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Genetic variability of Roma population in Serbia: The perspective from autosomal STR markers

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Summary - *Genetic variability of Roma population was shaped by the strong influence of genetic drift and gene flow during the migrations from their ancestral homeland in Indian subcontinent towards Europe. In addition, social stigmatization in many European countries, as a consequence of different cultural heritage and social practices, induced further genetic differentiation and sub structuring within the population. Although many populations genetic studies on European Roma were carried out, the genetic structure of the Serbian Roma has not been described yet, since only the modest number of individuals from this territory was analyzed. The main aim of this study was the characterization of genetic variability of the Roma and the assessment of intrapopulation genetic differentiation based on the analysis of 21 autosomal STR loci of 259 self-identified unrelated individuals from Serbia. Intrapopulation analysis revealed divergence of Roma groups illustrating the effect of the historical events after their arrival on Balkan Peninsula and emphasizing significance of the religious affiliation on admixture with autochthonous population. Genetic distance analysis showed the greatest similarity of the studied population with the Middle Eastern populations, while South Asian and European population were more distant. Our results demonstrate that Roma groups in this region of Balkan Peninsula do not represent completely isolated, but rather admixed populations with different proportion of gene flow with other Roma and non-Roma groups.*

Keywords - *Genetic structure, Roma population, Population genetics, Autosomal STRs.*

Introduction

The Roma population represents the largest transnational ethnic minority in the European continent. According to ethnostatistical data, there are currently between 10 and 12 million of Roma people in Europe (European Commission 2020), however, different international human rights organizations as well as many Roma organizations affirm that the more accurate number is 14 million (Ena et al. 2022). Determining the precise number is complicated by the fact that the subjective criteria is dominant during the official population censuses and members of Roma population often declare themselves as members of ethnic communities which share their living area, or they change attitude about their ethnicity (ethnomimicry). The official demographic analysis on the territory of the Republic of Serbia from 2022 shows that the Roma form 1,98% of the population with 131,936 members, with estimation that that is only one third of the accurate number (Statistical Office of the Republic of Serbia 2022).

Linguistic, cultural, and historical studies first indicated South Asian origin of the Roma (Liégeois 1994; Fraser 1995; Matras 2002; Kalaydjieva et al. 2005). In absence of their own written historical data, church and historical records of the nations they had a contact with were the only source of information. Comparative linguistic studies implied similarity between Roma and languages in the Punjab and Kashmiri regions in northern India (Matras

2002), while cultural studies revealed almost identical social organization as in Indian ethnic groups such as caste system and endogamic habits (Fraser 1995; Marushiakova and Popov 1997). The first evidence appointing to genetic relationship with the Indian subcontinent was identification of shared disease-causing mutations with Indian and Pakistani patients (Piccolo et al. 1996; Kalaydjieva et al. 2001; Morar et al. 2004; Bouwer et al. 2007), followed by many studies on uniparental Y-chromosome and mitochondrial DNA (mtDNA) markers, which clarified any further doubt about the origin of the Roma population (Gresham et al. 2001; Martínez-Cruz et al. 2016). Although the similar results of the dates of exodus from India were obtained from analysis of paternal and maternal lineages, there were discordances about the region of origin in South Asia. While mtDNA studies of M haplogroup pointed north-western India as the originating region (Medizabal et al. 2011), studies of the Y-chromosome H haplogroup suggested that it was probably Southern India (Regueiro et al. 2012). Diverse genome-wide SNPs and whole genome sequencing data analysis shed a light on this question, confirming that the Roma are admixed ethnic group with West Eurasian and South Asian ancestry whose ancestral homeland is north-western region of India, the states of Punjab, Gujarat, Jammu and Kashmir (Mendizabal et al. 2012; Moorjani et al. 2013). Melegh and co-workers (2017) strengthened these assumptions in their work and appointed that, besides India, Pakistani region could be also important source of ancestry for Roma people. Altogether, about 20-35% of South Asian ancestry is harboured in Roma genomes, and the rest is derived from West Eurasian ancestry. High levels of the latter could be explained by South Asian genetic ancestry component, named Ancestral North Indian (ANI), which emerged from admixture events with West Eurasians in this region before start of the Roma diaspora (Moorjani et al. 2013; Font-Porterias et al. 2019).

Both historical and genetic evidence coincide that the proto-Roma exodus from India occurred approximately around 5th century. Some authors suggest that the Roma common ancestors split from the North-Western Indian population some generation before diaspora started, pointing out that it is not a process that occurs at one generation (Bianco et al. 2020), while other authors equalize these two events (Mendizabal et al. 2012; Font-Porterias et al. 2019). A single initial founder population migrated through the Persia (the Iranian Plateau) towards the Caucasus region (Armenia). Historical records suggest that later they followed the route to the west towards Anatolia and more into Europe, alongside the Armenians, due to the Mongol and Seljuq invasions (Fraser 1995; Iovita et al. 2004). Although the Roma were first mentioned in the Balkans in written sources from the early 12th century, it is believed that most of them came to this region with Turks during the Ottoman conquest in the 14th century (Marushiakova and Popov 2001). After arrival on European soil, the Roma population was divided into four different groups. The first group stayed in Balkan (Balkan Roma) and settled in the Ottoman Empire. Vlax Roma continued their journey towards Wallachian Principalities (present-day Romania) where they soon were forced into slavery and divided into smaller groups. The third group of Romungro Roma emigrated to Habsburg monarchy and further to central Europe, while the fourth group, North/Western Roma, migrated further to Northern and Western Europe. Historical records from Iberian Peninsula claim that Roma came to this region in 15th century (Fraser 1995). Wars, persecutions and political changes in Europe during the 19th and 20th centuries led to new waves of migration of Roma toward western regions. The most significant recent migrations encompassing Balkan Peninsula are the migration from Romanian Old Kingdom after the abolition of Roma slavery in the late 19th century, economical and refugee migrations from Yugoslavia in the second half of the 20th century and refugee migrations from Kosovo to the other parts of the Serbia in the end of the 20th century (Liégeois 1994; Fraser 1995; Marushiakova and Popov 2001; Iovita et al.

