Croatian Genetic Heritage: Renewed Y Chromosome Story a Decade Later

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Abstract

Aim: To analyze additional set of Y-Chromosome genetic markers to acquire a more detailed insight into the diversity of the Croatian population.

Methods: The total number of 518 Yfiler™ Plus profiles was genotyped. Allele, haplotype frequencies and haplotype diversity, were calculated using the STRAF software package v2.0.4. Genetic distances were quantified by *R*st using AMOVA online tool from the YHRD. The evolutionary history was inferred using the neighbor-joining method of phylogenetic tree construction in MEGAX software. Whit Athey's Haplogroup Predictor v5 was used for additional comparison with selected European populations.

Results: The total of 507 haplotypes were used for genetic STR analysis. The interpopulation comparison with the original 27 Y-STR markers shows the lowest genetic diversity between Croatian and Serbian population, and the highest between Croatian and Spanish population. Interpopulation study on 17 Y-STR markers shows the lowest genetic diversity between Croatian and Bosnian-Herzegovinian population, and the highest between Croatian and Irish population. Total of 518 haplotypes were used in the determination of haplogroup diversity. Haplogroup I with its sublineage I2a expressed the highest prevalence. Haplogroup R, with its major sublineage R1a, is the second most abundant in the studied Croatian population, except for the subpopulation of Hvar, where E1b1b is the second most abundant haplogroup. Rare haplogroups also confirmed in this study are L, T and Q. G1 is detected for the very first time in Croatian population.

Conclusion: New insight into differences between examined subpopulations of Croatia and their possible (dis)similarities with neighboring abroad populations was notified.

The Y chromosome (~ 60 Mb) is relatively small and inherited from father to son unchanged (except for occasional mutations). The length of the Y chromosome is 57 Mb. Except for the small pseudoautosomal regions PAR, there is no recombination between the X and Y chromosome (1-3). This event is the reason why haplotype inheritance through the male lineage that can be tracked and analyzed (2, 4-6).

The Y chromosome mostly consists of repetitive sequences (around 50%), which are single-base substitutions, Alu elements, and LINEs. Short tandem repeats, as repetitive elements are the base of population genetic studies. Their average mutational frequency is ~0.2% per generation (7, 8).

Y haplogroup can be defined as a part of the Y chromosome family related by ancestry and determined by a specific set of Y-SNP. It is of great importance to better understand of the demographic processes that shaped modern populations (8-10). A relatively new approach related to Y haplogroup prediction uses a set of Y-STR markers (haplotypes) for the determination of a haplogroup of an individual.

The main aim of this research is to update information about Croatian Y chromosome diversity by using additional Y STR loci to compare new results with the previously published results generated using Y-STR and Y-SNP markers (11-14) and to restart the Croatian Y chromosome settlement scenario established in one of our previously published studies (15). Additionally, one of the goals was to analyze the subpopulation genetic structure of five Croatian regional subpopulations (with local center in Osijek, Pula, Varaždin, Split, and Hvar Island) identifying the most common haplogroups, as well as genetic differences between these five subpopulations, and analyzing potential (dis)similarity of those regional subpopulations with neighboring countries.

Materials and methods

For this study, buccal swab samples were obtained from 518 adult male individuals from five different regions of Croatia: Hvar (n=104), Osijek (n=110), Pula (n=99), Varaždin (n=100) and Split (n=105).

DNA extraction was performed on QIAsymphony instrument using the QIAsymphony DNA Investigator Kit and protocol (Qiagen, Hilden, Germany). DNA was quantified on Rotor-Gene Q real-time PCR cycler (Qiagen, Hilden, Germany) using Q-Rex Software and Investigator Quantiplex Pro RGQ Kit. Yfiler™ Plus PCR Amplification Kit (Applied Biosystems, Foster City, CA, USA) was used to simultaneously amplify 27 Y chromosome STR loci.

Amplification was carried out following the manufacturer's protocol. PCR amplification was performed on Mastercycler® nexus SX1 PCR thermal cycler (Eppendorf AG, Hamburg, Germany) according to the manufacturer's instructions. PCR-amplified products were separated and detected using standard protocols for electrophoresis on 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Allele calling was performed with GeneMapper™ ID-X Software v1.4 (Applied Biosystems, Foster City, CA, USA) using custom panel and bin sets.

A total number of 507 fully genotyped Y-STR profiles of the present study were submitted to Y Chromosome Haplotype Reference Database – YHRD with the accession numbers assigned as follows: Hvar (n=104; YA004742), Osijek (n=109; YA004743), Pula (n=94; YA004744), Varaždin (n=98; YA004746), and Split (n=102; YA004745).

Statistical analysis

The number of alleles and different haplotypes, allele and haplotype frequencies, and gene and haplotype diversity were estimated in order to assess the intrapopulation diversity.

Haplotype diversity was calculated using Nei's formula: HD = $(1-\sum p_i^2)*n/(n-1)$, where n is the sample size and p_i is the ith haplotype frequency. Gene diversity was calculated as $1-\sum p_i^2$, where p_i is the allele frequency. The formula $\sum p_i^2$ was used to calculate match probability (MP), where p_i is the frequency of the ith haplotype. Discrimination capacity (DC) was calculated by dividing the number of haplotypes by the number of individuals in the population (16, 17). Allele and haplotype frequencies, as well as gene and haplotype diversity were calculated using the STRAF software package v2.0.4 (18, 19).

Genetic distances between groups of males and between populations were quantified by R_{st} using AMOVA online tool from the Y Chromosome Haplotype Reference Database – YHRD (20, 21). In addition, associated probability values (*P* values) with 10,000 permutations were included for the studied populations. Genetic distances were used to generate MDS plots for the comparison of population haplotype data from YHRD.

AMOVA analysis was done with two population groups. The first comparison for analysis was made using the original number of the Yfiler™ Plus kit (27 Y-STR loci). The second comparison was made with a reduced number of markers using the Yfiler™ marker set (17 Y-STR loci). The reason for the two analyses was because of the increased number of markers in

the analyzed population and the reduced number of markers in the populations that were available for comparison. The first group of populations selected for comparison with the population of Croatia using the Yfiler™ Plus marker set included: Croatia (n = 507, present study), Slovenia (n = 194, (22)), Belgium (n = 160, (23-26)), Hungary (n = 218, (27-33)), Austria (n =392, (34-38)), Germany (n = 495, (39-49)), Italy (n = 689, (50-67)), North Macedonia (n = 295, (68-71)), Serbia (n = 183, (72-74)), Denmark (n=177, (75)), Ethiopia (n=290, (76)), French Polynesia (n=81), (77, 78)), Ghana (n=584, (79)), India (n=541, (80-98)), Lithuania (n=251, (99-101)), Mexico (n=354, (102-107)), Nigeria (n=337, (108)), Pakistan (n=280, (109-121)), Poland (n=612, (122-130)), Russian Federation (n=958, (131-143)), Saudi Arabia (n=156, (144, 145)), Spain (n=316, (146-164)), Switzerland (n=724, (19, 165, 166)), and United Kingdom (n=115, (167, 168)).

The second group of populations selected for comparison with the population of Croatia using reduced 17 Y-STR Yfiler™ marker set includes: Tiroler Unterland, Austria (n=547, (37)), Antwerpen, Belgium (n=309, (26)), Bosnia and Herzegovina (n=574, (169, 170)), Bulgaria (n=91, (31, 171)), Rostock, Germany (n=598, (25, 49)), Greece (n=191, (172)), Hungary (n=303, (30)), Italy (n=147, (65)), Warsaw, Poland (n=491, (122)), Serbia (n=567, (74)), Albania (n=315, (58, 66, 69, 173)), Czech Republic (n=109, (174)), Estonia (n=123, (99)), Ireland (n=863, (75, 168)), Lithuania (n=531, (100, 101)), North Macedonia (n=493, (68, 70, 71)), Norway (n=1555, (175, 176)), Slovenia (n=294, (22)), Sweden (n=296, (177, 178)), and Ukraine (n=212, (179)).

The evolutionary history was inferred for both sets of markers using the neighbor-joining (NJ) method of phylogenetic tree construction (180) in MEGAX (181), whereby the optimal tree is shown.

Y-chromosomal haplogroup prediction using allele frequencies on 518 Yfiler™ Plus profiles was performed using Whit Athey's Haplogroup Predictor v5 (182), an algorithm based on the Bayesian-allele-frequency approach (182, 183).

