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Original paper

Distribution of eleven markers in South Romanian (Walachia region) population

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Abstract The South Romanian population has not been well studied at the genetic level. This study aims to investigate the genetic distribution of di-allelic DNA polymorphisms associated with different diseases in the South Romanian population. We investigated eleven di-allelic DNA polymorphisms in blood samples from 648 healthy Caucasian subjects (age 18-66 years old) from Bucharest and eight South Romanian districts. All markers present no significant deviation from expected Hardy–Weinberg equilibrium even if subjects were stratified according to the birth place, gender or age group. The frequency of allelic variants and genotypes in South Romanian areas situates in the range of values found with other Caucasian populations. When testing the distribution between the tested districts only VDR Fok was found to present a different distribution in samples from Bucharest and in those from Calarasi, Dambovita or Teleorman ($p < 0.00008$). Excepting MTHFR and MTR no other difference was detected between the age grouped subjects. MTHFR 677T allele increases progressively from subjects of 59-66 years old to those with 18-26 years (21.7% vs. 41%, $p = 0.003$). The highest difference of heterozygosity was found between the subjects in Bucharest and Giurgiu or Olt.

Keywords Di-allelic markers, heterozygosity, MTHFR 677T allele.

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Introduction

The large scale resequencing projects and improvement of the bioinformatics tools accelerate the characterization of human genome. Genomic similarities between individuals are helpful for defining a species whereas genetic differences (e.g. polymorphisms, mutations) are useful for different research areas. Autosomal markers have been used more frequent for biomedical diagnosis, forensics or testing the population structure whereas heterosomal and mitochondrial markers have been preferred for genealogical, evolutionary and phylogeography studies. Di-allelic markers (e.g. SNPs, InDels) provide a more reduced statistical power compared to multiallelic markers (e.g. microsatellites) but frequency and distribution throughout the entire genome turn them into useful tools for genetic studies.

Characterization of population at the genetic level remains a difficult problem, despite the theoretical and technological innovations in the last decades. So far, only some markers relevant for forensics or for estimation of gene flow have been tested in Romanian population. Thus, analysis of Y chromosome markers indicated the role of Carpathian Mountain on gene flow between East and West [1] whereas investigation of 15 STR loci suggested that the population in South Romania is genetically more similar to Slavic populations of Croatia and Serbia than to other surrounding populations [2]. In some studies the association between genetic markers and different diseases has been tested in our populations. However, the results obtained in these studies may be influenced by the selection criteria of subjects [3-7].

The aim of this study was to assess the distribution of eleven common di-allelic markers in healthy population living in South Romania. These markers are associated with some common human diseases in different human populations [8-10].

Materials and Methods

Investigated area and Subjects. A total of 648 clinically healthy subjects from Bucharest and Calarasi, Dambovita, Dolj, Giurgiu, Ialomita, Ilfov, Olt and Teleorman districts (formally known as Walachia region) were selected for this study. Both genders are equally represented in each district (36 males and 36 females) (Table 1).

Table 1. The main data of subjects selected from the area of interest situated in South Romania

District characteristics			**Sample characteristics**						
Name	Surface (Km^2)	Population*	Age (M±SD)	Fasting plasma glucose (mg/dl)	Total cholesterol (mg/dl)	Triglycerides (mg/dl)	Smokers (Current/ Former)	Drinkers (Current/ Former)	Education: college/ faculty/ other
Bucharest	228	1926334	44±8.7	91±8 (70-109)	163±18.1 (121-215	107±16.2 (65-139)	18/12	15/14	57/7/8
Calarasi	5088	324617	41±8.93	92±7.14 (72-107)	161±18.05 (120-196)	104±19.35 (53-145)	8/8	8/4	62/3/7
Dambovita	4054	541763	42±9.93	89±9.37 (71-108)	171±22.32 (123-217)	111±17.01 (73-147)	7/7	3/3	59/4/9
Dolj	7414	734231	43±10.42	91±7.93 (70-108)	164±20.36 (136-211)	108±16.5 (63-141)	11/10	5/3	56/5/11
Giurgiu	3526	297859	51.7±9.1	92±9.51 (71-109)	160±18.06 (122-193)	108±17.34 (69-146)	8/10	8/7	58/4/10
Ialomita	4453	296572	43±11.19	94±6.72 (76-109)	162±18.64 (123-197)	110±15.5 (69-150)	10/9	4/5	58/3/11
Ilfov	1583	300123	42±10.26	93±6.96 (81-109)	161±20.38 (114-198)	95±16.1 (61-143)	17/14	7/7	57/6/9
Olt	5498	489274	41±10.74	89±10.17 (72-108)	170±22.35 (115-228)	108±19.06 (68-145)	8/9	5/2	61/2/9
Teleorman	5790	436025	42±11.3	93±7.72 (73-109)	162±21.71 (112-210)	106±16.26 (58-132)	9/9	7/4	64/2/6

*Data according to national census published in 2002

All subjects identified themselves as Romanian Caucasians and as dwellers of these districts for at least three generations. Other criteria for selection were: age between 18-66 years, normal blood pressure (≤ 130/90 mmHg after five minutes of rest in supination) and fasting glycaemia level (< 110 mg/dl). Smoking (more than five cigarettes per day, for at least one year) and drinking (at least 25 g of alcohol per day, for at least one year) habits and education levels were also recorded. Subjects with ages above 66 were not included in this study because the allele frequencies in elderly populations may be changed as a result of survival bias.