2004). Groups of Roma in different territories went through the process of assimilation and different proportions of admixture with autochthonous populations, which, in addition to a very complex demographic history, resulted in novel cultural, religious and linguistic barriers between them (Iovita et al. 2004).

The majority of Roma do not follow official dogma and customs, and their religious affiliations usually depend on the dominant religion in their surroundings. However, according to official census result in Republic of Serbia, majority of Roma (57.3%) declared themselves as Orthodox Christians, followed by Muslims (24.9%) and Catholic Christians (3.3%) (Statistical Office of the Republic of Serbia 2022). Roma who came to Balkan Peninsula along with Turks have predominantly become Muslims. Historical records report that during the rule of the Ottoman Empire in Serbia, Muslim Roma had privileged position in comparison to Christian Roma, especially during the rule of Mehmed II in the 15th century, since he abolished tax payment for Muslims and prohibited mixing between groups with different religion (Marushiakova and Popov 2001). These circumstances caused mass conversions to Islam and further separation between these two Roma groups. Two events during the 19th century were essential for present-day dominance of orthodox religion among Roma in Serbia: migrations of orthodox Roma from Romania and the end of the Ottoman domination on the Balkans (Marushiakova and Popov 2001; Cvorovic 2011).

Short tandem repeats (STR) are characterized by a high mutation rate (10^{-6}-10^{-2} per generation) and high level of polymorphism, which makes them an indispensable tool in forensic and population genetics studies (Fan and Chu 2007). Microsatellite markers stay the golden standard in these two fields, and their analysis still represents one of the most effective procedures for assessment of the genetic structure of populations (Hardy et al. 2003; Ehler and Vanek 2017) in spite of the expansion of the new generation sequencing (NGS) methods and genome-wide data in population genetics.

The main aim of this study was the examination of the genetic structure of the Roma population in Serbia based on data obtained from the analysis of the autosomal STR loci and the assessment of the genetic differentiation within the subpopulations that inhabit different areas on the territory of the Republic of Serbia and have diverse religion and cultural practices, as well as their relationship with other Roma populations on the European continent and general European populations.

Material and methods

Participants

In this study 259 self-identified unrelated Roma individuals from different regions of the Republic of Serbia were analyzed. All participants signed informed consent and ethical approval for the study was granted by the ethical committee of University of Belgrade - Institute for molecular genetics and genetic engineering (O-EO-027/2021/1). Also, all experiments were performed in accordance with relevant guidelines and regulations. Samples were classified into 5 different groups, based on the geographical region of origin of the previous generation: the central group (CR) with 75 examinees, the northern group (NR) with 40 examinees, the western group (WR) with 27 examinees, the southeastern group (SER) with 51 examinees and the southern group (SR) with 56 examinees (Supplementary material S1). These regions are defined in concordance with (Statistical Office of the Republic of Serbia 2023), except for Belgrade region which is, based on its position, unified with municipalities from central region. Also, we have formed groups based on the religion of the participants, assuming it's the same as the parental: the Orthodox Christians group (OC) with 158 examinees, the Catholic Christians group (CC) with 41 examinees, and the Muslims group (M) with 60 examinees. Difference in number of samples between religion and regional groups was obtained due to few participants that are part of the present-day Serbian Roma community but have origin in other countries. In addition,

we analysed group of 500 individuals from Serbia (unpublished data), regardless of their ethnicity, as a representative sample of general Serbian orthodox population (S).

DNA analysis

DNA isolation from buccal swabs was performed using the GeneJET Whole Blood Genomic DNA Purification Mini Kit (*Thermofisher Scientific*, USA) and extracted DNA was quantified using the Qubit dsDNA HS Assay Kit (*Thermofisher Scientific*, USA), both according to manufacturer's instructions. The Investigator 24plex QS kit (*Qiagen*, Germany) was used to accomplish simultaneous amplification of 21 autosomal STR loci: TH01, D3S1358, vWa, D21S11, TPOX, D1S1656, D12S391, SE33, D10S1248, D22S1045, D19S433, D8S1179, D2S1338, D2S441, D18S51, FGA, D16S539, CSF1PO, D13S317, D5S818 and D7S820. Detection of the amplified products was conducted on the ABI 3500 Genetic Analyser (*Applied Biosystems*, USA) and all samples were analysed in the GeneMapperTM ID-X 1.4/1.5 software (*Applied Biosystems*, USA).

Statistical analysis

The standard forensic (observed heterozygosity (Hobs), gene diversity (GD), match probability (MP), power of discrimination (PD), polymorphism information content (PIC), power of exclusion (PE) and typical paternity index (TPI)) and population genetic parameters (number of alleles, allelic frequencies) for microsatellite loci were calculated using STRAF 2.1.5. (Gouy and Zieger 2017). The Hardy–Weinberg equilibrium was tested using STRAF 2.1.5. with 10 000 permutations, after which the Bonferroni correction for multiple testing was performed. The Arlequin ver. 3.5.1.3 software (Excoffier and Lischer 2010) was used for assessment of genetic differentiation among different groups through analysis of molecular variance (AMOVA) as well as the estimation of the pairwise population Fst, and obtained results were visualized using the R functions connected with this software. The statistical significance of all performed tests in Arlequin ver. 3.5.1.3. was assessed with 10 000 permutations. POPTREE2 software (Takezaki et al. 2010) was used for construction of the neighbour-joining dendrogram based on the Fst values. The number of genetic clusters represented in the sample was estimated with STRUCTURE v 2.3.4 software (Falush et al. 2003; 2007; Pritchard et al. 2000) using the admixture model with a burn length of 10,000 and a Markov chain Monte Carlo (MCMC) of 100,000 randomizations. The range of the possible number of clusters (K) was from 1 to 10, with a series of 10 runs for each K. Obtained results were also analyzed in STRUCTURE harvester (Earl et al. 2012), software that indicates which number of K groups best fits the data. Discriminant analysis of principal components (DAPC), performed in R, was used to present the observed distances among different groups (Jombart et al. 2010). This method consists of performing the linear discriminant analysis (LDA) on the principal components analysis' (PCA) transformed matrix. The number of retained PCs was estimated performing stratified cross-validation for DAPC with 100 iterations and calculating the number of PCs with the lowest mean squared error. For assessment of genetic differences between Roma population in Serbia and other Roma populations in Europe, as well as the other general populations, we used the data from literature (Kovatsi et al. 2006; Soták et al. 2008; Lowery et al. 2011; Rak et al. 2011; Hedjazi et al. 2013; Shan et al. 2016; Barrio et al. 2019; Aliyeva et al. 2021; Kumar et al. 2021) and data obtained in personal correspondence with authors of the (Casals et al. 2017; Bozman et al. 2018; Pilav et al. 2020).