Results and discussion

A total of 518 haplotypes were detected and used for haplogroup prediction. Eleven haplotypes were considered newly detected microvariants, which require additional analysis for

confirmation. Therefore, the remaining 507 haplotypes (the ones without newly detected microvariants) were used for additional statistical analysis. On a sample of fully genotyped 507 Y-STR profiles, a total of 502 different haplotypes were detected in the study, with 497 unique haplotypes and 5 haplotypes appearing twice. In addition, 196 alleles at 27 Y-STR loci were detected (Table 1). Apart from the DYS385a/b double locus, the largest number of alleles was recorded on DYS481 with 14 detected alleles. Three loci had the smallest number of alleles, namely DYS393, DYS437, and Y-GATA-H4 with four alleles each.

Haplotype diversity was calculated to be 1.0000 ± 0.0014 with DC of 1.00 and MP of 0.01. The average genetic diversity for the study population was 0.656 across all loci, ranging from 0,886 for DYS481to 0,251 for DYS392. At the population level, the most common allele is allele 11 at locus DYS392 with a frequency of 0.862. This was not surprising considering that DYS392 is one of the least polymorphic loci in the studied population with six detected alleles and the lowest genetic diversity.

[Table 1. here]

In order to determine additional genetic differences within the analyzed population of Croatia, an interpopulation analysis was done between five regions: Hvar (n=104), Osijek (n=109), Pula (n=94), Split (n=102) and Varaždin (n=98). The lowest genetic diversity observed for the population of Hvar was compared to population of Split (*R*st=0.0009, *P*=0.3240). The greatest genetic diversity observed for the population of Hvar was compared to the population of Varaždin (*R*st=0.0979, *P*=0.0000), the population of Pula (*R*st=0.0284, *P*=0.0042) and the population of Osijek (*R*st=0.0210, *P*=0.0097). The lowest genetic diversity observed for the population of Osijek was compared to population of Split (*R*st=0.0063, *P*=0.1199) and the population of Pula (*R*st=0.0069, *P*=0.1138). The greatest genetic diversity observed for the population of Osijek was compared to the population of Varaždin (*R*st=0.0551, *P*=0.0000) and the population of Hvar (*R*st=0.0210, *P*=0.0097). The lowest genetic diversity observed for the population of Pula was compared to the population of Osijek (*R*st=0.0069, *P*=0.1138). The greatest genetic diversity observed for the population of Pula was compared to the population of Hvar (*R*st=0.0284, *P*=0.0042), the population of Split (*R*st=0.0180, *P*=0.0233) and the population of Varaždin (*R*st=0.0166, *P*=0.0260). The lowest genetic diversity observed for the population of Split was compared to population of Hvar (*R*st=0.0009, *P*=0.3240) and the population of Osijek (*R*st=0.0063, *P*=0.1199). The greatest genetic diversity observed for the

population of Split was compared to the population of Varaždin (*R*st=0.0821, *P*=0.0000) and the population of Pula (*R*st=0.0180, *P*=0.0233). The lowest genetic diversity observed for the population of Varaždin was compared to the population of Pula (*R*st=0.0166, *P*=0.0260). The greatest genetic diversity observed for the population of Varaždin was compared to the population of Hvar (*R*st=0.0979, *P*=0.0000), the population of Split (*R*st=0.0821, *P*=0.0000) and the population of Osijek (*R*st=0.0551, *P*=0.0000).

[Table 2. here]

In order to compare the studied population with a large number of worldwide published population data, interpopulation analyses were completed by comparing the analyzed population with two groups of countries. The first group of selected countries used the original set of markers 27 Y-STRs; the selection was limited since this is an expanded panel of Y-STR markers.

By comparing the population from the present study with previously published data of the first selected group on 27 Y-STR markers for 23 populations, the lowest genetic diversity was observed between the currently analyzed population of Croatia and the previously published Serbian population (*R*st=0.0097, *P*=0.0055), and the population of Slovenia (*R*st= 0.0297, *P*=0.000). Other populations with low genetic diversity values when compared to the present results include those from Hungary (*R*st=0.0482, *P*=0.000), North Macedonia (*R*st=0.0720, *P*=0.0000), Russian Federation (*R*st=0.0779, *P*=0.0000), Pakistan (*R*st=0.0854, *P*=0.0000), Poland (*R*st=0.0905, *P*=0.0000), India (*R*st=0.0961, *P*=0.0000), and Saudi Arabia (*R*st=0.0990, *P*=0.0000). The highest genetic distance was observed when the study population was compared with the populations of Spain (*R*st= 0.3283, *P*=0.000), Ghana (*R*st=0.2787, *P*=0.0000), Nigeria (*R*st=0.2414, *P*=0.0000), Italy (*R*st=0.2043, *P*=0.0000), Belgium (*R*st=0.2031, *P*=0.0000), Switzerland (*R*st=0.1999, *P*=0.0000), Lithuania (*R*st=0.1914, *P*=0.0000), French Polynesia (*R*st=0.1878, *P*=0.0000), Mexico (*R*st=0.1785, *P*=0.0000), Ethiopia (*R*st=0.1704, *P*=0.0000), Germany (*R*st=0.1461, *P*=0.0000), Denmark (*R*st=0.1290, *P*=0.0000), Austria (*R*st=0.1255, *P*=0.0000), and United Kingdom (*R*st=0.1122, *P*=0.0000).

[Table 3. here]

The second group of populations selected for comparison with the population of Croatia using reduced 17 Y-STR set of markers included 20 populations. The lowest genetic diversity was

observed between the currently analyzed population of Croatia and previously published population of Bosnia and Herzegovina (R_{st}=0.0076, P=0.0002), and the population of Serbia (R_{st}= 0.0186, P=0.000). Other populations with low genetic diversity values when compared to the present results include those from Bulgaria (R_{st}=0.0144, P=0.000), Ukraine (R_{st}=0.0195, P=0.000), Slovenia (R_{st}=0.0204, P=0.000), Hungary (R_{st}=0.0238, P=0.0000), Greece (R_{st}=0.0241, P=0.0000), North Macedonia (R_{st}=0.0375, P=0.0000), Italy (R_{st}=0.0659, P=0.0000), Albania (R_{st}=0.0728, P=0.0000), Czech Republic (R_{st}=0.0767, P=0.000), and Austria (R_{st}=0.0795, P=0.0000). The highest genetic distance was observed when the study population was compared with the populations of Ireland (R_{st}= 0.3178, P=0.000), Estonia (R_{st}=0.1877, P=0.0000), Lithuania (R_{st}=0.1706, P=0.0000), Belgium (R_{st}=0.1429, P=0.0000), Norway (R_{st}=0.1270, P=0.0000) Poland (R_{st}=0.1216, P=0.0000), Sweden (R_{st}=0.1209, P=0.0000), and Germany (R_{st}=0.1036, P=0.0000)

[Table 4. here]

Genetic relationships between investigated populations are also represented through MDS plots (Figures 1-3). The results of such comparisons confirm the general trends that were observed in Tables 2-4.

[Figures 1-3 here]

To further investigate molecular evolutionary relationships between the populations of Croatia and selected populations, NJ phylogenetic trees were constructed based on *R*st values for different regions of Croatia and both compared groups (Figures 4- 6). Generally, the population comparisons point towards an overall conclusion that geographically closer populations show a higher degree of genetic relatedness.

[Figures 4-6 here]

The constructed NJ tree shows the comparison between the five regions of the analyzed population of Croatia (Figure 4a). The populations of the Hvar and Split regions are clustered together; the populations of the Osijek and Pula regions are presented in a separate cluster. The population of the Varaždin region is on a separate cluster from other regions, which could

indicate the potential genetic specificity (probably linked with geographical position) of this subpopulation compared with the other four examined regional subpopulations.

Additionally, the expanded NJ tree of the analyzed population from five Croatian regions together with the neighboring abroad population (Figure 4b) shows expected genetic similarity between the populations of the Hvar, Split, and Osijek regions. The populations of the Pula region are also located in the same macro-cluster. The eastern neighboring Serbian population is also included in that macro-cluster, with the closest genetic similarity with Pula, then the other three regional subpopulations. However, the population of the Varaždin region is in a relatively distanced cluster from the other four regions. The Varaždin population shows potential genetic closeness with neighboring Slovenian and Hungarian populations. These results prove again that the Y-chromosome is expected to show greater geographical clustering than other population markers (2, 15), but also could potentially mark significant migrations from the eastern neighboring countries to the Pula region especially in the second half of the 20th century.

In the NJ tree constructed for comparison of the group with the original set of 27 Y-STR markers, the Croatian population is clustered together with the populations of Serbia and Slovenia. The populations of Lithuania, Poland, and the Russian Federation are clustered together on the same branch with populations of Croatia, Serbia, Slovenia. The population of North Macedonia is positioned on the same branch as well. The populations of French Polynesia and Mexico are clustered together. The populations of Hungary and India clustered together. Populations of Belgium and Spain are clustered together on the same branch with populations of Switzerland, Italy, Germany, Austria, Denmark, and Pakistan. The populations of Ghana and Nigeria are clustered together with the populations of Ethiopia, Saudi Arabia, and the United Kingdom (Figure 5).

