DNA Analysis of Early Mediaeval Individuals from Zvonimirovo Burial Site in Northern Croatia: Investigation of Kinship Relationships by Using Multiplex System Amplification for Short Tandem Repeat Loci

Jadranka Boljunčić

Institute of Archaeology,
Zagreb, Croatia

Aim To perform initial DNA analysis of four selected early mediaeval individuals from the Zvonimirovo burial site in Northern Croatia.

Methods Investigation of genetic matching of individuals from a "double burial" and of individuals with shared cranial non-metric/metric traits from 2 single inhumations, located in another block of the cemetery complex, was carried out. DNA from four teeth samples was extracted, quantified, and amplified by polymerase chain reaction (PCR) for short tandem repeat loci, using AmpFlSTR Profiler™ PCR Amplification Kit.

Results Autosomal short tandem repeat (STR) genotyping generated high parentage probability (PP) as to the matching of the two individuals from the "double burial" (PP 98.63%), and of two women with shared cranial non-metric/metric traits from neighboring single burials (PP 90.07%). Parentage probability calculations of a possible genetic matching of the subadult from a "double burial" with the adults from single burials 4 and 3 were significantly lower (PP 60.45% and 38.52%). DNA typing for amelogenin confirmed the sex of the 3 female individuals, estimated previously by morphology. The unknown sex of a the subadult was also determined as female.

Conclusion Increased parentage probability for autosomal STR loci matches and the presence of a rare allele shared among matched individuals support their possible kinship relationship, in accordance with bioarchaeological data. We assume an intentional double burial based on a close familial relationship, ie two single neighboring inhumations based on consanguinity, rather than a strong social relationship. The kinship lineages remain unknown at this point.

> **Correspondence to:**
Jadranka Boljunčić
Institute of Archaeology
Ulica grada Vukovara 68
10000 Zagreb, Croatia
jadranka.boljuncic@iarh.t-com.hr

> **Received:** June 18, 2007
> **Accepted:** July 16, 2007

> **Croat Med J. 2007;48:536-46**

DNA analysis has recently become one of the most advanced tools broadly employed in the investigation of relatedness within burial groups (1-5). Genetic relationships within and between burial sites are of help in understanding both the organization of inhumation places and the origin of unearthed individuals in reference to a single or limited number of family groups (5). Kinship analyses of double burials are particularly interesting in terms of the possibility of uncovering consanguinity or strong social relationships among such individuals (6-8).

However, except for a few cases of ancient DNA studies (9-11), the full advantage of DNA analysis is still not being taken in Croatian bioarchaeology (12). The major problem appears to be the cost of chemicals used in the procedure, along with other priorities for DNA typing in Croatia, such as the identification of war victims from mass graves (13-17) and in forensic medicine (9,14,18,19). A further problem is DNA degradation, contamination, or the presence of inhibitors (11-19). Damaged DNA templates in very old bones/teeth with a minute amount of cells occasionally lead to the elimination of a single or, in the worst case, all alleles, providing only non-reproducible results (20). Water or soil can also damage genomic DNA and make personal identification extremely difficult (18).

As in forensic DNA testing (18), genetic analysis of bioarchaeological samples requires the use of short sized loci, amplified by polymerase chain reactions (PCR), with short tandem repeat (STR) loci. Autosomal STRs have been used in the study of close parentage relationships due to excellent discrimination power (5,18) and are suitable markers for ancient DNA typing because of their small size and recognition of sample contamination by modern DNA (21). Because of the possibility of simultaneous amplification and reduction to an absolute minimum of the amount of sample material necessary for kinship analysis (5,9), we gave priority to STRs in performing an investigation of possible kinship relationships of mediaeval individuals from the Zvonimirovo site in Northern Croatia. To the best of our knowledge, this work represents the only attempt in Croatian bioarchaeology to perform an investigation of possible kinship relationships among individuals from a burial site.

Materials and methods

Skeletal material

Zvonimirovo is an early mediaeval cemetery arranged in rows, located in Virovitičko-Podravska County. The excavation of the site started in 1993. The findings, ie grave enclosures recovered, indicate a chronological classification to the Bijelo Brdo Culture of the 11th century (22).

The study was focused on initial DNA typing of four selected individuals, because we could not perform DNA typing of the entire Zvonimirovo burial series. The investigation of autosomal STR loci matching included the 2 individuals from Zvonimirovo burial 7 (or burial 22, according to the new corresponding designation; Ložnjak Dizdar, personal communication). This was supposed to be a "double burial," although it was considered a single burial by Tomičić (22). Two sets of remains with partially preserved cranial and absent or scanty postcranial inventories, belonging to an adult female and a subadult were unearthed from a burial with a scanty archaeological inventory. Sex of the adult was estimated according to Krogman and Işcan (23) and Bass (24). Sex of the subadult could not be estimated by standard morphological procedures because of very young age and consequent lack of morphological indicators of sex. Adult age at death was estimated according to Meindl and Lovejoy (25) and Lovejoy (26). The subadult's age at death was estimated according to dental eruption criteria after Ubelaker (27).