Quality control

All experiments were conducted at the Center for Forensic and Applied Molecular Genetics in Faculty of Biology at the University of Belgrade, and the quality control was ensured by the successful participation in GEDNAP proficiency tests. Furthermore, control DNA 9948 from the Investigator 24plex QS kit (*Qiagen*, Germany) and ddH2O were used as a positive and negative control in each PCR reaction.

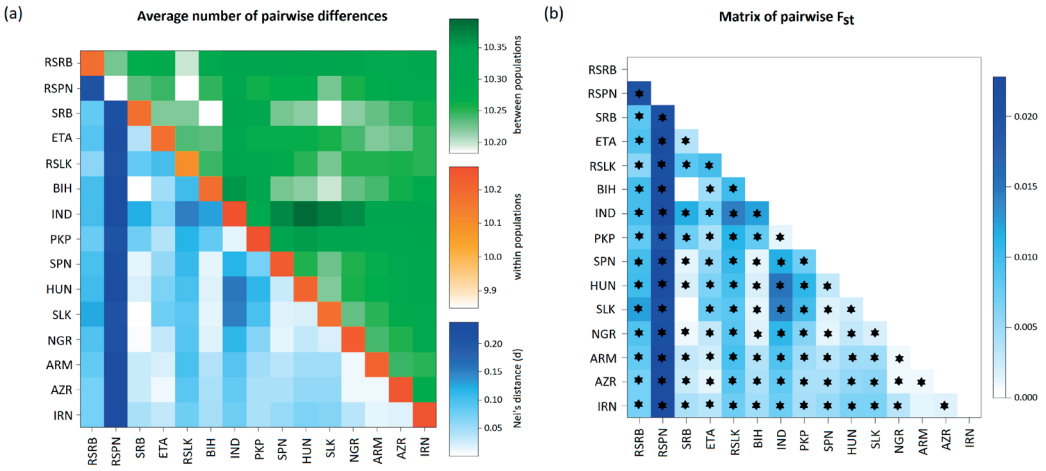

***Fig. 1** - (a) Matrix of average number of pairwise differences for different populations. The average number of pairwise differences between the populations, within the populations and Nei's distances are presented above, on and below diagonal respectively. (b) Matrix of pairwise Fst based on the analysis of the 13 autosomal marker on different populations. Marked Fst values show statistical significance. RSRB: Serbia (Roma); RSPN: Spain (Roma); SRB: Serbia; ETA: Eastern Turkey (Anatolia); RSLK: Eastern Slovakia (Roma); BIH: Bosnia and Herzegovina; IND: North-Western India; PKP: Pakistan (Punjab); SPN: Spain; HUN: Hungary; SLK: Eastern Slovakia; NGR: Northern Greece; ARM: Armenia; AZR: Azerbaijan; IRN: Iran (Fars).*

Results

Interpopulation genetic analysis

Comparison of the examined Roma population, Roma populations from Europe (Spanish and Slovakian) and general populations from European and Asian countries, which Roma had contact with during their diaspora, was based on analysis of 13 STR loci (TH01, D3S1358, vWa, D21S11, TPOX, D8S1179, D18S51, FGA, D16S539, CSF1PO, D13S317, D5S818 and D7S820) for which data were available from other studies. Average number of pairwise differences and matrix of Pairwise Fst values, obtained in Arlequin software, are displayed in the Figure 1 and Supplementary material S2, and the Fst values with the corresponding p-values are shown in Supplementary material S3. For better visualization of results, we also constructed the neighbor-joining dendrogram based on the Fst values which is presented in the Supplementary material S4. Pairwise Fst values ranged from 0.02279 (Spain Roma and Armenia) to 0.00076 (Northern Greece and Serbia), and only three values didn't show statistical significance (Serbia and Bosnia and Herzegovina, Serbia and Eastern Slovakia, and Armenia and Iran (Fars)). Based on Fst analysis, the Roma from Spain exhibited greatest divergence from other population with values ranging from 0.01955 (Pakistan (Punjabi)) to 0.02279 (Armenia), and in the same time that was a population with the lowest within population pairwise differences (9.84705). When comparing out studied Roma population with others, the highest average number of pairwise differences between population observed was 10.32809 (North-Western India (Rajasthan)) and the lowest 10.19794 (Eastern Slovakia Roma). Results of Nei's genetic distances showed highest similarity of Serbian Roma and Eastern Slovakian Roma population (0.06016) and greatest divergence from Spanish Roma population (0.2087). Overall, we can observe that European and Middle Eastern populations cluster together based on Nei's genetic distances, while samples from South Asia and all three Roma population were more divergent. Our Roma population showed the greatest similarity with the Middle Eastern countries, and approximately same distance from North-Western Indian population and European cluster.

