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Population Genetic Analysis of Haplotypes Based on 17 Short Tandem Repeat Loci on Y Chromosome in Population Sample from Eastern Croatia

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Aim To investigate the population genetics of 17 short tandem repeat (STR) loci on the Y chromosome in the population of eastern Croatia.

Methods We carried out a statistical analysis of the data from previously performed genetic analysis collected during routine forensic work by the Forensic Science Centre "Ivan Vučetić". A total of 220 unrelated healthy men from eastern Croatia were selected for the purpose of this study. Genomic DNA was extracted by Chelex from FTA® cards. Y-chromosomal STRs were determined using the AmpFISTR Yfiler PCR amplification kit. The haplotype frequencies were determined by direct counting and analyzed using Arlequin 3.1 and analysis of molecular variance calculated with the Y-chromosome haplotype reference database online analysis tool.

Results A total of 207 haplotypes were recorded, 197 of which were unique (90%). Haplotype diversity was 0.9993, with the most frequent haplotype found in 4 of 220 men (1.8%). Average locus diversity was 0.600, and it ranged from 0.256 for DYS392 to 0.780 for DYS458. Our results were compared with the pattern of Y-chromosome variability in publicly available population samples based on a minimal European haplotype set of 9 STRs and the greatest resemblance was found with samples from the Croatian capital of Zagreb, from Bosnia and Herzegovina, and from Serbia.

Conclusion This is the first description of Y chromosome haplotyping of the population of eastern Croatia, which may serve as a basis for genetic epidemiology and forensic studies. Further studies are needed for characterization of the genetic structure of the Y-chromosome in the modern Croatian population.

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The Y chromosome is much smaller than the X chromosome and is largely composed of repeating sequences (1). It makes up only about 2% of the total haploid genome and spans approximately 60 Mb. The non-recombining region (NRY) of the human Y chromosome comprises approximately 95% of the chromosome (1). Most of the genes present on the Y chromosome have their homologues on the X chromosome and these genes on the X chromosome are not subjected to X inactivation. The NRY is flanked on both sides by pseudoautosomal regions, where X-Y crossing-over is a normal and frequent event in male meiosis.

Human Y-chromosome short tandem repeats (Y-STR) are tandemly repeated arrays of 2-7 base pair units on the NRY. They are present only in men and are transmitted from father to son unchanged, as a haplotype, except for occasional mutations. Therefore, Y-STR haplotyping is of great importance for forensic applications, such as identification of unknown persons; sexual assault cases, where Y-STRs provide a male-specific DNA profile; paternity testing, even in cases when the alleged father is deceased; verification of amelogenin Y-deficient men; human migration and evolutionary studies; and historical and genealogical research (2). The frequency of individual haplotype is important in a relevant population, especially in forensic cases when the evidence and suspect match and the frequency of the Y-STR haplotype is needed to calculate a match probability (3). The increased use of human Y-STR markers in forensic analysis and in anthropological and archeological research has prompted a collaborative effort to collect haplotype data from different populations and to create the Y Chromosome Haplotype Reference Database (YHRD, www.yhrd.org) (4).

For Croatia, the YHRD previously contained haplotypes only from the capital city of Zagreb. We have expanded the YHRD to include Y-STR data covering all Croatian regions, so we have recently added a total of 1100 haplotypes from eastern, western, southern, northern, and central Croatia (220 haplotypes for each region). As a first step in analyzing these new data, the present study investigated the population genetics of the 17 STR loci on the Y chromosome in the eastern Croatian population.

PARTICIPANTS AND METHODS

Study sample

We carried out a statistical analysis of the data from previously performed genetic analysis collected during routine forensic work by the Forensic Science Centre "Ivan Vučetić." A total of 220 samples from the following 5 counties in eastern Croatia were evaluated: Virovitičko-podravska, Požeško-slavonska, Brodsko-posavska, Osječko-baranjska, and Vukovarsko-srijemska County. The participants were not related and the samples were of sufficient quality and quantity to be included in the statistical analysis. The study was approved by the Ethics Committee of the Institute for Medical Research and Occupational Health, Zagreb, Croatia.