Compared to population of Croatia and selected populations with a reduced marker set, the NJ tree shows the population of Croatia is positioned on the same branch with the cluster of populations of Bosnia and Herzegovina and Serbia. The populations of Albania and North Macedonia are clustered together on the branch with the populations of Greece and Bulgaria. The populations of Slovenia and Ukraine are clustered together. The populations of Poland, Estonia, and Lithuania are clustered together. On a different cluster, positioned together are the populations of Germany, Belgium, and Ireland. In addition, population comparisons using

comparative analysis methods presented above show very low to no genetic differentiation between the currently analyzed population and the Western Balkan populations.

For the calculation of Y-chromosomal haplogroup prediction and intrapopulation variability between the five subpopulations, the total number of 518 Yfiler™ Plus profiles was used: Hvar (n=104), Varaždin (n=100), Split (n=105), Pula (n=99), and Osijek (n=110). Regarding the haplogroup diversity between these five subpopulations of Croatia, successful haplogroup assignment was obtained for all 518 Y-STR profiles (Table 5). The results of Y haplogroup prediction using Whit Athey's Haplogroup Predictor tool (182) are summarized in Figure 7. Prediction accuracy was estimated to be 100% in 492 cases. For the remaining 26 samples the prediction accuracy was 5%. Prediction accuracy varied between 63,1% and 99.58%. Out of a total of 14 detected haplogroups, the most prevalent one is I2a, which accounts for 39% of all samples, followed by R1a (24,32%) and E1b1b (10,18%). The remaining eight haplogroups were less frequent.

[Figure 7. here]

[Table 5. here]

Four of five subpopulations of Croatia showed expected results (Figure 8a-c). High frequency of haplogroup I has been reported with its known sublineage I2a in the subpopulations Hvar 52,88%, Split 50,48%, Osijek 40,91%, and Pula 31,31%. Previously published reports demonstrate similar results (11, 13, 14). However, different results were obtained in the subpopulation of Varaždin (Figure 8d). R1a was the most frequent haplogroup in this subpopulation with the frequency of 38%, while the frequency for I2a haplogroup was 18%. It is interesting to note that R1a was also the dominant haplogroup within Slovenian population, which is the closest neighboring abroad population to Varaždin county (184).

[Figure 8 here]

In summary, sublineage I2a was generally the most frequent haplogroup in the populations of Croatia, Bosnia and Serbia, according to this study, but also to all previously studied data (11, 15, 185).

Arrival of haplogroup I in this part of Europe is approximated to around 25,000 years ago from the Middle East through Anatolia in the area of Balkan (15, 186). One scenario suggests the possibility of population expansion from one of the post-Glacial refuges into the rest of the Balkan Peninsula (169). There is also another possibility that this haplogroup could be connected with more recent population movements from the eastern Europe, but this idea still has to be examined (9). Definitively, when comparing to the other populations in Europe, the I2a haplogroup sublineage is considered the characteristic Southeast European haplogroup (187).

The R1a as a leading sublineage of haplogroup R was the second most frequent haplogroup in the studied population of Croatia, with an overall frequency of 24,32%. The prevalence of haplogroup R1a in the subpopulations of Croatia reported frequencies for Varaždin 38%, Pula 28,28%, Osijek 26,36% and Split 19,05%. In the subpopulation of Hvar a small genetic deviation in the frequency of haplogroups R1a and E1b1b was reported. The R1a haplogroup accounted for 10,58% just slightly lower than haplogroup E1b1b with frequency 11,54% (Figure 8a). This is most likely due to the founder effect which is expected for island populations. In previously reported studies on mainland population of Croatia, haplogroup R reported a frequency of 22,9% (11, 185). Migration theories of R1a origins indicates outflow of haplogroup R from West Asia to the region of Balkan as a post-Last Glacial Maximum (LGM) event, during the Mesolithic time (15, 188).
Sublineage R1b presented a lower frequency in the studied population of Croatia. The overall frequency of the R1b sublineage for the population of Croatia accounts of 6,37%. The highest frequency of R1b haplogroup was reported in the subpopulation of Varaždin with a prevalence of 9% and in Pula with a frequency of 8,08%. Similar results were obtained in the Bosnian population based on 481 Y-STR profiles, whereby R1b accounted for 8,75% of the samples (189).

Sublineage E1b1b is the most frequent “neolithic haplogroup” for the males in this part of Europe (15). In the present study E1b1b was detected with a frequency of 10,81%. The highest prevalence of this haplogroup was reported in the subpopulation of Varaždin, with a frequency of 16%. In the other four subpopulations the frequency of haplogroup E1b1b was estimated: Hvar 11,54%, Pula 11,11%, Split 10,48% and Osijek 5,45%. In previously published results, this haplogroup was also less frequent in the population of Croatia (5.5%), Serbia (18.6%) and Bosnia and Herzegovina (14,58%) (11, 185, 189). There are two suggested theories about

E1b1b arrival in Europe. One theory is a post-LGM event from Asia and Africa during Neolithic period of time, while the other evidence suggests that this haplogroup is Balkan-specific, and originated around 8,000 years ago as a part of Greek colonization among the northern part of the Peninsula (15, 186). This ancient European haplogroup shows its possible dual origin from two different source populations, during recolonization of Europe from Iberia and from West Asia (15, 185).

Rare haplogroups discovered in this study were Q, T, L and G1 each present in 1,93%, 0,58%, 0,19% and 0,19% of all samples, respectively. Haplogroup L is associated with South Asia and India but is also found in low frequencies in Central Asia, Southwest Asia, and Southern Europe. With its alternative phylogenetic name K1a, haplogroup L is closely related to haplogroup T (190). Haplogroup T, also known by its phylogenetic name K1b, possibly originated in Western Asia and spread to East Africa, South Asia, and Southern Europe (191, 192). Finally, haplogroup Q represents the only Pan-American haplogroup and confirms the Asian origin of Native Americans, and provides insight into the main Asian-American migrations (193). Haplogroup G1 is detected for the very first time within Croatian population. This haplogroup is found predominantly in Iran, and is very rare in Europe. Its origin is still not clearly described.

The present analyses generally confirmed previous results and provided a more detailed insight into the genetic diversity of the subpopulations of Croatia. Analysis of 27 Y-STR loci on the currently studied population of Croatia was quantified through *R*st values and calculated based on the results of the same set of markers from other populations from the YHRD database. The results indicate that the Croatian population does not deviate significantly from the neighboring populations of Bosnia and Herzegovina, Serbia, and Slovenia. This proves that the Y chromosome genetic marker has noticeable geographical background (2, 15) and this analysis resulted with expected geographic clustering.

A previous study (15) concluded that most of Croatian men ("owners" of HgI, R1a and R1b) are harboring the ancestral genetic impact of Old Europeans people who settled in Europe approximately 25.000 – 30.000 years ago and survived the LGM in several different refugium. Results of our studies confirm that more than 78% of the contemporary Croatians are included in that group. The rest of the population relate to the people who arrived mostly during the Neolithization process. A small portion of examined population originated from the "owners" of rare haplogroups in the term of European genetic diversity, of which the origin is still not

clarified. Usage of additional Y-STR loci revealed detailed insight and supplementary information within the highly diverse and significant genetic diversity of the Croatian population.

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TABLE 1. Allele frequencies for the 27 Y-STR loci in the population of Croatia.

Allele/Locus	DYF387S1a	DYF387S1b	DYS19	DYS385a	DYS385b	DYS389I	DYS389II	DYS390	DYS391	DYS392	DYS393	DYS437	DYS438	DYS439	DYS448	DYS449	DYS456	DYS458	DYS460	DYS481	DYS518	DYS533	DYS570	DYS576	DYS627	DYS635	YGATAH4
7													0,002														
8																			0,006								
9			0,002						0,020				0,079	0,006					0,097			0,012					
10				0,028					0,485				0,602	0,162					0,584			0,014					0,039
11				0,270	0,006	0,002			0,483	0,862			0,256	0,235					0,262			0,124					0,531
12			0,002	0,028	0,002	0,144			0,010	0,041	0,081		0,057	0,333			0,006		0,051			0,517					0,361
13			0,126	0,114	0,036	0,704			0,002	0,065	0,854	0,002	0,004	0,235			0,020	0,008				0,321					0,069
14			0,134	0,394	0,320	0,146				0,012	0,059	0,432		0,030			0,077	0,032				0,012					
15			0,249	0,059	0,406	0,002				0,018	0,006	0,465					0,509	0,249					0,006	0,022	0,014		
16			0,400	0,085	0,049	0,002				0,002		0,101					0,223	0,231					0,041	0,095	0,077		
17			0,083	0,022	0,075										0,002		0,150	0,331					0,178	0,280	0,136		
18			0,004		0,081										0,008		0,016	0,112					0,391	0,416	0,063		
19					0,018										0,357			0,036					0,217	0,128	0,105		
20					0,008										0,556			0,002		0,004			0,110	0,045	0,306	0,024	
21								0,004							0,073					0,083			0,041	0,010	0,187	0,116	
22								0,069							0,002					0,150			0,016	0,004	0,073	0,235	
23								0,148							0,002					0,178					0,032	0,499	
24								0,527												0,077					0,008	0,103	
25								0,237								0,002				0,063						0,022	
26								0,016								0,002				0,016						0,002	
27							0,004									0,014				0,037							
28							0,075									0,073				0,045							
29							0,178									0,144				0,059							
30							0,329									0,168				0,164							
31							0,321									0,233				0,103							
32							0,087									0,191				0,018							
33							0,004									0,128				0,004							
34	0,018						0,002									0,043											
35	0,144	0,034														0,002					0,002						
36	0,150	0,049																			0,018						
37	0,274	0,089																			0,051						
38	0,353	0,363																			0,124						
39	0,059	0,391																			0,292						
40	0,002	0,063																			0,254						
41		0,012																			0,160						
42																					0,067						
43																					0,028						
44																					0,004						