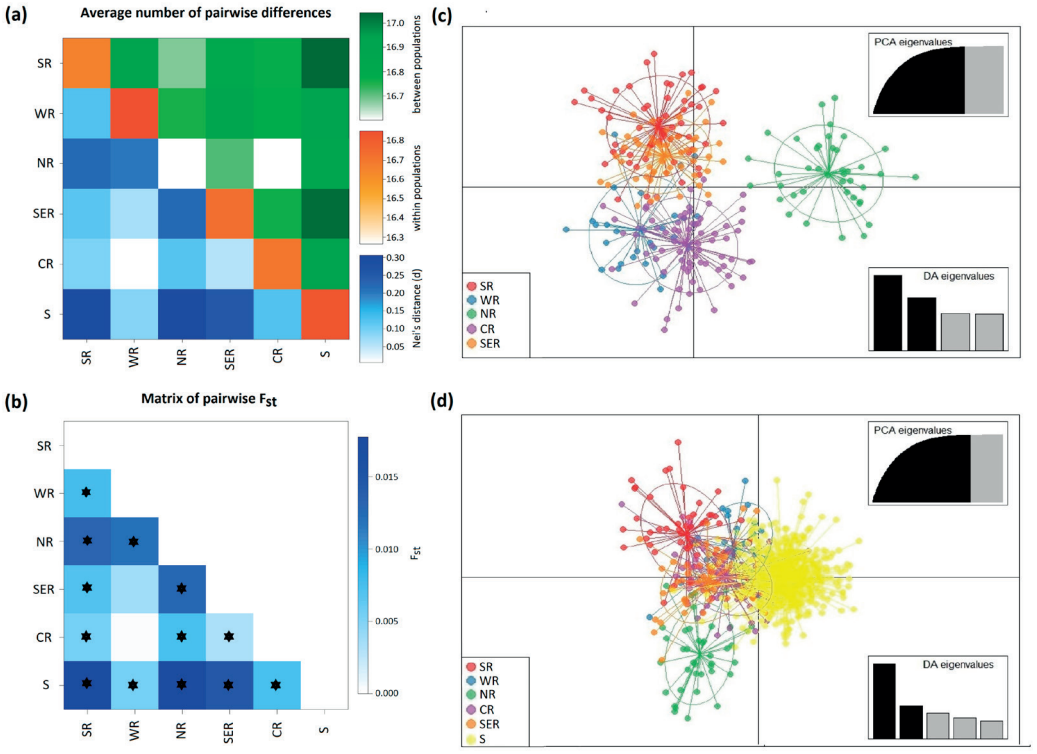

***Fig. 2** - (a) Matrix of average number of pairwise differences for region groups. The average number of pairwise differences between the populations, within the population and Nei's distances are presented above, on and below diagonal respectively. (b) Matrix of pairwise Fst based on the analysis of the 21 autosomal markers on region classified groups. Marked Fst values show statistical significance. Discriminant analysis of principal components where different Roma groups (c) and general Serbian population (d) are presented separated by the first and second linear discriminant.*

Genetic structuring within Roma population in Serbia

The average number of pairwise differences between and within different region groups along with Nei's distances are present in the Figure 2a and Supplementary material S5. The highest values of between populations differences are noted between Roma region groups and general Serbian (S) population (on average 16.94864), while the highest intrapopulation differences are observed for WR (16.85185) group.

The Figure 2b and the Supplementary material S6 display a matrix of pairwise Fst with statistically significant p values pointed out. The greatest Fst values are observed when comparing NR and SR with S group (0.01774 and 0.01733 respectively). As for the diverse Roma samples, highest Fst is calculated between southern and northern region (0.01323). The smallest difference, according to Fst testing is between central and western region (0.00039).

The results of DAPC analysis for different region groups of Roma population are present in the Figure 2c Analysis was performed on 138 PCs, obtained by cross-validation, which conserve 99,2% of total variance. Individuals from southern and southeastern region, as well as the ones from central and western region, are clustered together indicating similarity between these Roma populations. Samples from northern region of the country are separated from the other groups, suggesting the presence of the

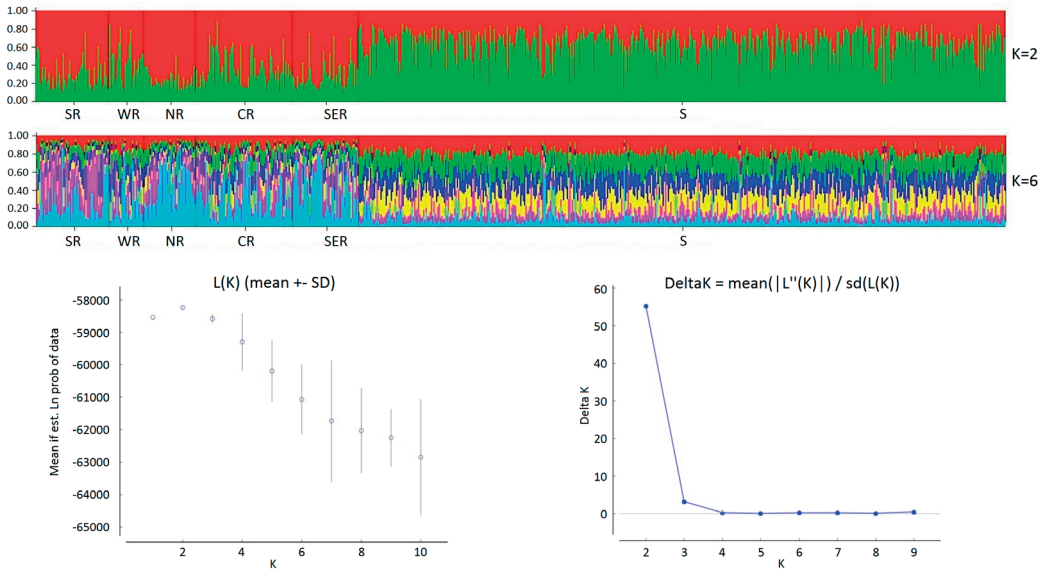

***Fig. 3** - Proportions of the inferred STRUCTURE clusters from the individuals and L(K) mean and ΔK for the assumed number of genetic clusters.*

distinct genetic variability in this group. After performing an analysis with general Serbian population (Fig. 2d), it can still be detected that the northern region is clearly separated from other regions, while the other regions are more similar with autochthonous population.