Two samples (1 and 2,) were processed for DNA typing in order to investigate whether the 2 individuals in a supposed "double burial" were

possibly related, or this was merely a case of randomly buried unrelated remains. No other double burials have yet been found in the available Zvonimirovo burial series.

The investigation of a possible genetic matching was also performed for female individuals from 2 neighboring single inhumations from the same grave row – Zvonimirovo 3 and 4 (28) with shared cranial non-metric traits. Metric traits, ie cranial phenotype affiliation shared between the 2 individuals also pointed to their possible relationship (28). Burials were located in another block of the same cemetery complex (22). Two samples (sample 3 and 4) were processed for STR genotyping in order to investigate whether the inhumation of the 2 females in the 2 neighboring burials was a consequence of their possible kinship relationship. Grave inventory pointed to a strikingly different social status (22).

Sex determination was performed using the amelogenin (AMEL) sex-typing locus for the 3 female individuals (Table 1), whose sex was estimated by morphology.

Table 1. Teeth samples used for DNA typing at 10 short tandem repeat (STR) loci, obtained from 4 individuals from Zvonimirovo early mediaeval burial site*

Sample	Burial No.	Age group/sex of the individual
1	7†	adult/female
2	7†	subadult/unknown sex
3	3‡	mature/female§
4	4‡	young adult/female§

*Zvonimirovo series 1993-1998 stored at the Institute of Archaeology in Zagreb, Croatia.
†Year of excavation 1995 – "the double burial."
‡Year of excavation 1993 – two neighboring single inhumations.
§Age and sex are taken from ref. 28.

Sample preparation

Teeth samples were used for DNA typing procedure, because teeth have been shown to be the best sources of DNA, as dental enamel, which is the hardest substance in the human body, protects the DNA rich pulp and dentin and ensures a good quality of isolated DNA (29,30). As the DNA of the pulp cells degrades faster in damaged teeth with the pulp opened and the tissue exposed to the environment (31), only best preserved teeth were processed in the analysis.

Teeth corresponding to four individuals (2 from "double burial" 7, 2 from single burials 3 and 4) were used for the sample preparation. The teeth were thoroughly cleaned. All traces of tissue in the teeth cavities were removed using razor blades and the teeth surfaces were cleaned by abrasion with a grinding tip (or drill bit) and sandpaper. The teeth were crushed into small fragments and stored in sterile polypropylene tubes at -20°C until analyzed.

About 1 g of the teeth was rinsed with deionized water several times and left to air dry. The teeth fragments were cleaned in a 5% sodium hypochlorite solution, in deionized water several times, then in 70% ethanol. They were then poured into clean and labeled weight boats and allowed to air-dry overnight in a laminar flow hood. The material was pulverized with a mill or frozen samples were crushed by hammering with liquid nitrogen. The material had to be pulverized into a very fine powder before adding the extraction buffer.

DNA extraction

The powdered teeth were transferred into 2 sterile 15 mmL conical tubes. Three mL of extraction buffer: 100 mmol/L Tris – (hydroxyl-methyl) amino methane/hydrochloride (Tris-HCl), pH 8.0; 1M sodium chloride; 100 mM ethylene-diamine-tetraacetic acid (EDTA), pH 8.0; 10% sodium dodecyl sulfate (SDS), and 100 μL proteinase K (20 mg/mL), was added to each tube. The tubes were incubated at 56°C for 48 hours with intermediate shaking. The solutions were then extracted 3 times (or until the upper aqueous layer was completely clear) with equal volumes of phenol/chloroform/isoamyl alcohol (25:24:1) and then centrifuged. The aqueous layer was extracted with water-saturated *n*-butanol and centrifuged. The aqueous phase was concentrated using Centricon-100 micro concentrator tubes and centrifuged. Afterwards, the residue was

washed 3 times with 2 mL of TE buffer (10 mM Tris-HCl pH 7.5; 1 mM EDTA pH 8). The recovered DNA was stored at 4°C (14,32,33).

DNA quantification

The total DNA was evaluated using agarose gel electrophoresis (ethidium bromide staining) and absorbencies at 260 and 280 nm were measured by spectrophotometer (Ultrospec 2000. Pharmacia Biotech). The A260/A280 ratios were used to evaluate the quality of the extracted DNA (33).

Before concluding on possible absence of amplifiable DNA in a sample, it was necessary to confirm that the inhibitors of Taq polymerase were not present in the preparation. QRT-PCR was performed in total volume of 25 μL containing 2 μL of DNA extract, Quantifiler human primer mix, and Quantifiler PCR reaction mix with thermal cycling conditions according to the manufacturer's protocols (34).

Data were collected using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Data analysis was performed with ABI PRISM 7000 Sequence Detector Software (SDS), version 1.0 to generate the individual standard curves from each experiment and to calculate the DNA amount from each unknown sample.

PCR amplification and typing

The repeatedly extracted and purified DNA was amplified on the Perkin-Elmer Thermal Cycler 9600, using the AmpFlSTR Profiler™ PCR Amplification Kit (Applied Biosystems, Foster City, CA, USA), following the recommended protocol. The typing of polymerase chain reaction (PCR) products was performed on ABI Prism 310 Genetic Analyzer (Applied Biosystems). The internal standard was Rox-350 (14,33,35). Nine autosomal short tandem repeat (STR) loci and the sex-determination locus amelogenin were simultaneously amplified.