The design of the study was in accordance with internationally agreed ethical standards (Declaration of Helsinki, 1964). Ethics Committee approval and participants' consent were obtained before commencement of the research.

DNA Analysis. DNA extracted with AxyPrep™ Blood Genomic DNA Miniprep Kit (Axygen Scientific, Inc., CA, USA) from 0.25 ml of blood samples.

The markers were selected based on three main criteria: they are di-allelic and have a minimum allele frequency (MAF) of 5% being located within or in tightly linkage with candidate genes for common disease. The selected

polymorphisms were: MTHFR C677T (rs1801133) (1p36.3), HSPG BamH1 T/G (rs3767140) (Intron 6) (1p36-p34), LECAM P213S (rs2229569) (1q23-q25), MTR A2756G (rs1805087) (1q43), ATR1 A1166C (rs5186) (3q21-q25), eNOS ID 4a / 5b (No rs number) (7q36), Insulin +1127Pst (rs3842752) (11p15.5), IGF2 Apa (rs680) (11p15.5), VDR Fok T/C, (rs10735810 has merged into rs2228570) (12q13), ACE ID (rs4646994) (Intron 16), TGF-beta -509C/T (rs1800469) (19q13). Genotyping was performed using PCR or PCR-RFLP and the allele and genotype frequencies were estimated by direct counting. Ten percent of random selected samples have been re-genotyped by a second researcher to assure a quality control of genotyping process.

Statistical analysis. Data are presented as mean ±SD. PowerMarker v3.25 was used to calculate summary statistics, deviation from Hardy Weinberg Equilibrium and significance of marker distribution between groups [11]. The p values ≤ 0.05 or Bonferroni corrected for adequate number of tests were considered significant.

Results and Discussion

A total of 22 allelic variants were observed at the 11 polymorphisms in samples from each South Romanian district (Table 2).

Table 2. Distribution of genotypes and allelic variants in South Romania

Marker	Geno-type	Calarasi		Dambovita		Dolj		Giurgiu		Ialomita		Ilfov		Olt		Teleorman		Bucharest	
		W	M	W	M	W	M	W	M	W	M	W	M	W	M	W	M	W	M
MTHFR C677T	CC	19	20	17	19	16	21	14	20	13	19	13	15	23	14	19	18	13	19
	CT	16	14	16	13	17	11	18	13	15	12	17	15	10	17	12	13	21	14
	TT	1	2	3	4	3	4	4	3	8	5	6	6	3	5	5	5	2	3
MTR A2756G	AA	25	26	21	27	24	23	23	28	25	24	23	21	24	23	21	24	21	20
	AG	11	10	13	9	10	13	12	7	11	11	12	15	12	10	10	11	13	15
	GG	0	0	2	0	2	0	1	1	0	1	1	0	0	3	5	1	2	1
HSPG Bam	TT	17	19	18	22	19	17	22	15	16	18	14	13	17	19	17	14	16	16
	GT	15	14	14	12	14	15	11	14	16	13	16	15	13	13	13	18	17	19
	GG	4	3	4	2	3	4	3	7	4	5	6	8	6	4	6	4	3	1
TGFb	CC	15	20	23	14	21	20	15	18	17	23	11	18	19	19	21	16	12	11
	CT	17	12	12	14	11	16	13	15	15	10	22	16	13	13	12	15	20	19
	TT	4	4	1	8	4	0	8	3	4	3	3	2	4	4	3	5	4	6
LECAM P213S	CC	19	25	25	23	26	21	21	25	28	21	22	24	22	24	28	23	20	20
	CT	14	9	9	12	9	14	12	10	7	13	13	12	12	10	7	12	13	15
	TT	3	2	2	1	1	1	3	1	1	2	1	0	2	2	1	1	3	1
Insulin Pst	AA	27	22	23	27	28	22	25	22	21	25	20	18	23	23	23	24	23	21
	AT	7	14	10	7	8	12	10	11	14	8	14	17	13	10	12	10	12	15
	TT	2	0	3	2	0	2	1	3	1	3	2	1	0	3	1	2	1	0
IGF2 GA	GG	21	17	20	19	18	20	16	20	16	19	19	16	15	19	22	19	11	10
	AG	11	18	11	16	15	14	16	11	18	13	15	18	15	14	11	16	20	22
	AA	4	1	5	1	3	2	4	5	2	4	2	2	6	3	3	1	5	4
VDR Fok	CC	18	18	20	12	12	13	17	13	15	15	14	12	10	18	16	15	7	5
	CT	14	15	15	19	20	20	15	19	17	18	19	20	22	13	18	18	19	22
	TT	4	3	1	5	4	3	4	4	4	3	3	4	4	5	2	3	10	9
eNOS4a5b	Bb	21	24	23	23	25	22	25	21	23	24	21	19	25	26	26	24	21	17
	Ab	14	10	12	13	10	13	11	10	12	9	14	16	10	9	9	11	13	17
	Aa	1	2	1	0	1	1	0	5	1	3	1	1	1	1	1	1	2	2
ACE ID	DD	9	11	9	14	8	13	17	9	16	12	8	9	8	13	8	17	10	7
	DI	20	15	22	16	18	18	12	18	15	19	23	19	16	17	21	12	16	20
	II	7	10	5	6	10	5	7	9	5	5	5	8	12	6	7	7	10	9
ATR1	AA	25	16	21	26	24	26	19	26	18	23	20	14	27	21	27	19	16	18
	AC	9	16	14	8	11	10	16	9	15	11	13	20	9	14	7	17	18	17
	CC	2	4	1	2	1	0	1	1	3	2	3	2	0	1	2	0	2	1