TABLE 2. Interpopulation comparison of the current data within five different regions genetic distance *R*st values and *P* values. Data was obtained from the relevant publications and accessed through the YHRD database.

Population	Hvar	Osijek	Pula	Split	Varaždin
Hvar	*	0.0097	0.0042	0.3240	0.0000
Osijek	0.0210	*	0.1138	0.1199	0.0000
Pula	0.0284	0.0069	*	0.0233	0.0260
Split	0.0009	0.0063	0.0180	*	0.0000
Varaždin	**0.0979**	**0.0551**	0.0166	**0.0821**	*

TABLE 3. Interpopulation comparison of the current data with 23 previously published European populations using genetic distance *R*st values and *P* values. Data was obtained from the relevant publications and accessed through the YHRD database.

Population	Croatia	Austria	Belgium	Denmark	Ethiopia	French Polynesia	Germany	Ghana	Hungary	India	Italy	Lithuania	Mexico	Nigeria	North Macedonia	Pakistan	Poland	Russian Federation	Saudi Arabia	Serbia	Slovenia	Spain	Switzerland	United Kingdom
Croatia	-	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0055	0.0000	0.0000	0.0000	0.0000
Austria	0.1255	-	0.0000	0.0024	0.0000	0.0000	0.0052	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Belgium	0.2031	0.0197	-	0.0000	0.0000	0.0000	0.0027	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.4444	0.0000
Denmark	0.1290	0.0099	0.0412	-	0.0000	0.0000	0.0013	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Ethiopia	0.1704	0.2165	0.2801	0.2495	-	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
French Polynesia	0.1878	0.1419	0.2024	0.1934	0.2763	-	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Germany	0.1461	0.0045	0.0104	0.0102	0.2501	0.1709	-	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Ghana	0.2787	0.3710	0.4691	0.4133	0.2459	0.3321	0.4094	-	0.0000	0.0000	0.0000	0.0000	0.0000	0.0012	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Hungary	0.0482	0.0757	0.1484	0.0879	0.1303	0.1706	0.0947	0.3044	-	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0004	0.0000	0.0000	0.0000
India	0.0961	0.1023	0.1642	0.1154	0.1454	0.1681	0.1217	0.2779	0.0201	-	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Italy	0.2043	0.0249	0.0159	0.0575	0.2656	0.1578	0.0281	0.4213	0.1339	0.1529	-	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Lithuania	0.1914	0.1028	0.1330	0.0847	0.2836	0.2703	0.0922	0.4656	0.1106	0.1226	0.1454	-	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Mexico	0.1785	0.0949	0.1173	0.1287	0.2192	0.1469	0.1094	0.3530	0.1398	0.1410	0.1291	0.2279	-	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Nigeria	0.2414	0.3309	0.4230	0.3709	0.2023	0.2829	0.3721	0.0054	0.2563	0.2394	0.3853	0.4213	0.3107	-	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
North Macedonia	0.0720	0.0374	0.0848	0.0627	0.1212	0.1627	0.0548	0.3156	0.0348	0.0656	0.0780	0.1377	0.1012	0.2687	-	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Pakistan	0.0854	0.0531	0.1030	0.0519	0.2216	0.1213	0.0644	0.3449	0.0381	0.0503	0.1003	0.0963	0.1256	0.2998	0.0718	-	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Poland	0.0905	0.1294	0.1886	0.1076	0.2569	0.2681	0.1225	0.4045	0.0687	0.0972	0.2007	0.0684	0.2363	0.3703	0.1198	0.0762	-	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Russian Federation	0.0779	0.0677	0.1188	0.0648	0.1586	0.1833	0.0794	0.2947	0.0303	0.0593	0.1229	0.0704	0.1350	0.2609	0.0471	0.0689	0.0635	-	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Saudi Arabia	0.0990	0.1150	0.1891	0.1464	0.0732	0.1506	0.1523	0.2084	0.0562	0.0604	0.1695	0.2070	0.0956	0.1639	0.0577	0.1029	0.1896	0.0794	-	0.0000	0.0000	0.0000	0.0000	0.0002
Serbia	0.0097	0.1259	0.2101	0.1415	0.1231	0.2006	0.1525	0.2604	0.0449	0.0847	0.2057	0.2023	0.1647	0.2159	0.0517	0.1014	0.1189	0.0743	0.0669	-	0.0000	0.0000	0.0000	0.0000
Slovenia	0.0297	0.0671	0.1355	0.0615	0.1703	0.1821	0.0781	0.3378	0.0142	0.0514	0.1387	0.0932	0.1533	0.2887	0.0418	0.0334	0.0310	0.0275	0.0899	0.0423	-	0.0000	0.0000	0.0000
Spain	0.3283	0.1183	0.0490	0.1695	0.4077	0.3475	0.0887	0.5812	0.2931	0.2865	0.0760	0.2378	0.2156	0.5509	0.2032	0.2321	0.2990	0.2268	0.3483	0.3590	0.2795	-	0.0000	0.0000
Switzerland	0.1999	0.0138	-0.0003	0.0299	0.2845	0.1833	0.0075	0.4310	0.1421	0.1632	0.0180	0.1283	0.1143	0.3997	0.0840	0.1026	0.1816	0.1170	0.1850	0.2052	0.1314	0.0581	-	0.0000
United Kingdom	0.1122	0.0813	0.1442	0.1122	0.0780	0.1429	0.1167	0.2236	0.0562	0.0558	0.1344	0.1718	0.0994	0.1752	0.0438	0.0865	0.1744	0.0756	0.0181	0.0808	0.0809	0.3041	0.1438	-

TABLE 4. Interpopulation comparison of the current data with 20 previously published European populations using genetic distance *R*st values and *P* values. Data was obtained from the relevant publications and accessed through the YHRD database.