The results of the analysis in STRUCTURE v 2.3.4 software and STRUCTURE harvester are present in the Figure 3 and Supplementary material S7, indicating most likely the presence of two clusters in our data. When K=2, we can clearly distinguish data for Roma and general Serbian population. As for the different region groups of Roma population, only slight differences can be detected when K=6, where you can detect a similarity between WR and CR, NR and SER and divergence of the SR.

The Figure 4a and Supplementary material S8 represent the average number of pairwise differences between and within different religion groups along with Nei's distances. The maximum value between populations (17.04245) and Nei's distances (0.29714) is observed among M and S groups, while the maximum number of pairwise differences within Roma population is detected for OC group (16.76983). For assessment of the effect of unequal sample size in religion groups on results, we performed analysis again reducing the OC group to similar size as the other groups (60 examinees). Although the obtained results (Supplementary material S9) were slightly different, the same trend was observed: the maximum values of Nei's distances were between all three Roma groups and S group, while the highest values for parameters among Roma groups were calculated when comparing the CC group and M group. Figure 4b and Supplementary material S10 display a matrix of pairwise Fst with marked statistically significant p values. The smallest values are detected when comparing other groups with OC group (0.00647, 0.00885 and 0.00886), whilst the highest values are obtained by comparing M and CC group with general Serbian population (0.01733 and 0.01635 respectively). The matrix of pairwise Fst values for similarly sized groups is present in Supplementary material S11. Again, the obtained Fst values were fairly different but analogous to the previous ones.

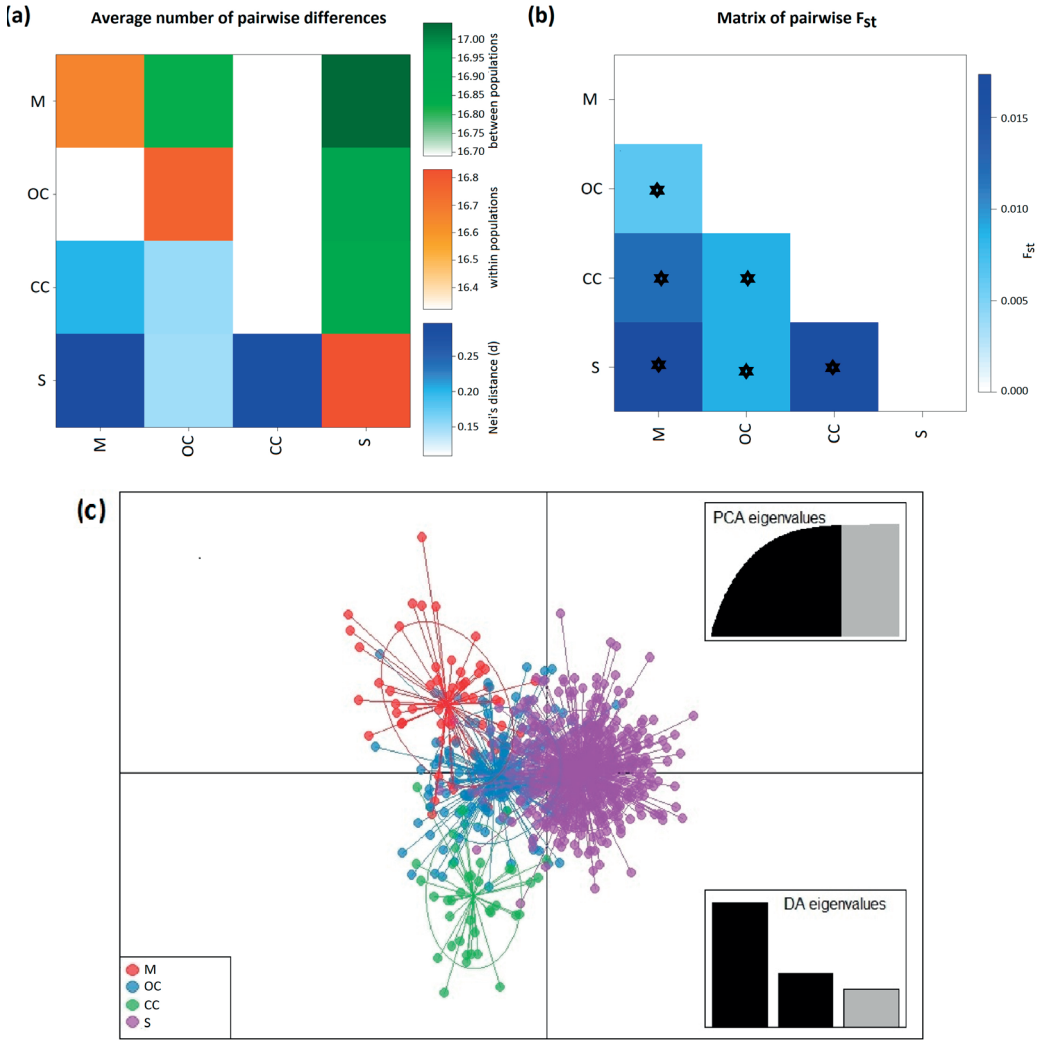

***Fig. 4** - (a) Matrix of average number of pairwise differences for religion groups. The average number of pairwise differences between the populations, within the population and Nei's distances are presented above, on and below diagonal respectively. (b) Matrix of pairwise Fst based on the analysis of the 21 autosomal markers on religion classified groups. Marked Fst values show statistical significance. (c) Discriminant analysis of principal components where different Roma religion groups and general Serbian population are presented separated by the first and second linear discriminant.*

The results of DAPC analysis for different religion groups of Roma and general Serbian population are present in the Figure 4c. Analysis was performed on 173 PCs, obtained by cross-validation, which describe 99,3% of total variance. The middle of the plot is occupied with OC group, overlapping with all three other groups, which are found to be more diverse with each other. The DAPC analysis wasn't performed after the reduction of the OC group since the previous two analysis showed no significant differences after change in the number of samples.