DNA analysis

Genomic DNA from all samples of the materials expertise was extracted from FTA cards (Whatman, Maidstone, Kent, UK) using Chelex (5). After isolation, the genomic DNA content of each sample was determined by quantitative real-time polymerase chain reaction (PCR) using the Quantifiler™ Human Male DNA Quantification kit (Applied Biosystems, Foster City, CA, USA), which includes an internal positive control to test for the presence of PCR inhibitors in the DNA extracts. Quantitative real-time PCR was performed on a 7500 Real-Time PCR System (Applied Biosystems). Genomic DNA (1 ng) was amplified using the AmpFlSTR Yfiler PCR amplification kit (Applied Biosystems), which coamplifies 17 Y-STRs: DYS19, DYS389I, DYS389II, DYS390, DYS391, DYS392, DYS393, DYS385, DYS437, DYS438, DYS439, DYS448, DYS456, DYS458, DYS635, and GATAH4. These amplification reactions were performed using a GeneAmp PCR System 9700 (Applied Biosystems). Y-STR amplification products were analyzed on 3130xl Genetic Analyzer (Applied Biosystems). Analysis of the data was performed using Genemapper® software (version 3.2, Applied Biosystems). Amplicon sizing was performed using an internal size standard (GeneScan-500 LIZ, Applied Biosystems), and the amplicons were compared with the AmpFlSTR Yfiler allelic ladder for unambiguous allele designation.

Statistical analysis

Allelic and haplotype frequencies were estimated by direct counting. Locus and haplotype diversities were calculated using Arlequin 3.1 (6), according to the formula:

$$H = \frac{n}{n-1}\left(1-\sum_{1}^{k} p_i^2\right)$$

where n is the population size and p_i is the frequency of the i-th haplotype. Locus diversity was calculated

according to the same formula, using allele frequencies instead of haplotype frequencies.

Furthermore, we used the YHRD (www.yhrd.org) to characterize the Y-STRs in the samples and compare them with the countries in the region. Due to the limited number of markers reported in other publicly available samples, the comparative analysis was performed on a minimal European Y-STR haplotype comprising 9 loci: DYS19, DYS389I, DYS389II, DYS390, DYS391, DYS392, DYS393, and DYS385ab (7). A total of 10 population samples were included in this analysis, to give a total of 1469 samples. The samples were from the following areas (the YHRD designation is given in parentheses): Budapest, Hungary ("Hungarian," n = 200 haplotypes); Doboj-Banja Luka-Bijeljina, Bosnia and Herzegovina ("Bosnian," n = 31); Ljubljana, Slovenia ("Slovenian," n = 180); Mostar, Bosnia and Herzegovina ("Bosnian," n = 34); Novi Sad, Serbia ("Serbian," n = 215); Romania ("Romanian," n = 104); Sarajevo, Bosnia and Herzegovina ("Bosnian," n = 35); Szeged, Hungary ("Hungarian," n = 100); Zagreb, Croatia ("Croatian," n = 150); and the study sample from eastern Croatia, Croatia ("Croatian," n = 220). Since analyses conducted in the YHRD are limited to 10 populations per analysis, we also performed a second-stage analysis, with a number of other more geographically and historically distant populations. These comparisons were also made using the minimal haplotype set of 9 loci and the publicly available population sets in the YHRD. This analysis yielded significant differences from the eastern Croatian sample: Albania ("Albanian," n = 111), Republic of Macedonia ("Macedonian," n = 250); Graz, Austria ("Austrian," n = 65); Bonn, Germany ("German," n = 90); and the Czech Republic ("Czech," n = 69).

We calculated population pairwise genetic distances (Rst). This is an extension of the commonly used Fst measure (8), defined as Rst = (Sb - Sw)/Sb, where Sw is the sum over all loci of twice the weighted mean of the within-population variances V(A) and V(B), and Sb is the sum over all loci of twice the variance V(A+B) of the combined population (8). *P* values were calculated using analysis of molecular variance (AMOVA), with 10 000 permutations, using an online tool of the YHRD. Significance was set at $P < 0.05$.

RESULTS

The initial analysis indicated a wide extent of variance of the number of alleles in different loci, with the most recorded alleles in DYS458 (Table 1). Locus diversity for the entire sample was 0.600, ranging from 0.256 for DYS392 to 0.780 for DYS458. A total of 207 haplotypes were recorded, 197 of which were unique (90%). Total haplotype diversity was 0.9993, with the commonest recorded haplotype found in 4 of 220 men (1.8%) (www.yhrd.org/YA003594). Discrimination capacity was 94.1%.

AMOVA indicated that our eastern Croatian sample was closest to populations from the Croatian capital of Zagreb, Bosnia and Herzegovina, and Serbia. The remaining pair-wise comparisons showed significant differences (Table 2). A second-stage analysis yielded significant differences between our eastern Croatian sample and the following samples ($P < 0.001$ in all cases): Albania ("Albanian," $R_{st} = 0.052$), Republic of Macedonia ("Macedonian," $R_{st} = 0.034$), Graz, Austria ("Austrian," $R_{st} = 0.102$), Bonn, Germany ("German," $R_{st} = 0.101$), and the Czech Republic ("Czech," $R_{st} = 0.144$).