Population	Croatia	Austria	Belgium	Bosnia and Herzegovina	Bulgaria	Germany	Greece	Hungary	Italy	Poland	Serbia	Albania	Czech Republic	Estonia	Ireland	Lithuania	North Macedonia	Norway	Slovenia	Sweden	Ukraine
Croatia	*	0.0000	0.0000	0.0002	0.0089	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0001
Austria	0.0795	*	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0035	0.0000	0.0000	0.0000	0.0079	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Belgium	0.1429	0.0188	*	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0007	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Bosnia and Herzegovina	0.0076	0.0988	0.1671	*	0.0034	0.0000	0.0000	0.0000	0.0000	0.0000	0.0002	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Bulgaria	0.0144	0.0649	0.1467	0.0186	*	0.0000	0.1307	0.0001	0.0000	0.0000	0.0095	0.0041	0.0000	0.0000	0.0000	0.0000	0.0706	0.0000	0.0001	0.0000	0.0003
Germany	0.1036	0.0214	0.0173	0.1382	0.1206	*	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.2638	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Greece	0.0241	0.0519	0.1187	0.0269	0.0048	0.1025	*	0.0001	0.0008	0.0000	0.0001	0.0024	0.0000	0.0000	0.0000	0.0000	0.0785	0.0000	0.0000	0.0000	0.0000
Hungary	0.0238	0.0252	0.0623	0.0451	0.0354	0.0321	0.0283	*	0.0001	0.0000	0.0000	0.0000	0.0024	0.0000	0.0000	0.0000	0.0000	0.0000	0.0973	0.0000	0.0130
Italy	0.0659	0.0144	0.0441	0.0744	0.0465	0.0546	0.0223	0.0273	*	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Poland	0.1216	0.1049	0.1305	0.1687	0.1603	0.0580	0.1555	0.0679	0.1542	*	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Serbia	0.0186	0.1087	0.1870	0.0068	0.0145	0.1612	0.0182	0.0612	0.0735	0.2021	*	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Albania	0.0728	0.0960	0.1714	0.0585	0.0258	0.1723	0.0159	0.0865	0.0527	0.2372	0.0336	*	0.0000	0.0000	0.0000	0.0000	0.0112	0.0000	0.0000	0.0000	0.0000
Czech Republic	0.0767	0.0156	0.0252	0.1086	0.0875	0.0012	0.0704	0.0176	0.0390	0.0519	0.1330	0.1324	*	0.0000	0.0000	0.0000	0.0000	0.0018	0.0002	0.0000	0.0000
Estonia	0.1877	0.1038	0.0940	0.2198	0.2054	0.0623	0.1630	0.1043	0.1226	0.1052	0.2477	0.2263	0.0693	*	0.0000	0.0080	0.0000	0.0000	0.0000	0.0000	0.0000
Ireland	0.3178	0.1360	0.0852	0.3403	0.3446	0.1221	0.3169	0.2276	0.2137	0.2475	0.3677	0.3590	0.1671	0.2125	*	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Lithuania	0.1706	0.1162	0.1109	0.2114	0.2008	0.0615	0.1754	0.0977	0.1484	0.0555	0.2415	0.2548	0.0652	0.0121	0.2139	*	0.0000	0.0000	0.0000	0.0000	0.0000
North Macedonia	0.0375	0.0747	0.1458	0.0312	0.0067	0.1358	0.0036	0.0528	0.0419	0.1887	0.0165	0.0067	0.1028	0.2029	0.3271	0.2178	*	0.0000	0.0000	0.0000	0.0000
Norway	0.1270	0.0391	0.0394	0.1616	0.1473	0.0160	0.1183	0.0500	0.0715	0.0862	0.1797	0.1913	0.0178	0.0553	0.1700	0.0670	0.1535	*	0.0000	0.0000	0.0000
Slovenia	0.0204	0.0376	0.0887	0.0463	0.0297	0.0466	0.0284	0.0025	0.0439	0.0639	0.0606	0.0868	0.0269	0.1196	0.2641	0.1046	0.0511	0.0589	*	0.0000	0.0281
Sweden	0.1209	0.0427	0.0447	0.1437	0.1301	0.0400	0.0894	0.0545	0.0449	0.1360	0.1546	0.1406	0.0363	0.0646	0.2141	0.0956	0.1176	0.0147	0.0674	*	0.0000
Ukraine	0.0195	0.0603	0.1135	0.0461	0.0405	0.0599	0.0384	0.0079	0.0619	0.0530	0.0640	0.1005	0.0396	0.1254	0.2892	0.1021	0.0632	0.0845	0.0060	0.0950	*

TABLE 5. Haplogroup composition in the five regions of Croatia.

	Haplogroup/number of haplotypes													
Region	I1	I2a	I2b	J1	J2a	J2b	R1a	R1b	G1	G2a	E1b1b	Q	T	L
Hvar	3	55			4	1	11	4		6	12	8		
Varaždin	8	18	3	1	4	3	38	9			16			
Split	5	53	2		2	3	20	5	1		11	1	2	
Pula	9	31	1	1	3	2	28	8		3	11		1	1
Osijek	12	45	1	1	5	2	29	7		1	6	1		
Total number of haplotypes	37	202	7	3	18	11	126	33	1	10	56	10	3	1

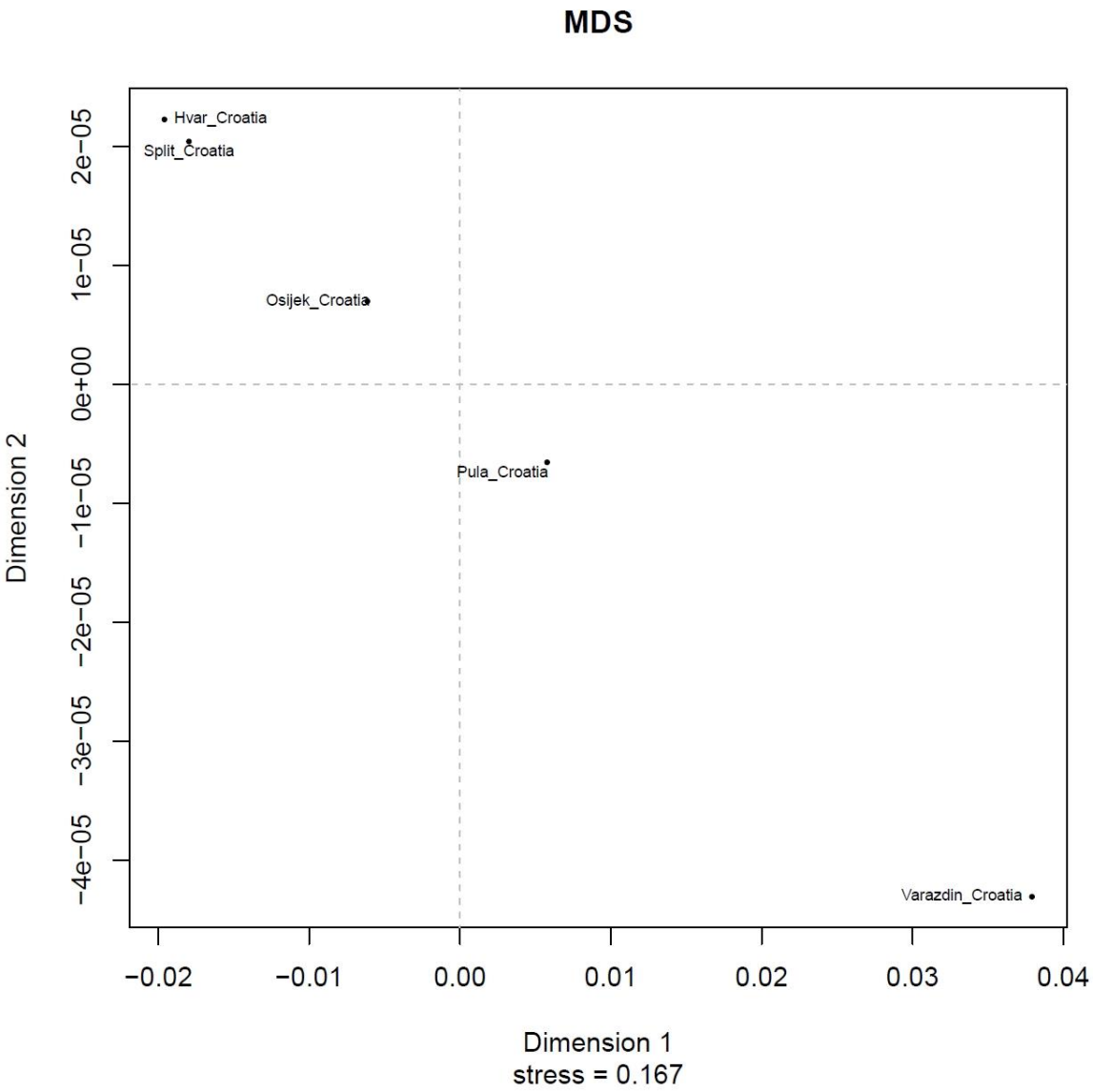

FIGURE 1. MDS plot showing genetic differentiation between five different regions of the analyzed populations of Croatia in two dimensions with original 27 Y-STR markers.