Statistical analysis

Microsoft Excell 2000 (Microsoft, Seattle, WA, USA) was used for statistical calculation. Calculations for statistical probability of matching were performed according to the cited protocols (18). Parentage probability was calculated by combining the data on the population frequency estimates for a forensic paternity case and forensic identity case for individual loci and their combinations (18). The allelic frequencies quoted in the Tables are from the database on general Croatian population obtained from the Department of Pathology, Forensic Medicine and Cytology, Split University Hospital (Split, Croatia).

Results

Investigation of a possible relatedness among 2 individuals (samples 1 and 2) from burial 7 by DNA typing for autosomal STRs generated very high matching probability at 6 STR loci alleles, with high parentage probability (PP 98.63%) (Table 2). Two sets of partially preserved human remains were unearthed from a supposed "double burial." They belonged to an adult female and a subadult (Table 1). The increased paternity index (PI) obtained for the matched individu-

Table 2. Parentage probability calculation for genetic matching at 6 short tandem repeat (STR) loci in samples 1 and 2 (double burial)

				Allele frequency*			
Locus	Sample 1	Shared alleles	Sample 2	a	b	total	Paternity index†
vWa	16	16	16;17	0.16667	0	0.166667	2.999994
FGA	20	20	20;23;24;25	0.07937	0	0.079365	6.300006
TH01	6	6	6;8;9.3	0.23810	0	0.238095	2.100002
TPOX	8	8	8;11	0.50397	0	0.503968	0.992127
CSF1PO	10	10	10;12	0.28175	0	0.281746	1.774648
D5S818	10;13	10;13	10;12;13	0.05952	0.18254	0.242064	1.032785

*Allele frequencies in the general Croatian population (data from the Department of Pathology, Forensic Medicine and Cytology, Split University Hospital, Split, Croatia).
†Paternity index was calculated after ref. 18. Using the known allelic frequencies of the general Croatian population, the probability of parentage for this case was calculated to be 98.63%.

als (Table 2) was apparently related to the presence of a shared rare allele 20 of the FGA locus. This allele has the frequency of only 7.94% in the general Croatian population (Table 2), and sharing such a rare allele increases the probability of relatedness among 2 individuals (18). Although the allele 10 of the D5S818 locus had an even lower frequency in the general Croatian population (5.95%), its conjunction with the allele 13 (18.25%), and consequent increased summarized allelic frequency of 24.21%, decreased the respective paternity index calculation (Table 2). The probability of biological relationship was calculated using the allelic frequencies of the general Croatian population. As for random man not excluded (RMNE) – the proportion of the population that could contribute all obligate alleles and cannot be excluded (18) – there was a 99.70% chance of non-exclusion of a random man from contributing obligate alleles.

We had a few cases of a loci dropout during the PCR procedure, ie the failure to detect an allele within a sample or to amplify an allele. A single locus dropout was obtained for sample 2 (locus D7S820), and 2 loci dropout for sample 1 (loci D3S1358 and D13S317). In addition, multiple allele presence was detected at some loci (Table 2).

DNA typing for amelogenin confirmed the female sex for both the adult and subadult in the supposed double burial.

The shared cranial metric traits, ie cranial phenotype affiliation (28) of the 2 female individuals from the single neighboring burials 3 and 4, suggested a possible relationship between the 2 individuals. The same is the case with the following shared cranial non-metric variants: lambdoid ossicles, epipteric bone, ossicle at asterion, supraorbital foramen, complete, ie incomplete. In addition, ossicle at lambda was present in the woman from a single burial 4 and a variant of the 3-part Inca bone in the female from a single burial 3, in conjunction with lambdoid ossicles (28). Besides, sagittal ossicles, mastoid foramen, posterior condylar canal, condylar facet double, and precondylar tubercle were present additionally in the young woman from a single burial 4. One has to point out that in terms of some given bilateral traits, the individuals exhibited asymmetry in their expression. The side exhibiting the maximum expression was closest to the true underlying genotype for the trait (36). One also has to point out that no biological relatedness in reference to non-metric traits was calculated for the Zvonimirovo summarized cranial series due to both severe fragmentation and damage, mainly in reference to the Zvonimirovo 1994 series (28), in addition to the Zvonimirovo 1995 series yielding just a few complete crania. Besides, a sample comprising just the 2 mentioned female individuals included in the study was too small to perform a biodistance study. Although suggestive in reference to a possible relationship between the individuals in question, the obtained morphological results could not yield reliable information as results of molecular study.

Examination of the 2 female individuals (samples 3 and 4) for possible STR loci matches generated high matching probability at 7 STR loci alleles with elevated calculated parentage probability (PP: 90.07%) (Table 3). The increased paternity index (PI) obtained for the 2 females in reference to the presence of a shared rare allele 20 of the FGA locus pointed to possible kinship relationship between the matched individuals. There was a 99.34% chance of not excluding a random man from contributing obligate alleles. During PCR a single locus dropout was obtained for sample 3 (locus CSF1PO) as well as sample 4 (locus D7S820) (Table 3).