DISCUSSION

This study is the first attempt to characterize the genetic structure of the eastern Croatian population in terms of Y-chromosome STRs. Such information is of crucial importance for any forensic work, for genetic epidemiology and population genetic purposes.

Nowadays, a great effort is made in order to improve the discrimination power of Y chromosome haplotypes as an increased number of Y-STRs is used and introduced in commercially available typing kits. The Y-STR analysis has been facilitated when commercial kits, such as the AmpFlSTR Yfiler, has become commercially available (9-11,20).

We have recently expanded the YHRD to include Y-STR data from all 5 regions of Croatia (western, northern, southern, central, and eastern). Our results from the eastern region of Croatia can be directly compared with those published for Splitsko-dalmatinska County in the southern region (12), because this study examined the same Y-STR polymorphic loci using the same AmpFlSTR Yfiler amplification kit. These two studies showed the same pattern of most frequent alleles across all loci, except DYS391 and DYS385 (12). The different results at locus DYS385 are consistent with the fact that DYS385 is one of the most polymorphic Y-STR markers.

A study in western Croatia (Primorsko-goranska County) analyzed fewer loci than our study (13). Nevertheless, they found the same pattern of the most frequent alleles for DYS389I, DYS390, DYS19, DYS393, DYS391, and DYS392 loci,

but not for DYS389II and DYS385. Together with the results from eastern and southern Croatia, this study shows that the DYS385 locus diversity values differ between the three regions of Croatia.

DYS458 locus is a heterogeneous locus composed of a polymorphic number of tetranucleotide repeats (GAAA) (14). A large deletion in the Yp11.2 band of at least 1.13 Mb was reported to include amelogenin, the NRY1 minisatel-

TABLE 1. Allele frequencies and locus diversity values at Y chromosome short tandem repeat (Y-STR) loci in the eastern Croatian population*

	STR locus on Y chromosome															
Allele	DYS456	DYS389I	DYS390	DYS389II	DYS458	DYS19	DYS393	DYS391	DYS439	DYS635	DYS392	YGATAH4	DYS437	DYS438	DYS448	DYS385*
9								2.27	0.45			0.91		8.18		0.45 (10-10)
10								51.36	12.73			1.36		63.64		0.45 (10-13)
11								45.00	23.18		85.91	55.45		20.45		2.27 (10-14)
12	0.91	14.55				0.45	14.55	1.36	36.36		2.73	35.91	0.45	7.27		0.45 (10-20)
13	0.91	71.82			0.45	10.00	77.27		24.09		7.73	5.45	0.45	0.45		1.36 (11-11)
14	9.55	13.64			3.18	21.82	7.73		2.73		1.82	0.91	40.45			5.00 (11-13)
15	59.09				23.64	21.82	0.45		0.45		0.45		49.55			13.64 (11-14)
16	17.73				21.82	34.55					1.36		8.18			1.36 (11-15)
17	10.45				30.91	11.36							0.91			0.45 (11-16)
18	1.36				15.00										3.18	0.45 (11-18)
19					1.82					0.45					44.55	1.82 (12-14)
20										3.64					43.64	0.91 (12-15)
21										17.73					7.73	1.36 (12-18)
22			6.82							24.09					0.91	4.09 (13-14)
23			16.36							44.09						0.91 (13-15)
24			53.64							7.73						1.82 (13-16)
25			21.36							2.27						0.45 (13-17)
26			1.36													0.45 (13-18)
27			0.45	0.91												0.45 (13-19)
28				10.00												5.00 (14-14)
29				15.91												30.00 (14-15)
30				29.09												6.36 (14-16)
31				38.64												2.27 (14-17)
32				5.45												1.82 (14-18)
12.2†					0.45											0.91 (14-19)
16.2†					0.45											2.73 (15-15)
17.2†					0.45											0.91 (15-17)
19.2†					1.82											0.45 (15-19)
																0.45 (15-20)
																0.45 (16-17)
																5.00 (16-18)
																0.45 (16-19)
																0.91 (16-20)
																1.82 (17-17)
																1.36 (17-18)
																0.45 (17-19)
																0.45 (17.2-17.2)
Locus diversity value	0.602	0.447	0.638	0.731	0.780	0.766	0.378	0.535	0.742	0.712	0.256	0.563	0.587	0.544	0.607	0.721

*The table shows allele frequencies for each investigated locus except DYS385, for which genotype frequencies are reported as percentages. These genotype frequencies were calculated for the combination of two alleles indicated in parentheses.
†Variant alleles at the DYS458 locus are denoted as "0.2."