Tab. 1 - Analysis of molecular variance in different Roma groups.

	SOURCE OF VARIATION	SUM OF SQUARES	VARIANCE COMPONENTS	PERCENTAGE OF VARIATION
Regional groups	Among populations	55.568	0.05687	0.67884* (p=0.02356)
	Among individuals within populations	2043.620	0.05522	0.65913 (p=0.16297)
Religion groups	Among populations	35.490	0.06707	0.79793* (p=0.00000)
	Among individuals within populations	2154.906	0.07946	0.94533 (p=0.07089)

*p<0.05

The results of analysis of molecular variance (AMOVA) across all loci showed low but statistically significant value of genetic variance between regional as well as religion groups (Tab.1). The percentage of variation between Roma subgroups which differed in religion (0.8 %) was slightly higher than the one between the subgroups from different regions (0.68 %). The degree of within-subpopulation genetic variation was not significant in either of groups.

Genetic diversity of Roma population in Serbia

Allelic frequencies for total Roma population and each of Roma groups are listed in the Supplementary material S12, while the calculated standard diversity and forensic parameters for analyzed loci are presented in the Table 2 and Table 3. The SE33 locus exhibited the highest number of alleles (27) in Roma population in Serbia, while the D10S1248, D3S1358, TH01 and TPOX were the loci with the smallest number of alleles (6). In each of the formed groups, the locus with the highest number of alleles was also SE33, while the loci with the lowest number varied. GD values (Tab.2) in Roma population ranged from 0.685333 (TPOX) to 0.912632 (SE33) with the average of 0.796649 for all 21 loci. This value is only marginally different from the average of GD for general Serbian population (0.801439). Average GD (Supplementary material S13) for regional groups ranged from 0.774427 (NR group) to 0.802469 (WR group) while the one for religion groups ranged from 0.777104 (CC group) to 0.798563 (OC group). The locus with the minimal GD in SR, NR, SER, CC and M groups, was D5S818, while in WR, CR and OC groups was TPOX, like in total Roma and general Serbian population. Similar results regarding the GD values are obtained in studies of previously mentioned populations from Europe and Asia. Among 13 shared analyzed loci, TPOX was the locus which exhibited the lowest GD values, with exception in Spanish Roma population, while the FGA was the locus with the highest GD value only in our studied population and populations of Iran, Pakistan and India, as well as the Spanish Roma population (Kovatsi et al. 2006; Soták et al. 2008; Lowery et al. 2011; Rak et al. 2011; Hedjazi et al. 2013; Shan et al. 2016; Casals et al. 2017; Bozman et al. 2018; Barrio et al. 2019; Pilav et al. 2020; Aliyeva et al. 2021; Kumar et al. 2021).

Hobs values ranged from 0.662651 (D5S818 and TPOX) to 0.927711 (SE33) with an average of 0.785045, which is a slightly lower than the average GD value. The locus with the lowest Hobs values in all populations, except in Northwestern Indian and Spanish Roma, was again TPOX, while the results for the locus with the highest Hobs values were not so uniform across different populations. FGA, which was the locus with the greatest Hobs in Serbian

Tab. 2 - Gene diversity for 21 analyzed autosomal STR loci in Roma population.

LOCUS	N	NALL	GD
CSF1PO	498	8	0.696767
D10S1248	498	6	0.786074
D12S391	498	15	0.859591
D13S317	498	8	0.779472
D16S539	498	7	0.80697
D18S51	498	13	0.857967
D19S433	498	15	0.783779
D1S1656	498	16	0.863761
D21S11	498	12	0.829838
D22S1045	498	8	0.704807
D2S1338	498	11	0.873199
D2S441	498	9	0.763464
D3S1358	498	6	0.751036
D5S818	498	7	0.689753
D7S820	498	7	0.799706
D8S1179	498	9	0.826889
FGA	498	18	0.888294
SE33	498	27	0.912632
TH01	498	6	0.797597
TPOX	498	6	0.685333
vWA	498	7	0.772709
Average		10.52381	0.796649

* N: total number of alleles; Nall: number of different alleles; GD: gene diversity

Roma on the 13 selected loci, was also observed to have the highest values in Eastern Slovakian Roma, Spanish Roma and Pakistani Punjab population. The highest PD was observed on SE33 (0.981371) while the lowest was for CSF1PO (0.839664) and combined TPI index for this set of 21 STR markers was 2,12E+08. When comparing our results with other populations, TPOX was again the locus with lowest PD in all populations, except in Spanish Roma (D18S51), while the FGA was the locus with the highest PD only in our studied population and populations of Iran, Pakistan and India as well as in Spanish and Eastern Slovakian Roma population. Overall, obtained values of all analyzed parameters suggest a diverse population structure.

The Supplementary material S14. displays p-values associated with evaluation of the Hardy-Weinberg equilibrium (HWE) for each locus. After the analysis with 10 000 permutations, three loci (D21S11, D5S818 and TH01) demonstrated deviation from frequency expectations under HWE, but the significance was annulated after applying Bonferroni correction for multiple testing ($p<0.002381$).

Discussion

Complex demographic history of the Roma population shaped their genetic variability through the strong influence of genetic drift, as they faced series of bottlenecks and founder effects during their migrations from Indian subcontinent towards Europe. Concurrently, Roma groups experienced different levels and sources of admixture depending on the area they settled. In addition, their specific cultural heritage and social practices, along with the nomadic lifestyle, led to social stigmatization in most European countries and consequently to further internal genetic differentiation and sub structuring. All these factors have created a unique genetic landscape, which represents a great challenge for researchers due to incomplete integration in many countries and subsequently underrepresentation of the Roma population in large genomic projects. The present study aims to characterize Serbian Roma population and to assess genetic sub structuring by performing analysis on the 21 autosomal STR loci for 259 individuals.