Homozygosity was found in the individual from grave 4 (sample 4) at the allele 11 of the D13S317 locus and at allele 12 of the CSF1PO locus (Tables 3-4).

The sex of the individuals of apparently different social status – the older person was found with a few simple grave goods (jewelry), while the

young person had rich luxury artifacts (22) – was substantiated by DNA typing for amelogenin.

On the other hand, calculation of the matching probability at 8 STR loci for the subadult from burial 7 (the "double burial") and the young adult female from the single burial 4 (corresponding samples 2 and 4), generated a low parentage probability (60.45%) (Table 4). The latter was due to increased summarized allelic frequency (19.05%) in reference to the presence of a shared rare allele 20 of the FGA locus, in conjunction with allele 23, and decreased paternity index (PI). The same was the case with the allele 10 of the D5S818 locus. Although with a low frequency in the general Croatian population (5.95%), its conjunction with the common allele 12 of the D5S818 locus (35.71%), and consequent increased summarized allelic frequency (41.67%), decreased the respective paternity index calculation (Table 4). There was a 97.84% chance of not excluding of a random man (RMNE) from contributing obligate alleles.

Calculation of the matching probability at 7 STR loci for the subadult from the "double burial" 7 and the mature woman from the single burial 3 (corresponding samples 2 and 3) provid-

Table 3. Parentage probability calculation for genetic matching at 7 short tandem repeat (STR) loci in samples 3 and 4 (single burials)

				Allele frequency†			
Locus	Sample 3	Shared alleles	Sample 4*	a	b	total	Paternity index‡
D3S1358	14;15;16	14;16	14;16	0.11111	0.24206	0.353173	0.7078684
vWa	17;18	17	16;17	0.26587	0	0,265873	1.8805971
FGA	18;19;20	20	20;23;25	0.07937	0	0.079365	6.3000063
TH01	9.3	9.3	6;8;9.3	0.26984	0	0.269841	1.8529430
TPOX	8;9;11	8;11	8;11	0.50397	0.27381	0.777778	0.3214285
D5S818	11;12;13	12	10;12	0.35714	0	0.357143	1.3404250
D13S317	8;11	11	11;11	0.36905	0	0.369048	1.3548373

*Zvonimirovo female individual from a single burial 4 homozygous at the D13S17 site (allele 11).
†Allelic frequencies in the general Croatian population (data from the Department of Pathology, Forensic Medicine and Cytology, Split University Hospital, Split, Croatia).
‡Paternity index was calculated after ref. 18. Using the known allelic frequencies of the general Croatian population, the probability of parentage for this case was calculated to be 90.07%.

Table 4. Parentage probability calculation for genetic matching at 8 short tandem repeat (STR) loci in samples 2 and 4

				Allele frequency†				
Locus	Sample 2	Shared alleles	Sample 4*	a	b	c	total	Paternity index‡
D3S1358	14;16;17	14;16	14;16	0.11111	0.24206	0	0.35317	0.70786838
vWa	16;17	16;17	16;17	0.16667	0.26587	0	0.43254	1.15596245
FGA	20;23;24;25	20;23	20;23;25	0.07937	0.11111	0	0.19048	2.62501641
TH01	6;8;9.3	6;8;9.3	6;8;9.3	0.23810	0.16270	0.269841	0.67063	0.74556315
TPOX	8;11	8;11	8;11	0.50397	0.27381	0	0.77778	0.64285696
CSF1PO	10;12	12	12;12	0.27381	0	0	0.27381	1.82608378
D5S818	10;12;13	10;12	10;12	0.05952	0.35714	0	0.41667	1.19999904
D13S317	8;11	11	11;11	0.36905	0	0	0.36905	0.67741866

*Zvonimirovo female individual from a single burial 4 homozygous at the CSF1PO site (allele 12) and the D13S17 site (allele 11).
†Allelic frequencies in general Croatian population (data from the Department of Pathology, Forensic Medicine and Cytology, Split University Hospital, Split, Croatia).
‡Paternity index was calculated after ref. 18. Using the known allelic frequencies of the general Croatian population, the probability of parentage for this case was calculated to be 60.45%.

Table 5. Parentage probability calculation for genetic matching at 7 short tandem repeat (STR) loci in samples 2 and 3

				Allele frequency*			
Locus	Sample 2	Shared alleles	Sample 3	a	b	total	Paternity index†
D3S1358	14;16;17	14;16	14;15;16	0.11111	0.24206	0.353174	0.7078664
vWa	16;17	17	17;18	0.26587	0	0.265873	1.8805971
FGA	20;23;24;25	20	18;19;20	0.07937	0	0.079365	6.3000063
TH01	6;8;9.3	9.3	9.3	0.26984	0	0.269841	1.8529430
TPOX	8;11	8;11	8;9;11	0.50397	0.27381	0.777778	0.6428570
D5S818	10;12;13	12;13	11;12;13	0.35714	0.18254	0.539683	0.4632349
D13S317	8;11	8;11	8;11	0.13095	0.36905	0.500000	0.1354108

*Allelic frequencies in the general Croatian population (data from the Department of Pathology, Forensic Medicine and Cytology, Split University Hospital, Split, Croatia).
†Paternity index was calculated after ref. 18. Using the known allelic frequencies of the general Croatian population, the probability of parentage for this case was calculated to be 38.52%.

ed the significantly lower parentage probability (38.52%; Table 5), in comparison with the above cases. This was due to high total frequencies of shared more or less common alleles, the latter being present individually or in conjunction with other alleles. Consequently, low paternity index (PI) values were obtained, except for the individually present shared rare allele 20 of the FGA locus (Table 5). There was a 98.93% chance of non-exclusion of a random man (RMNE) from contributing obligate alleles.