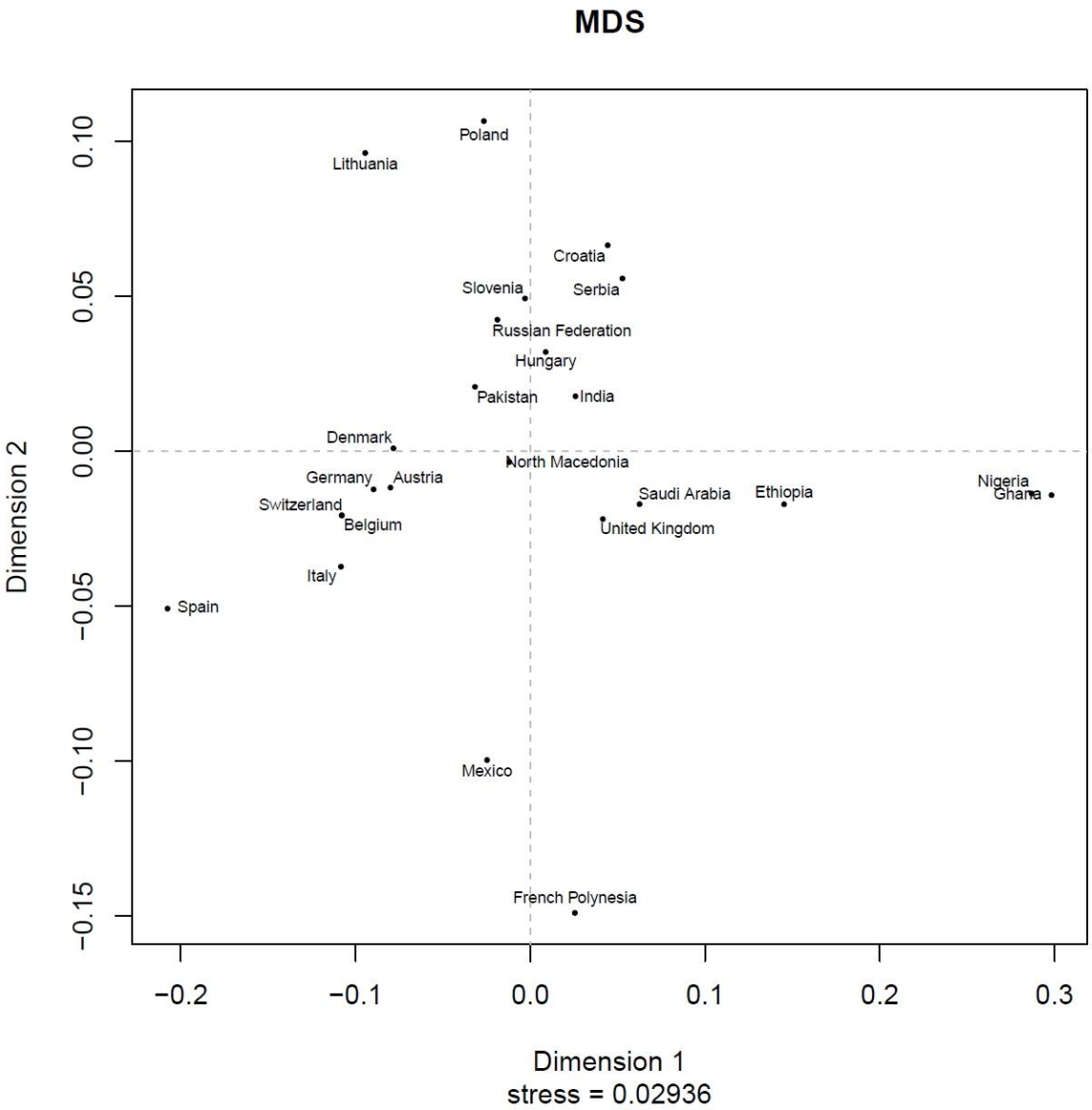

FIGURE 2. MDS plot showing genetic differentiation between 24 analysed populations in two dimensions with original 27 Y-STR markers.

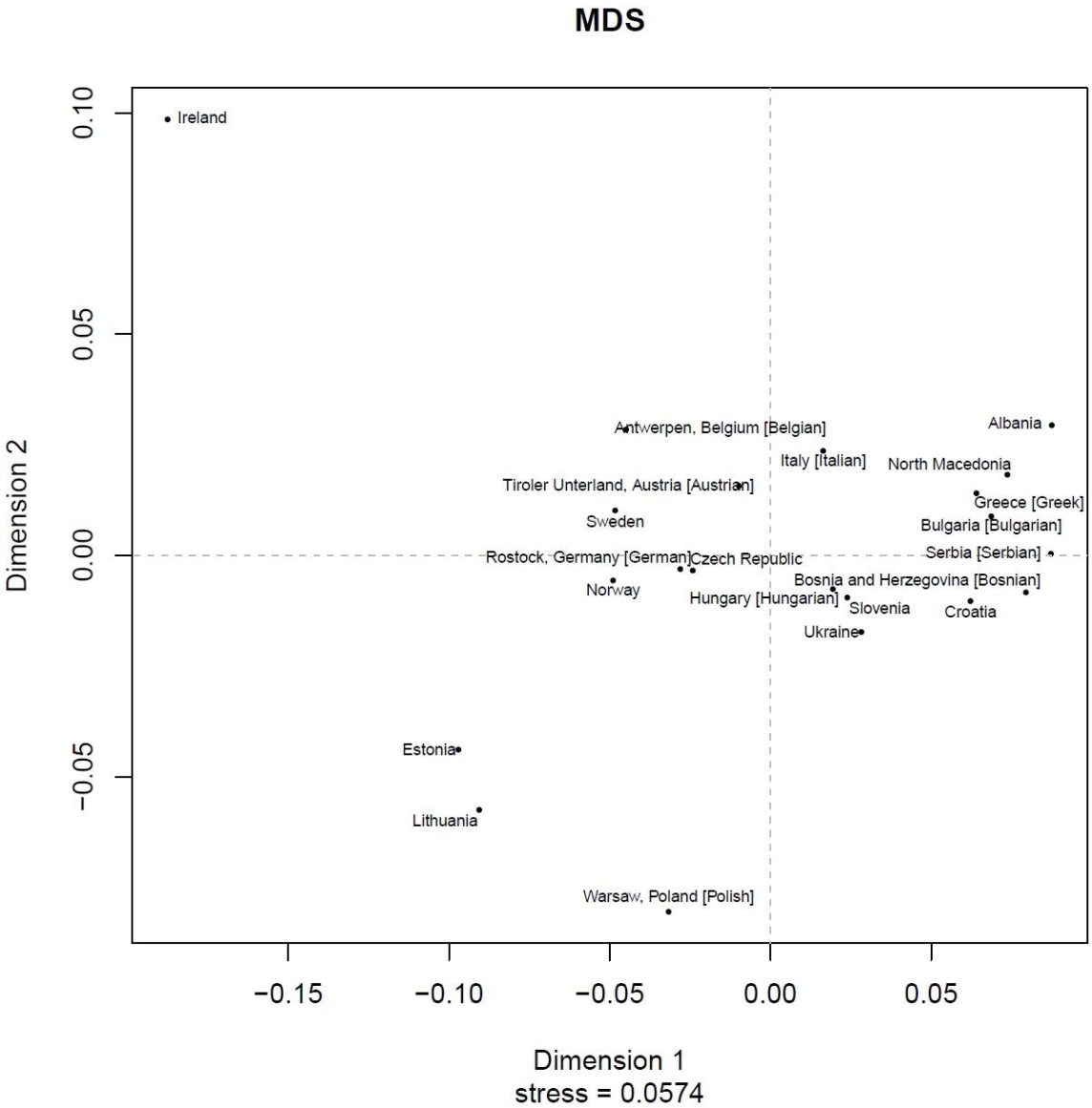

FIGURE 3. MDS plot showing genetic differentiation between the 21 analyzed populations in two dimensions with reduced number of 17 Y-STR markers.