Several genetic studies confirmed former linguistic and historical evidence about origin of the proto-Roma population, emphasizing the

Tab. 3 - Forensic parameters for 21 analysed autosomal STR loci for Roma population.

LOCUS	HOBS	MP	PD	PE	TPI
CSF1PO	0.714859	0.160336	0.839664	0.451621	1.753521
D10S1248	0.827309	0.088595	0.911405	0.650667	2.895349
D12S391	0.86747	0.03937	0.96063	0.729573	3.772727
D13S317	0.759036	0.078596	0.921404	0.525353	2.075
D16S539	0.799197	0.076725	0.923275	0.59755	2.49
D18S51	0.835341	0.038564	0.961436	0.666188	3.036585
D19S433	0.759036	0.078208	0.921792	0.525353	2.075
D1S1656	0.835341	0.035209	0.964791	0.666188	3.036585
D21S11	0.831325	0.055531	0.944469	0.65841	2.964286
D22S1045	0.674699	0.128046	0.871954	0.390216	1.537037
D2S1338	0.879518	0.034274	0.965726	0.7538	4.15
D2S441	0.722892	0.087531	0.912469	0.464556	1.,804348
D3S1358	0.75502	0.106337	0.893663	0.518394	2.040984
D5S818	0.662651	0.150401	0.849599	0.372878	1.482143
D7S820	0.779116	0.072305	0.927695	0.560873	2.263636
D8S1179	0.827309	0.056177	0.943823	0.650667	2.895349
FGA	0.883534	0.025725	0.974275	0.761922	4.293103
SE33	0.927711	0.018629	0.981371	0.852303	6.916667
TH01	0.767068	0.075241	0.924759	0.539417	2.146552
TPOX	0.662651	0.155981	0.844019	0.372878	1.482143
vWA	0.714859	0.08437	0.91563	0.451621	1753521

Hobs: observed heterozygosity; MP: match probability; PD: power of discrimination; PE: power of exclusion; TPI: typical paternity index.

role of Punjab province in North-Western India (Mendizabal et al. 2012; Moorjani et al. 2013; Melegh et al. 2017; Font-Porterias et al. 2019; Bianco et al. 2020). Previously reported genetic heterogeneity among the different regions of India, which is a consequence of linguistic and socio-cultural demarcations (Tamang et al. 2012), justifies observed Fst values and divergence of the Serbian Roma and population of Rajasthan (North-Western India) (Kumar et al. 2021) despite its geographical proximity to the ancestral homeland of the proto-Roma. Concurrently, closeness of Pakistan population of the Punjab region to our studied population could indicate importance of this region to genetic legacy of Roma as it was suggested by Melegh and coworkers (2017), but it could also be explained by the heterogeneity of present-day Punjabi population due to repeated invasions from different sources after Roma exodus (Shackle 1970).

According to the historical data, before entering the European continent, Roma passed through Middle East (Fraser 1995; Iovita et al. 2004). Linguistic analysis revealed strong influence of Armenian and Georgian on Romani language and indicated extended settlement in this region (Matras 2002), nevertheless genuine amount of admixture with Middle Eastern

populations is still a debate. Based on analysis of genome wide and uniparental data, some authors suggest that Caucasus region is significant source of the genetic legacy for Roma people (Bánfai et al. 2018), while others support moderate gene flow (Medizabal et al. 2011, 2012; Font-Porterias et al. 2019). Although the Fst analysis results show that our studied population has the greatest genetic similarity with Middle Eastern population, the real proportion of the gene flow during diaspora could be overestimated for two reasons. First, Middle Eastern populations are admixed with high percent of European component, which is also portrayed in low Fst values between these populations. Second, Middle Eastern ancestry could be acquired during the Ottoman rule in Europe, and not during the diaspora as it was showed by Bánfai and coworkers (2019), since they were already the largest ethnic minority in the area.

Dispersal of Roma population in Europe was rapid since only a modest period of time as one century had passed from their arrival in the Balkan Peninsula until they have been documented in most parts of the European continent. Some authors suggest that out-of-Balkans colonialization, that started around 900 years ago, occurred in several waves, during which small Roma groups, according to historical records from Western Europe of 50-300 individuals led by an elder, settled in new places from where often new migrations were initiated (Marushiakova and Popov 1997). In their study, Bianco and coworkers (2020) allegated that more than 50% of Roma ancestral component was replaced by European admixture. Extended stay in the Balkan Peninsula, which has been appointed as the cradle of Roma people currently living in Europe (Bianco et al. 2020), has left trace on Roma genetic variability. Font-Porterias and co-workers (2019) in their study of genome-wide data reported that the proportion of Balkan genetic component in European Roma genomes decreases with the distance from this region, from 45% in Bulgarian, Greek and Serbian Roma to 25% in Lithuanian, Estonian and Iberian Roma. Our results correlate with these allegations, as the Fst values obtained by comparing Serbian Roma and Eastern Slovakian Roma with Balkan populations are lower than the ones of Spanish Roma. Reduction in population sizes and extensive effects of genetic drift after out-of-Balkan migrations could explain observed genetic differentiation between our sample and the Roma sample from Spain. However, Font-Porterias and coworkers (2019) in their study pointed out vast within-country heterogeneity of Roma in Iberian Peninsula, most likely due to different demographic patterns caused by different laws in various parts of that area, which probably also contributed to these results. Low Fst value between Eastern Slovakian Roma and Serbian Roma imply more recent common origin or admixture of these two populations, considering the nearness of those geographical regions.

Our results from the STRUCTURE software portrayed significant differences between the Roma and the general Serbian population, which is further confirmed by DAPC and Fst analysis. However, when comparing regional Roma groups with general Serbian population, it can be observed that diverse groups have different proportion of similarity: western and central groups the largest followed by southeastern group. Divergence of the southern and northern groups from Serbian group suggests greater enclosure of these groups. That is also supported by the fact that significantly higher Fst values are obtained by comparing Muslims and Catholic Cristian groups with general Serbian population, as well as the position of these groups on DAPC plot, which implies that effect of religious affiliations is not negligible when it comes to admixture with predominant population.