Due to the match in only 4 loci, no probability percentages of a possible genetic matching were calculated for other sample combinations (1 and 3, 1 and 4), because they would not provide statistical significance.

Discussion

In terms of autosomal STRs, the results of DNA analysis obtained in the present study showed statistically high parentage probability as to the genetic matching between individuals from a supposed "double burial" (burial 7) at the early mediaeval Zvonimirovo site. The same was true for the case with 2 females from the single burials 3 and 4 located in another block of the same cemetery complex. Parentage probabilities in terms of a possible genetic matching of a subadult from a "double burial" with the adult, ie mature woman from single burials 4 and 3, were significantly lower. The latter fact indicates possible authentic relatedness of genetically matched individuals with increased parentage probabilities (18). However, one has to bear in mind that in STR genotyping of bioarchaeological material, the results of genetic matching can easily be misinterpreted ("false matching") without the inclusion of additional data, as is also the case in forensic medicine (16). On the basis of the obtained alleles at STR loci of the individuals (samples) processed in DNA typing, the Zvonimirovo mediaeval series fits into the general Croatian population.

The testing of a "double burial" hypothesis was performed on the basis of bioarchaeological data indicating that 2 individuals were interred in Zvonimirovo burial 7. The latter was apparently considered by Tomičić (22) to be a single inhumation. The procedure was carried out in order to investigate whether the adult female and the subadult were possibly related, or it was merely a case of randomly buried unrelated skeletal remains in a grave pit, with the given scanty archaeological inventory (22). On the basis of the obtained elevated probability percentages in terms of STR loci matching we can assume that it is apparently a question of a double burial, ie an intentional common inhumation of the 2 individuals, due to possible consanguinity.

As for the case of 2 female individuals from the individual Zvonimirovo burials 3 and 4, shared cranial non-metric data matched with high parentage probability calculations.

As early as in 1967, Berry and Berry (37) pointed to major genetic, ie epigenetic determination of discontinuous variants. In more recent studies cranial non-metric variants have been successfully used to assess biological relatedness (36) and evolutionary relationships (38-41), in addition to the most recent identification of familial relationships (42). However, Berry and Berry (37) made no attempt to distinguish between ossicle at lambda, and the true "interparietal" Inca bone formed from the membraneous part of the occipital bone. On the other hand, a variant of a classical 3-part Inca bone observed in the individual from a single burial 3 in our study is considered an anomaly as a result of irregular ossification of the upper portion of the occipital squama (28). It was present in association with the lambdoid ossicles (28).

Although some studies suggest artificial cranial deformation having influence on discontinuous morphological traits (43), specifically on the increased complexity of the lambdoid suture (44), the research of Konigsberg et al (45) also showed that biodistances derived by using non-

metric traits of deformed crania differ little from distances calculated without such traits. The presence of lambdoid ossicles in both female individuals from the Zvonimirovo site, is not combined with any form of cranial deformation, and it is most likely a question of inherited variants (37). The obtained results of genomic DNA analysis matching with the shared non-metric variants suggest a possible genetic relationship between the 2 individuals in question.

A single or several loci dropout, or multiple alleles obtained in the present study, is usually considered to be the result of DNA degradation or bacterial DNA contamination from the soil. In our case the extremely cautious handling of the material prior to DNA typing and analysis performed after repeated DNA extraction and purification, in addition to the fact that no one from the staff/investigators matched the multiple allele genotypes, suggests authentic DNA amplification. It has to be emphasized that matching of multiple alleles was performed by a separate investigator. If any of the multiallelic profiles matched those of the staff/investigators, we would consider them likely a case of contamination (5) and they would be excluded from the study. We also employed the necessary procedure to confirm that the inhibitors of Taq polymerase (11) were not present in the preparation. In addition, the molecular and morphological sex was in accordance for 3 female samples supplementing the authenticity of early mediaeval DNA (5). However, environmental factors and the taphonomic conditions may induce destructive processes in the teeth DNA, resulting in multiple allele presence ("shadow bands") at some loci, as reported by Ricaut et al (46). The latter is considered to be an indicator of significant degradation of the DNA target (46), which is not unusual for ancient samples.