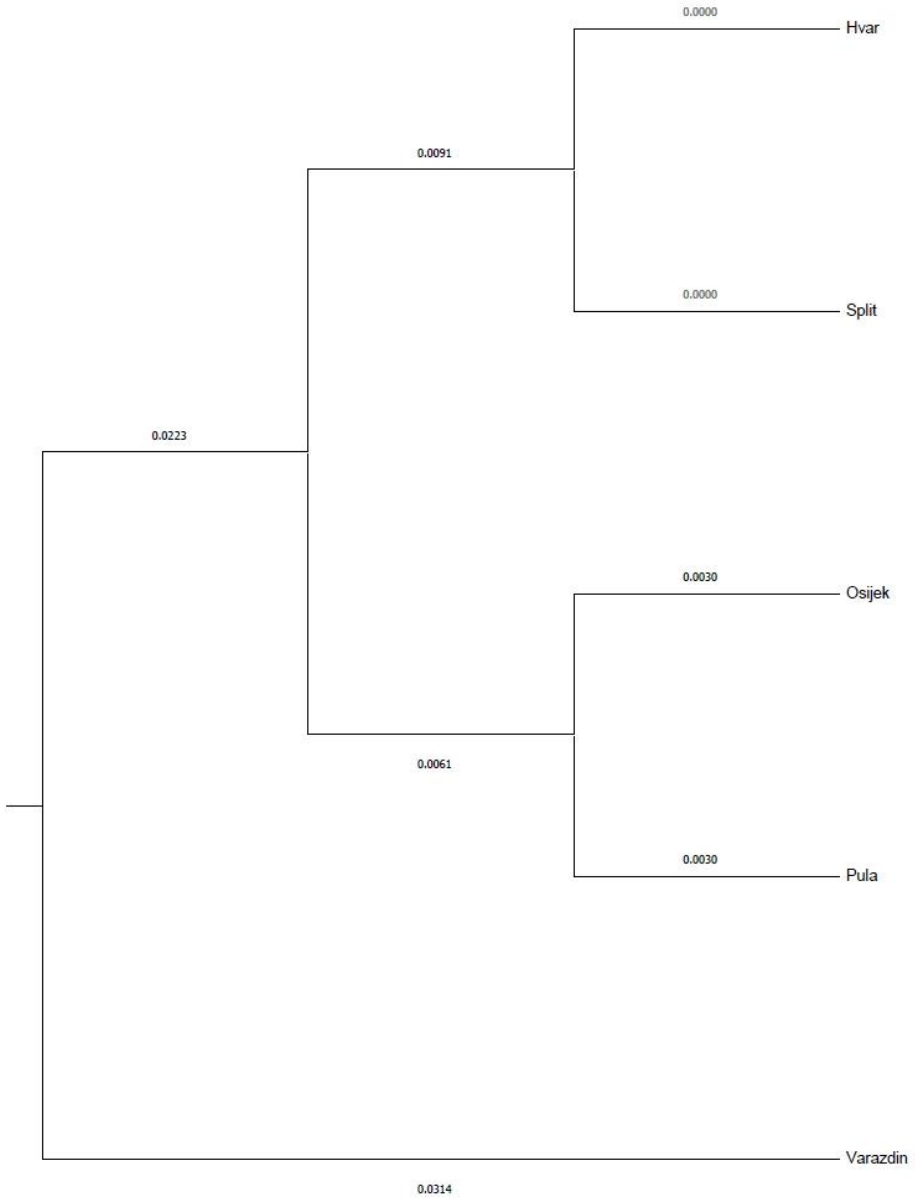

FIGURE 4a. Neighbour-joining phylogenetic tree showing genetics relationships and clustering between five different regions of the studied population of Croatia with original 27 Y-STR markers.

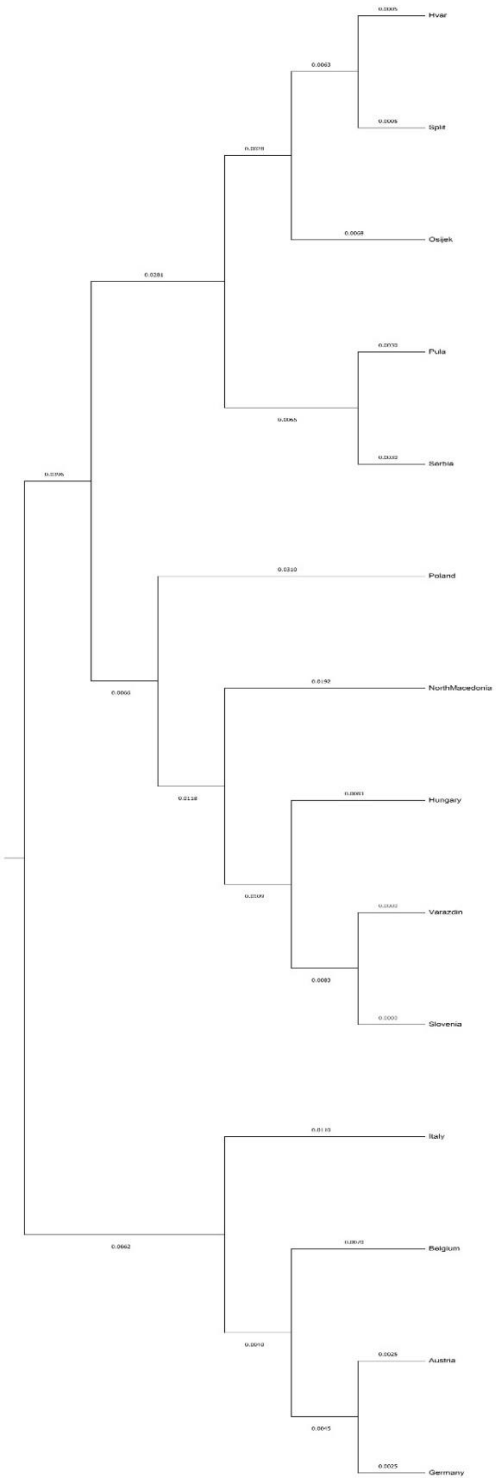

FIGURE 4b. Neighbor-joining phylogenetic tree showing genetics relationships and clustering between five different regions of the studied population of Croatia with original 27 Y-STR markers and selected neighboring countries.

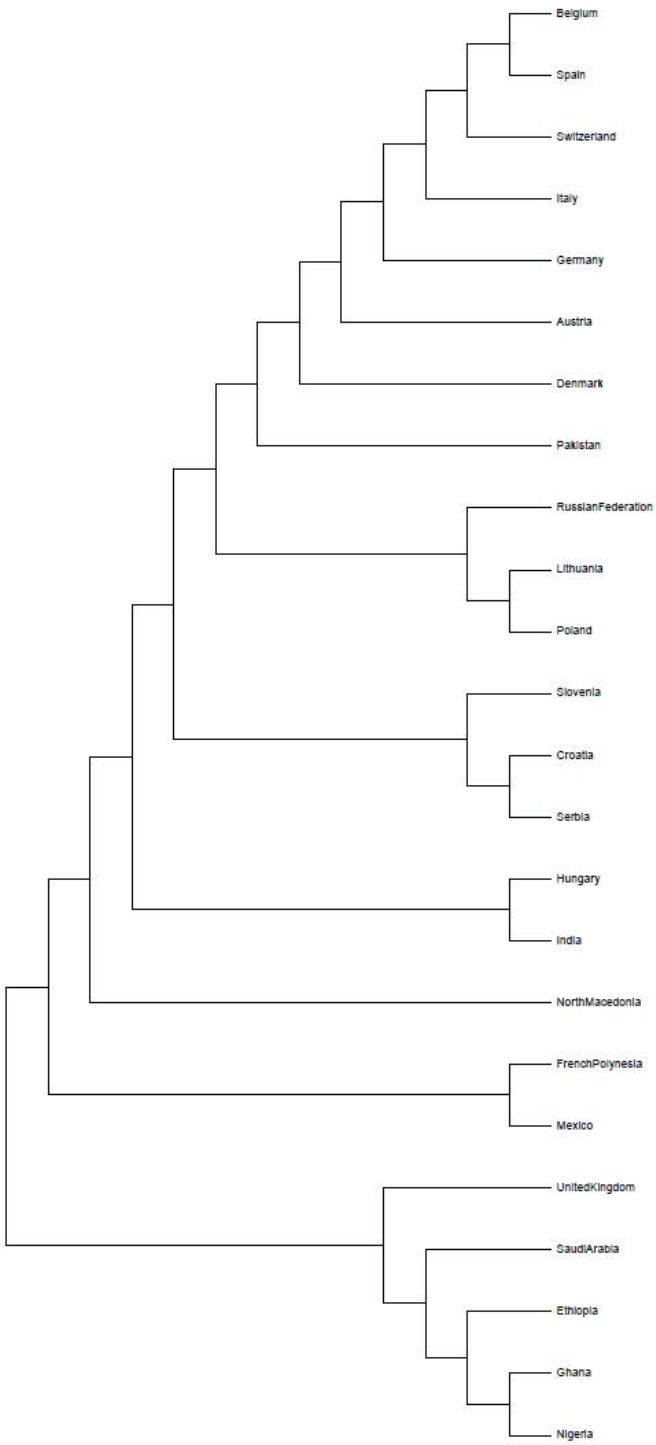

FIGURE 5. Neighbor-joining phylogenetic tree showing genetics relationships and clustering between the 24 studied populations with original 27 Y-STR markers.

