factors would actually only decrease, and not increase the effects of inbreeding, and are thus not relevant for this study.

Since each individual has only two alleles on each locus (out of 10–15 different alleles present in the population), it is far more likely that its descendant will have the same combination of alleles, than any other possible combination of alleles (each ancestor acts as a bottleneck for an available gene pool). We have hypothesized that this will be visible through increased frequency of individual combinations (mini-haplotypes) much sooner that any disturbance in heterozygosity or the frequency of individual alleles would be observed. Analysis of individual mini-haplotypes in our virtual populations strongly supported this hypothesis. After 10 generations of mating in subpopulations of 100 individuals, each mini-haplotype was on average three times more likely than expected (Table 3). However, since there was a random loss of alleles, different number of different mini-haplotypes was created in each population. When combined into a 15-loci haplotype (which is a sole basis of determining parenthood in a single parent situation), each member of the subpopulation would be on average 250 times more likely to have a specific 15-loci haplotype, than what would be expected from the frequency of individual alleles. The situation would be much worse if two individuals would share some of the mini-haplotypes that were more overrepresented. As shown in Table 3, in each subpopulation of 100 people there were nearly 300 mini-haplotypes whose frequency was more than 10 times higher than expected. In worst-case scenario, the probability that two independent members of the same subpopulation share the same 15-loci haplotype (combination of 5 three-loci haplotypes) would be several orders of magnitude higher than that expected from the frequency of individual alleles.

Recent comparison of different methods to estimate effects of population subdivision concluded that they are of the order of a factor of 10 (26). Our results indicate that in fact these effects can be up to several orders of magnitude larger. In most forensic cases when genotypes of a suspect and a sample are being compared, likelihood ratios are so high that even inbreeding coefficients of up to 10^4 or 10^5 would not make a significant difference, but in the process of identification of missing people this might not be the case. Missing people are frequently being identified solely on the basis of comparison with a single relative, and in that case likelihood ratios are rarely higher than 10^7 (with 15 analyzed STR loci). If substructure-related uncertainty in LR is combined with the fact that in a mass fatality event an observed correspondence between DNA profiles is generally a consequence of random comparisons of thousands of genotypes in the database (resulting in low prior probability), in some cases, evidential value of the calculated likelihood ratio might be too small to enable reliable identification. However, both these values (substructure-related uncertainty in LR and prior probability) are highly variable and cannot be defined for a specific case.

In some cases, the presence of population substructure has been clearly documented (27), but most of the time there are no methods to determine whether an unidentified body and a potential relative belong to a specific subpopulation and additional safeguards are needed to prevent errors in the identification process when likelihood ratio starts to approach threshold values. In our work on the identifications of war victims in Croatia, in addition to obtaining as much DNA evidence as possible (Y-STR, mitochondrial DNA), we heavily relied on other types of evidence (such as the information about time, place and other conditions of disappearance – although they can also, in some occasions, be quite misleading), as well as 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.

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References

1. Budimlija ZM, Prinz MK, Zelson-Mundorff A, Wiersema J, Bartelink E, MacKinnon G, et al. World Trade Center human identification project: experiences with individual body identification cases. Croat Med J 2003;44:259–63.
2. Williams ED, Crews JD. From dust to dust: ethical and practical issues involved in the location, exhumation, and identification of bodies from mass graves. Croat Med J 2003;44:251–8.
3. Primorac D, Anđelinović S, Definis-Gojanović M, Drmić I, Režić B, Baden MM, et al. Identification of war victims from mass graves in Croatia, Bosnia, and Herzegovina by use of standard forensic methods and DNA typing. J Forensic Sci 1996;41:891–4.
4. Brinkmann B, Pfeiffer H, Schurenkamp M, Hohoff C. The evidential value of STRs. An analysis of exclusion cases. Int J Legal Med 2001;114:173–7.
5. Calafell F. The probability distribution of the number of loci indicating exclusion in a core set of STR markers. Int J Legal Med 2000;114:61–5.
6. Thomson JA, Ayres KL, Pilotti V, Barrett MN, Walker JI, Debenham PG. Analysis of disputed single-parent/child and sibling relationships using 16 STR loci. Int J Legal Med 2001;115:128–34.
7. Lander E. DNA fingerprinting on trial. Nature 1989;399:501–5.
8. Lander E. Research on DNA typing catches up with courtroom application. J Hum Gen 1991;48:819–23.
9. Lander E. Fight erupts over DNA fingerprinting. Science 1991;254:1921–3.
10. Waits LP, Luikart G, Taberlet P. Estimating the probability of identity among genotypes in natural populations: cautions and guidelines. Mol Ecol 2001;10:249–56.
11. Henderson J. The use of DNA statistics in criminal trials. Forensic Sci Int 2002;128:83.
12. Tracey M. Short tandem repeat-based identification of individuals and parents. Croat Med J 2001;42:233–8.
13. Gornik I, Marcikić M, Kubat M, Primorac D, Lauc G. The identification of war victims by reverse paternity is associated with significant risks of false inclusion. Int J Legal Med 2002;116:255–7.
14. Beer Z, Džijan S, Lauc D, Csete K, Lauc G. Current methods for statistical and logical estimation of significance of genetic matches might not always be appropriate. Period Biol 2004;106:169–72.

15. Džijan S, Primorac D, Marcikic M, Andelinovic S, Sutlovic D, Dabelic S, et al. High estimated likelihood ratio might be insufficient in a DNA-lead process of war victim's identification. Croat Chem Acta 2005;78:393–6.
16. Lewontin RC, Hartl DL. Population genetics in forensic DNA typing. Science 1991;254:1745–50.
17. Balding DJ, Nichols RA. DNA profile match probability calculation: how to allow for population stratification, relatedness, database selection and single bands. Forensic Sci Int 1994;64:125–40.
18. Curran JM, Gill P, Bill MR. Interpretation of repeat measurement DNA evidence allowing for multiple contributors and population substructure. Forensic Sci Int 2005;148:47–53.
19. National Research Council. The evaluation of forensic DNA evidence. Washington, DC: National Academy Press, 1996.
20. Beer Z, Pénzes Z, Farkas-Hegyi E, Csete K, Varga T. Analysis of nine somatic STR loci in an isolated Székely population. Visegrad, Hungary: University of Szeged, 2002;17–20.
21. Ayres KL, Overall ADJ. Allowing for within-subpopulation inbreeding in forensic match probabilities. Forensic Sci Int 1999;103:207–16.
22. Buckleton JS, Curran JM, Walsh SJ. How reliable is the sub-population model in DNA testimony? Forensic Sci Int 2006;157:144–8.
23. Biruš I, Marcikić M, Lauc D, Džijan S, Lauc G. How high should paternity index be for reliable identification of war victims by DNA typing? Croat Med J 2003;44:322–6.
24. Weir B. Genetic data analysis II. Sauderland, MA: Sinauer Associates, Inc. Publishers, 1996.
25. Balding DJ, Nichols RA. A method for quantifying differentiation between populations at multi-allelic loci and its implications for investigating identity and paternity. Genetica 1995;96:3–12.
26. Curran JM, Buckleton JS, Triggs CM. What is the magnitude of the sub-population effect? Forensic Sci Int 2003;135:1–8.
27. Vitart V, Biloglav Z, Hayward C, Janicijevic B, Smolej-Narancic N, Barac L, et al. 3000 Years of solitude: extreme differentiation in the island isolates of Dalmatia, Croatia. Eur J Hum Genet 2006;14:478–87.

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