In reference to the previously mentioned Zvonimirovo series affiliation, Šlaus (12) also reports on the affiliation of the Zvonimirovo (and Josipovo) mediaeval sites with early mediaeval Croatian populations of Slavic ancestry, along with excavations in Stenjevac, Đelekovac, and Đakovo phase 1. On the other hand, a more recent study of the craniometric relationship among mediaeval populations of Central Europe has generated a classification of Zvonimirovo to the Bijelo Brdo population, and Josipovo to the early mediaeval Croatian population (47). However, the discriminating analyses in those studies were generated on a rather small sample, both for the Zvonimirovo/Josipovo site and beyond. In terms of the early mediaeval archaeological material recovered from Zvonimirovo, it was assigned by Tomičić to the Bijelo Brdo Culture (22). Both Slavs and Hungarians, with a rather heterogeneous Slavic component, have been defined as bearers of the Bijelo Brdo Culture (48).

Despite controversial data on the affiliation of the Zvonimirovo mediaeval series, our study showed that a certain gene flow could be presumed, in terms of the presence of alleles that were not common in the general Croatian population, due to migrations. The latter refers to allele 20 of the FGA locus, which has a low frequency in the general Croatian population. One also has to point out to the presence of allele 10 of the D5S818 locus, which has an even lower frequency in the general Croatian population. However, its presence in conjunction with other alleles generated the increased summarized allelic frequencies.

Recent results of the study of the Y chromosomal heritage of the Croatian population on an Adriatic island revealed relatively high frequencies of lineages/mitochondrial haplogroups unusual for European populations (49). They indicate connections of the Croatian population with central Asian populations, possibly derived from the Avars (49). This is particularly interesting in terms of the obtained cranial phenotype affiliation of the 2 female crania from the Zvonimirovo neighboring burials 3 and 4, as they differ somewhat from the rest of the Zvonimirovo early series. The earliest study of the Zvonimiro-

vo cranial series suggested the presence of cranial phenotypes with a mixture of Slavic and Mongoloid, ie Avar, characteristics (28). However, any further discussion on a "population" level at this point, in reference to the presence of alleles in the Zvonimirovo mediaeval series that are not characteristic of the general Croatian population, requires a more extensive Zvonimirovo genetic database. Furthermore, PI values over 6.00, in reference to presence of a shared rare allele 20 of the FGA locus point to high matching probabilities of the 2 pairs of samples processed for STR genotyping (samples 1 and 2, and 3 and 4). However, Biruš et al (16) have found that in some cases in DNA typing of war victims even a relatively high PI over 10.00 might not be sufficiently high for the correct determination of identity without bearing in mind the important role of some classical anthropological data besides molecular elements, likewise employed in the present study.

Low frequency of the allele 20 of the FGA locus and its presence, individually or in conjunction with other alleles, in all 4 individuals, points to possible local inbreeding. It is well known that the greater the presence of alleles with low frequency in a population, the higher the possibility of relatedness within a population (18). The observed clear case of homozygosis in the individual from a single burial 4 supports the possibility of local inbreeding, considering that inbreeding increases homozygosis. Inbreeding has been an important issue, especially when it is the question of small populations where the expectance of relationship between individuals is more likely. In terms of the mediaeval Zvonimirovo series, even some historical incidents (50) may have influenced local inbreeding at some point. However, final conclusion on the homozygosis frequency at the Zvonimirovo site will be possible after generating a more extensive database for molecular analysis.

In conclusion, the obtained elevated probability percentages for autosomal STR loci matches and high paternity index in reference to the presence of a rare allele shared among matched individuals supported their possible relatedness in accordance with bioarchaeological data. However, at this point the kinship lineages remain unknown. We assume that it is the question of an intentional double burial based on a close familial relationship, ie 2 single neighboring inhumations based on consanguinity, rather than a strong social relationship. In addition, results for sex-determination of the amelogenin locus were compatible with sex-estimation for the 3 female individuals whose sex could be estimated from the morphological analysis in the previous (28) and present studies. They substantiate the authenticity of early mediaeval DNA amplification (5). The next step in the analysis of the remains from the Zvonimirovo site will include the mitochondrial DNA analysis, as well as the generation of a more extensive Zvonimirovo database for molecular genetic analysis. Future studies will provide additional answers in reference to the kinship lineages within the cemetery complex and allow broader insights into the burial organization, ie funerary behavior in reference to possible relatedness or the social positions of early mediaeval individuals buried at the Zvonimirovo site.

Acknowledgments

This study was supported by Ministry of Science, Education, and Sports (grant No. 01970105). I thank Prof. Dr Š. Anđelinović, Head of the Department of Pathology, Forensic Medicine and Cytology, Split University Hospital, for their support and for allowing the bioarchaeological material to be processed for DNA analysis. I thank Dr D. Sutlović from the Department of Pathology, Forensic Medicine and Cytology, Split University Hospital for performing technical procedures in this study. I also thank Ms B. Režić for preparing the material for DNA typing.