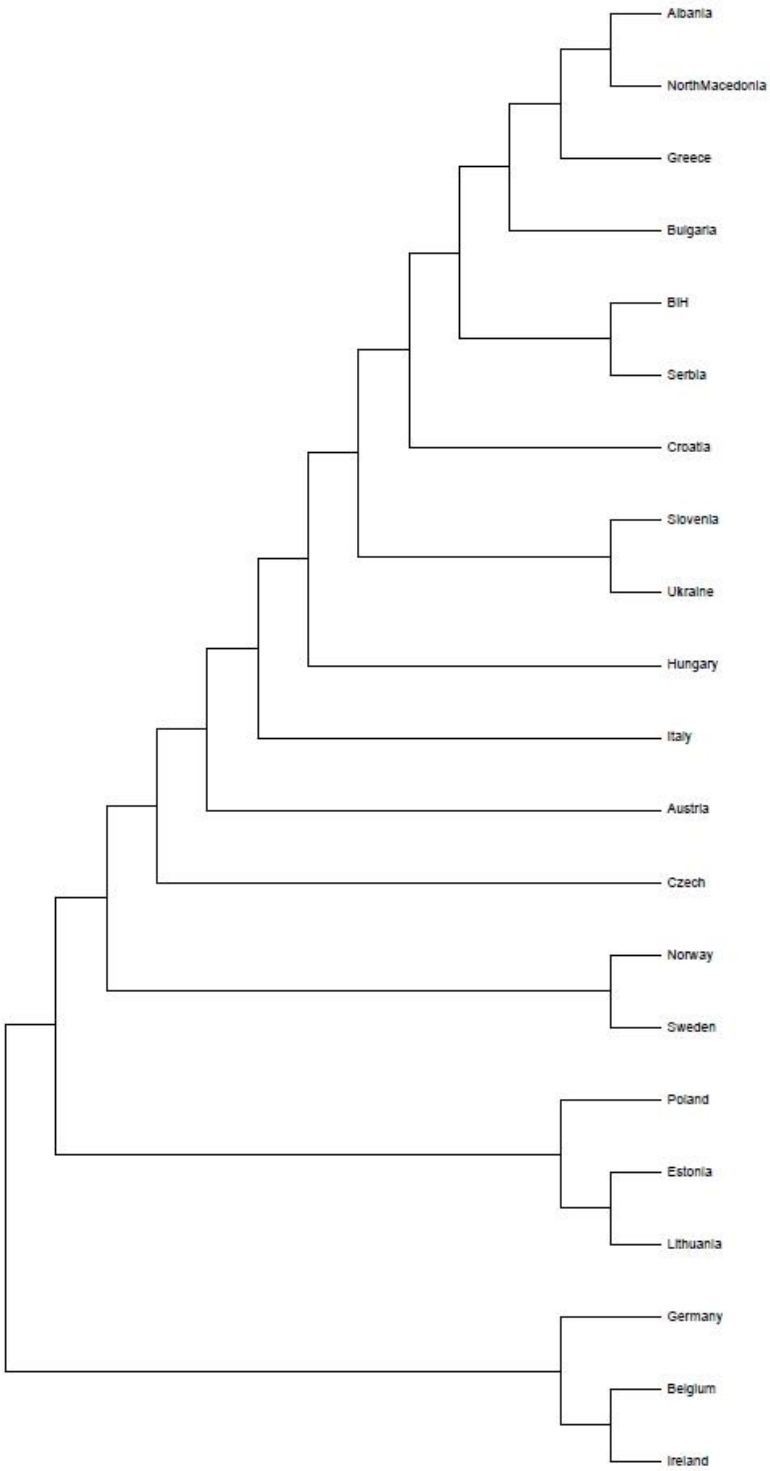

FIGURE 6. Neighbor-joining phylogenetic tree showing genetics relationships and clustering between the 21 studied populations with reduced number of 17 Y-STR markers.

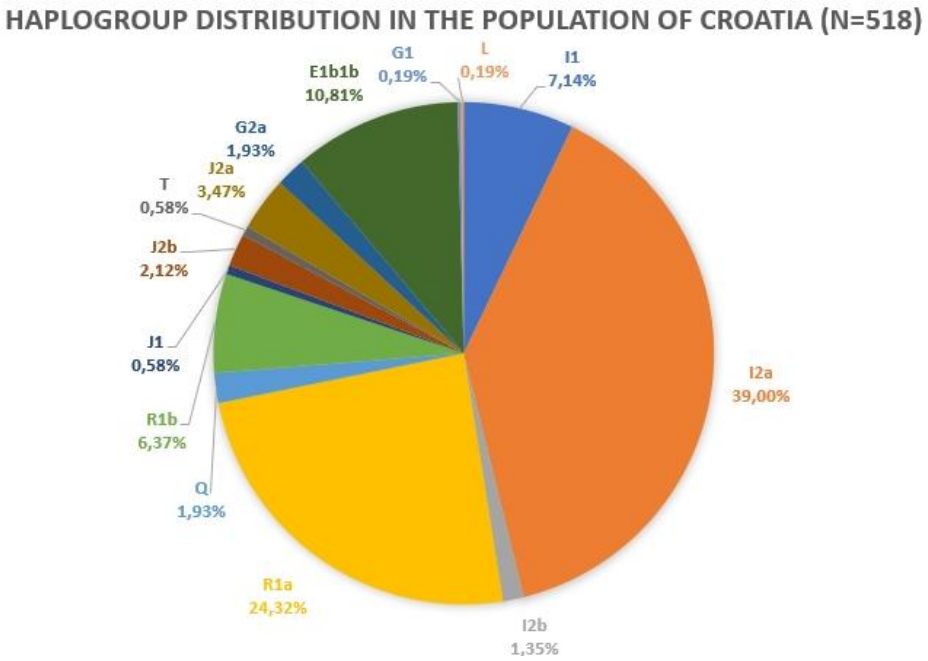

FIGURE 7. The results of Y haplogroup prediction in Croatian population (n=518).

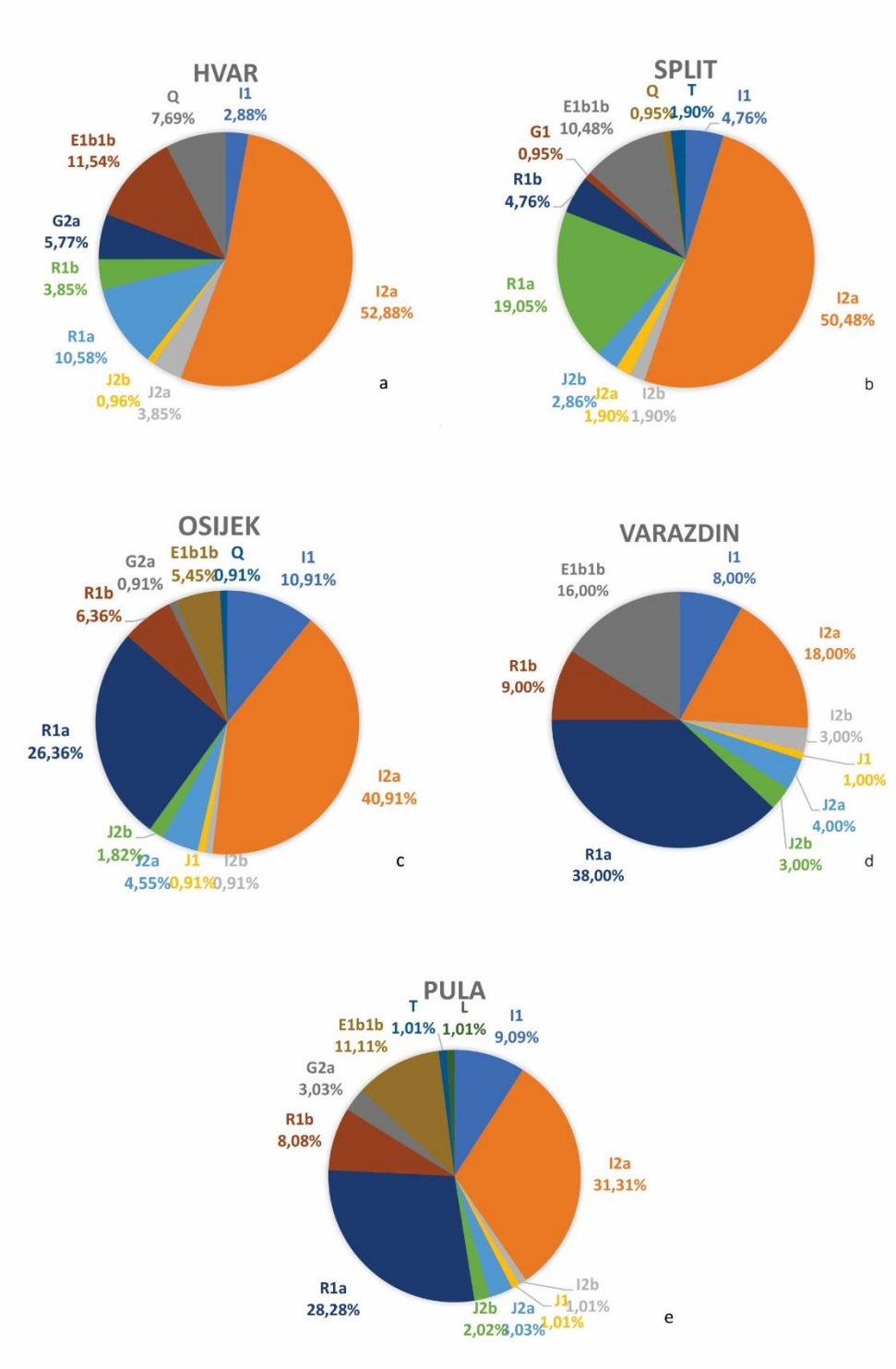

FIGURE 8. Y haplogroup frequency in five Croatian subpopulations: a. Hvar, b. Split, c. Osijek, d. Varaždin, e. Pula