In our study, we investigated internal genetic differentiation of Roma population from Serbia. We analyzed two different groups of datasets in which we classified samples by the origin region of the previous generation and by the practicing religion, with objective of pointing out the influence of geographical and religious barriers on genetic variability. Religion groups coincide with regional distribution of Roma, which explains the similar results for both. Muslim group is

formed from 98,88% of southern group, 0,74% of central group, 0.81% of western group and 1,02% of southeastern group. Catholic Cristian group is obtained by merging 99,6% of northern group and 0.51% of southeastern group, and the rest of the samples are joined into Orthodox Cristian group. When comparing only different regional Roma groups, the greatest genetic distance was observed between northern and the rest of the groups, whose main cause could be explained by historical information. Since the institution of the slavery in Wallachian Principalities in the early 14th century, there had been two migratory waves of Roma people from this region towards other parts of Europe: during the Austrian- Turkish wars in the late 17th- early 18th and after the abolition of slavery in the end of the 19th century (Marushiakova and Popov 2001). In both instances, groups of migrants left the country speaking different archaic dialects of Romanian language, since their mother language was prohibited during the period of slavery (Kalaydjieva et al. 2005). Although the examinees from northern region of our country confirmed that besides Serbian, they have their own language that differs from other Romani languages, but it's similar to Romanian language, which suggest that they represent the branch of Vlax Roma, the practice of Catholic religion is unusual since enslaved Roma were converted into Orthodox Christianity (Marushiakova and Popov 2001; Cvorovic 2011). This phenomenon could additionally be explained by the proximity of Eastern Slavonia, a province in Croatia from which a certain number of Roma migrated to the neighbouring areas at the end of the 20th century (Marushiakova and Popov 2001). As the religion of Roma groups usually depends on location or circumstances, it could be assumed that orthodox migrants from Romania firstly came to Eastern Slavonia where they have accepted Catholicism before novel migration wave. All this information suggests that Roma group from north of Serbia diverged from the other groups earlier, probably soon after their arrival in Balkans, as well as they are still isolated community. Central and western groups have the lowest values of pairwise differences with each population on average, including general Serbian, which in combination with low Fst values notated when comparing other religion groups with Orthodox Cristian group, can be explained with higher level of mixture of the Roma who accepted orthodox religion with other orthodox groups and autochthonous population. Southeastern part of Serbia could be the region with greatest percent of admixture of different Roma groups, since our results show that southeastern group has proportion of shared genetic variability with all other Roma groups. Results of AMOVA analysis corraborate the data obtained with Fst analysis and suggest lower levels of expected substructuring as an outcome of loss of isolation within present-day Roma population. Individuals from southeastern group are predominantly Orthodox, and its shared genetic legacy with southern group, whose individuals are predominantly Muslims, probably illustrates the historical events that succeeded the Turkish departure from Balkan Peninsula. Also, results from STRUCTURE (Fig. 3) indicate similarity between northern and one part of the southeastern group, which, in addition to the closeness of region of former Wallachian Principalities to Southeastern Serbia, straightness former allegations about Vlax Roma origins of the analyzed population from northern part of the country.

The STR loci are widely used in biological researches, especially in the field of forensic and population genetics. Although the analysis of single nucleotide variants (SNVs) is becoming more popular nowadays in genealogy, there are several reasons for why the analysis of the STR loci is still preferred. While analysis of slow-mutating SNVs sheds a light on the long-term subdivision among populations, the high mutation rates, as well as the stepwise changes of the number of repeats, give the STR markers an advantage when it comes to analysis of relatively recent divergence of population (Romualdi et al. 2002; Hardy et al. 2008). Also, databases with ancestry-informative SNVs are still not sufficiently developed, while the loci used in this study are well described in many populations, since they

represent the expanded CODIS (Combined DNA Index System) loci (Hares et al. 2015) and are used across the globe in forensic calculations. Additionally, more studies of STR loci with an ancestral context will lead to deeper analysis of diversity within and among different ethnic group, which could be of a crucial importance in forensics, since the mathematical theory underlying the calculations of the genotype frequencies assumes that the reference population is homogenous, thus the estimations without accounting the population structure could be inaccurate (Michaelis et al. 2008). However, assigning of the ethnicity of the sample in a database is often based of unreliable information provided by the investigators or individuals themselves, and admixed individuals will still be incorrectly categorized. Moreover, in most of the cases, forensic geneticists do not have information about the sample origin at all (Lowe et al. 2001; Graydon et al. 2009).

Conclusion

Our result suggest that in spite of the reproductive isolation and the increased frequency of endogamy due to the great cultural heterogeneity described in literature, Roma groups in this region of Balkan Peninsula do not represent completely isolated, but rather admixed populations, which correlates with previously reported result (Gresham et al. 2001; Morar et al. 2004; Mendizabal et al. 2012; Moorjani et al. 2013; Martínez-Cruz et al. 2016; Melegh et al. 2017; Bánfai et al. 2018; Bánfai et al. 2019; Font-Porterias et al. 2019; Bianco et al. 2020). Additionally, an increased gene flow to some Roma groups, as a consequence of modern changes in social rules, probably altered geographical and religion patterns and decreased dissimilarities between different subpopulations. Further studies, especially genome-wide and complete uniparental marker analysis on a larger number of samples, would provide new insight which is necessary for complete understanding of genetic diversity of Roma population in Serbia.

Data Sharing

Data from the current study are openly available in Zenodo at https://doi.org/10.5281/zenodo.1103346.

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Author Contributions

All authors contributed to the study concept and design. Experiments and analysis of the data were performed by V.T., M.V. and M.M.S. The first draft of the manuscript was written by V.T., and reviewed and edited by M.K.M., M.K. and D.K. All authors have read and agreed to the submitted version of the manuscript.

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