References

1 Oota H, Saitou N, Matsushita T, Ueda S. A genetic study of 2000-year old human remains from Japan using mitochondrial DNA sequences. Am J Phys Anthropol. 1995;98:133-45. Medline:8644875

2 Fily ML, Crubézy E, Courtaud P, Keyser C, Ebrard D, Ludes B. Analyse paléogénétique des sujets de la grotte sépulcrale d'Elzarreko Karbia (Bronze ancien, Pays Basque). C R Acad Sci III. 1998;321:79-85. Medline:9759357

3 Stone AC, Stoneking M. Analysis of ancient DNA from a prehistoric Amerindian cemetery. Philos Trans R Soc Lond B Biol Sci. 1999;354:153-9. Medline:10091255

4 Schultes T, Hummel S, Herrmann B. Ancient DNA-typing approaches for the determination of kinship in a disturbed collective burial site. Anthropol Anz. 2000;58:37-44. Medline:10816784

5 Keyser-Tracqui C, Crubézy E, Ludes B. Nuclear and Mitochondrial DNA Analysis of a 2,000-Year-Old Necropolis in the Egyin Gol Valley of Mongolia. Am J Hum Genet. 2003;73:247-60. Medline:12858290

6 Adachi N, Dodo Y, Ohshima N, Doi N, Yoneda M, Matsumura H. Morphologic and Genetic Evidence for the Kinship of Juvenile Skeletal Specimens from a 2,000 Year-old Double Burial of the Usu-Moshiri Site, Hokkaido, Japan. Anthropol Sci. 2003;111:347-63.

7 Clisson I, Keyser C, Francfort HP, Crubezy E, Samashev Z, Ludes B. Genetic analysis of human remains from a double inhumation in a frozen kurgan in Kazakhstan (Berel site, Early 3rd Century BC). Int J Legal Med. 2002;116:304-8. Medline:12376844

8 Adachi N, Suzuki T, Sakaue K, Takigawa W, Ohshima N, Dodo Y. Kinship analysis of the Jomon skeletons unearthed from a double burial at the Usu-Moshiri site, Hokkaido, Japan. Anthropol Sci. 2006;114:29-34.

9 Alonso A, Martin P, Albarrán C, García P, Primorac D, García O, et al. Specific quantification of human genomes from low copy number DNA samples in forensic and ancient DNA studies. Croat Med J. 2003;44:273-80. Medline: 12808718

10 Alonso A, Martín P, Albarrán C, García P, García O, de Simón LF, et al. Real-time PCR designs to estimate nuclear and mitochondrial DNA copy number in forensic and ancient DNA studies. Forensic Sci Int. 2004;139:141-9. Medline:15040907

11 Sutlovic D, Definis Gojanovic M, Andelinovic S, Gugic D, Primorac D. Taq polymerase reverses inhibition of quantitative real time polymerase chain reaction by humic acid. Croat Med J. 2005;46:556-62. Medline:16100758

12 Šlaus M. The bioarchaeology of continental Croatia. Oxford: Archaeopress; 2002.

13 Primorac D, Andelinovic S, Definis-Gojanovic M, Drmic I, Rezic 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. Medline:8789853

14 Alonso A, Andelinovic S, Martín P, Sutlovic D, Erceg I, Huffine E, et al. DNA typing from skeletal remains: evaluation of multiplex and megaplex STR systems on DNA isolated from bone and teeth samples. Croat Med J. 2001;42:260-6. Medline:11387635

15 Gornik I, Marcikic 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. Medline:12376832

16 Birus I, Marcikic M, Lauc D, Dzijan 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. Medline:12808726

17 Andelinovic S, Sutlovic D, Erceg Ivkosic I, Skaro V, Ivkosic A, Paic F, et al. Twelve-year experience in identification of skeletal remains from mass graves. Croat Med J. 2005;46: 530-9. Medline:16100755

18 Primorac D, Schanfield MS. Application of forensic DNA testing in the legal system. Croat Med J. 2000;41:32-46. Medline:10810166

19 Sutlović D, Definis Gojanović M, Anđelinović Š. Rapid extraction of human DNA containing humic acid. Croatica Chemica Acta. 2007;80:117-20.

20 Benecke M. DNA typing in forensic medicine and in criminal investigations: a current survey. Naturwissenchaften. 1997; 84:181-8.

21 Hummel S, Bramanti B, Schultes T, Kahle M, Haffner S, Herrmann B. Megaplex DNA typing can provide a strong indication of the authenticity of ancient DNA amplifications by clearly recognizing any possible type of modern contamination. Anthropol Anz. 2000;58:15-21. Medline: 10816781

22 Tomičić Ž. Early mediaeval graveyard Zvonimirovo-Veliko Polje, the municipality of Sopje. In: Tomičić Ž, editor. Zvonimirovo and Josipovo graveyards from the Croatian early mediaeval period in the Virovitica and Podravina County. Zagreb-Virovitica: Institut za arheologiju u Zagrebu; 1997. p. 37-43.

23 Krogman WM, Işcan MY. The human skeleton in forensic medicine. 2nd ed. Springfield, IL: Charles C Tomas; 1986.

24 Bass WM. Human osteology: a laboratory and field manual of the human skeleton. 3rd ed. Columbia, MO: Missouri Archaeological Society;1987.