TABLE 2. Analysis of molecular variance pair-wise distances based on Rst values between the eastern Croatian population in this study and selected comparison populations

	Population samples*									
Population samples†	Budapest, Hungary (Hungarian)	Doboj-Banja Luka-Bijeljina, Bosnia and Herzegovina (Bosnian)	Ljubljana, Slovenia (Slovenian)	Mostar, Bosnia and Herzegovina (Bosnian)	Novi Sad, Serbia (Serbian)	Romania (Romanian)	Sarajevo, Bosnia and Herzegovina (Bosnian)	Szeged, Hungary (Hungarian)	Zagreb, Croatia (Croatian)	East Croatia, Croatia (Croatian)
Budapest, Hungary (Hungarian)	–	0.010	<0.001	<0.001	<0.001	0.001	<0.001	0.535	0.002	<0.001
Doboj-Banja Luka-Bijeljina, Bosnia and Herzegovina (Bosnian)	0.047	–	<0.001	0.062	0.705	0.509	0.429	0.054	0.182	0.239
Ljubljana, Slovenia (Slovenian)	0.031	0.139	–	<0.001	<0.001	<0.001	<0.001	0.008	<0.001	<0.001
Mostar, Bosnia and Herzegovina (Bosnian)	0.085	0.044	0.133	–	0.035	0.005	0.029	0.009	0.175	0.181
Novi Sad, Serbia (Serbian)	0.048	-0.009	0.130	0.032	–	0.395	0.433	0.001	0.026	0.067
Romania (Romanian)	0.038	-0.005	0.133	0.064	0.000	–	0.330	0.006	0.013	0.020
Sarajevo, Bosnia and Herzegovina (Bosnian)	0.088	-0.005	0.200	0.061	-0.002	0.001	–	0.008	0.032	0.086
Szeged, Hungary (Hungarian)	-0.002	0.036	0.022	0.063	0.045	0.036	0.069	–	0.030	0.005
Zagreb, Croatia (Croatian)	0.024	0.011	0.070	0.010	0.013	0.023	0.035	0.018	–	0.712
East Croatia, Croatia (Croatian)	0.034	0.006	0.088	0.008	0.006	0.017	0.018	0.026	-0.003	–

*P values are shown above the diagonal and Rst values below.
†Names in parentheses refer to the YHRD designations (www.yhrd.org).

lite, and the DYS458 locus (15). Intermediate alleles, which contain incomplete repeat units, have been described at the DYS458 locus (16) and some of them are denoted with a "0.2." Such intermediate alleles arise by insertion/deletion events most probably caused by slipped-strand mis-pairing within the locus during spermatogenesis. These intermediate alleles were shown to be most frequent in north and east Africa, and in the Caucasus, while their occurrence in Europe is less widespread (16). This study found for the first time the variant alleles 12.2, 16.2, 17.2, and 19.2 at the DYS458 locus in the Croatian population. The 12.2 allelic variant at the DYS458 locus has not been previously reported in the YHRD (4). The 16.2 allelic variant at the DYS458 locus has been previously described in Germany, Poland, Portugal, and Russia (4,17,18). The 19.2 allelic variant at DYS458 locus has been found in the Republic of Macedonia, Poland, Russia, Greece, Italy, and Austria (4,17-19,21,22), while the 17.2 variant has been reported in Greece, Italy, Poland, Portugal, Germany, Albania, Republic of Macedonia, and Russia (4,17-19,21,23).

Our study showed a great similarity between our and the samples from the Croatian capital of Zagreb, Bosnia and Herzegovina, and Serbia, while there were difference from other neighboring countries (24-30). These results are in line with expectations, suggesting that neighboring populations shared similar Y-STR origins. Populations that were geographically distant from the eastern Croatia sample were also more genetically distant, while the second-stage analysis showed that even more geographically distant populations were considerably genetically different (31-33).

The limitations of this study include the possibility that the sample was not ethnically representative of the population of eastern Croatia. Furthermore, the comparison with the other populations was based on the samples available in the YHRD, which were occasionally rather small and thus perhaps not representative of their corresponding populations. In light of this, further studies are needed to characterize the genetic structure of the entire Croatian popu-

lation in order to provide the basis for future research in forensics, genetic epidemiology, and understanding of some major demographic and historical events in this region (34).

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