25 Meindl RS, Lovejoy CO. Ectocranial suture closure: a revised method for the determination of skeletal age at death based on the lateral-anterior sutures. Am J Phys Anthropol. 1985;68:57-66. Medline:4061602

26 Lovejoy CO. Dental wear in the Libben population: its functional pattern and role in the determination of adult skeletal age at death. Am J Phys Anthropol. 1985;68:47-56. Medline:4061601

27 Ubelaker DH. Human skeletal remains. 2nd ed. Washington, DC: Taraxacum Press; 1989.

28 Boljunčić J. Anthropological analysis of the early mediaeval graveyard Zvonimirovo-Veliko Polje. In: Tomičić Ž, editor. Zvonimirovo and Josipovo graveyards from the Croatian early mediaeval period in the Virovitica and Podravina County. Zagreb-Virovitica: Institut za arheologiju u Zagrebu; 1997. p.53-61.

29 Schwartz TR, Schwartz EA, Mieszerski L, McNally L, Kobilinsky L. Characterization of deoxyribonucleic acid (DNA) obtained from teeth subjected to various environmental conditions. J Forensic Sci. 1991;36:979-90. Medline:1680960

30 Pötsch L, Meyer U, Rothschild S, Schneider PM, Rittner C. Application of DNA techniques for identification using human dental pulp as a source of DNA. Int J Legal Med. 1992;105:139-43. Medline:1419874

31 Pfeiffer H, Hühne J, Seitz B, Brinkmann B. Influence of soil storage and exposure period on DNA recovery from teeth. Int J Legal Med. 1999;112:142-4. Medline:10048676

32 Hochmeister MN, Budowle B, Borer UV, Eggmann U, Comey CT, Dirnhofer R. Typing of deoxyribonucleic acid (DNA) extracted from compact bone from human remains. J Forensic Sci. 1991;36:1649-61. Medline:1685164

33 Burgi SB, editor. First European-American intensive course in PCR based clinical and forensic testing. Split (Croatia): Genomix; 1997.

34 Quantifiler™ Human DNA Quantification Kit, User's Manual. Foster City (CA):Applied Biosystems; 2003.

35 Butler JM, McCord BR, Jung JM, Wilson MR, Budowle B, Allen RO. Quantitation of polymerase chain reaction products by capillary electrophoresis using laser fluorescence. J Chromatogr B Biomed Appl. 1994;658:271-80. Medline:7820255

36 Sutter RC, Mertz L. Nonmetric cranial trait variation and prehistoric biocultural change in the Azapa Valley, Chile. Am J Phys Anthropol. 2004;123:130-45. Medline:14730647

37 Berry AC, Berry RJ. Epigenetic variation in the human cranium. J Anat. 1967;101:361-79. Medline:4227311

38 Sjøvold T. A report on the heritability of some cranial measurements and non-metric traits. In: Howells WW, editor. Multivariate statistical methods in physical anthropology. Boston: D. Reidel; 1984. p. 223-46.

39 Konigsberg LW. Migration models of prehistoric post-marital residence. Am J Phys Anthropol. 1988;77:471-82. Medline:3066223

40 Prowse TL, Lovell NC. Concordance of cranial and dental morphological traits and evidence for endogamy in ancient Egypt. Am J Phys Anthropol. 1996;101:237-46. Medline:8893087

41 Ishida H, Dodo Y. Cranial variation in prehistoric human skeletal remains from the Marianas. Am J Phys Anthropol. 1997;104:399-410. Medline:9408544

42 Slavec ZZ. Identification of family relationships by epigenetic traits. Anthropol Anz. 2005;63:401-8. Medline:16402590

43 Ossenberg NS. The influence of artificial cranial deformation on discontinuous morphological traits. Am J Phys Anthropol. 1970;33:357-72.

44 Gottlieb K. Artificial cranial deformation and the increased complexity of the lambdoid suture. Am J Phys Anthropol. 1978;48:213-4. Medline:637122

45 Konigsberg LW, Kohn LA, Cheverud JM. Cranial deformation and nonmetric trait variation. Am J Phys Anthropol. 1993;90:35-48. Medline:8470754

46 Ricaut FX, Keyser-Tracqui C, Crubézy E, Ludes B. STR-genotyping from human medieval tooth and bone samples. Forensic Sci Int. 2005;151:31-5. Medline:15935940

47 Slaus M, Tomicic Z, Uglesic A, Juric R. Craniometric relationships among medieval Central European populations: implications for Croat migration and expansion. Croat Med J. 2004;45:434-44. Medline:15311416

48 Demo Ž. Vukovar Lijeva Bara [in Croatian]. Zagreb: Arheološki muzej Zagreb; 1996.

49 Barac L, Pericic M, Klaric IM, Rootsi S, Janicijevic B, Kivisild T, et al. Y chromosomal heritage of Croatian population and its island isolates. Eur J Hum Genet. 2003;11:535-42. Medline:12825075

50 Boljunčić J. Archaeological populations from northern Croatia: a paleopathological survey. In: Andersson AC, Cornell P, Karlsson H, Kjärsgaard J, Rolöf M, editors. Book of abstracts of 4th EAA annual meeting; 1998 Göteborg (Sweden), Sep 23-27; Göteborg (Sweden): Göteborgs Länstryckeri AB; 1998, p.70.