The minor allele frequency (MAF) were estimated to be within 18.9% for the MTR A2756G and 44.98% for ACE ID (Table 3). For eNOS polymorphism we identified only two allelic variants which are specific for Caucasian populations; the allelic variants with two, three and six repeats found in African-American populations were not identified [12].

Table 3. Summary statistics and Classic F statistics for markers in the entire lots (n = 648 subjects) (PowerMarker v3.25)

Marker	MAF	Gene Diversity	Heterozygosity	PIC	Theta	F	*f*
MTHFR	0.3148	0.4314	0.4074	0.3384	0.0005	0.056	*0.056*
MTR	0.1890	0.3066	0.3164	0.2596	-0.0008	-0.031	*-0.03*
HSPG	0.3210	0.4359	0.4043	0.3409	0.0003	0.073	*0.073*
TGFb	0.3125	0.4297	0.4090	0.3374	0.0034	0.049	*0.046*
LECAM	0.1998	0.3198	0.3133	0.2687	-0.0025	0.021	*0.023*
Insulina	0.1991	0.3189	0.3148	0.2680	-0.0029	0.014	*0.016*
IGF2	0.2994	0.4195	0.4228	0.3315	0.0054	-0.007	*-0.012*
VDR	0.3650	0.4635	0.4985	0.3561	0.0171	-0.072	*-0.091*
eNOS	0.2029	0.3235	0.3287	0.2712	-0.0002	-0.015	*-0.015*
ACE	0.4498	0.4950	0.4892	0.3725	0.0001	0.012	*0.012*
ATR1	0.2238	0.3474	0.3611	0.2870	0.0078	-0.038	*-0.046*
Mean	0.2797	0.3901	0.3878	0.3119	0.003	0.007	*0.004*

The next step was to identify the distribution of genetic markers between districts. After correction of significance level for multiple tests only VDR Fok presented statistically significant differences between Bucharest and Calarasi, Dambovita or Teleorman (e.g. $p < 0.00008$) although other markers are no similarly represented in investigated districts ($p < 0.05$).

Multifactorial diseases associated with the investigated polymorphisms shows a different sex distribution. Starting from this observation we tested the distribution of polymorphisms correlated with the gender of subjects. The chi-square test was used to compare the distribution of genotypes (allelic variants) between man and women from the same district or age group (PowerMarker v3.25). The marginal significant difference between men and women from the same district was found in Dambovita (TGFb: $p = 0.003$, VDR: $p = 0.032$), Teleorman (ATR1: $p = 0.022$), Calarasi (ATR1: $p = 0.035$), Olt (MTHFR: $p = 0.045$), Giurgiu (HSPG: $p = 0.048$) district. These differences did not remain statistically significant after Bonferroni correction was applied.

The distribution of genetic markers with subjects stratified by age group has been performed. A progressively increase of MTHFR 677T allele with the middle age to the youngest subjects (18-26 vs. 43-66 years old, p value: 0.03-0.003) was detected. In sublots of subjects grouped according to the age, MTR was differently represented between 51-58 and 59-66 years old subjects (Table 4).

Table 4. The MAF distribution according to the age group. (2-tailed Z score) [a] $p < 0.05$; [b] $p < 0.003$; [c] $p < 0.002$

	Marker distribution in age groups					
	18-26	**27-34**	**35-42**	**43-50**	**51-58**	**59-66**
Subjects number	50	105	179	192	69	53
MTHFR	0.4100	0.3857	0.3492	0.2682 [a]	0.2536 [a]	0.2170 [b]
MTR	0.2000	0.2000	0.2039	0.1875	0.0870	0.2453 [c]
HSPG	0.2600	0.2714	0.3296	0.3229	0.3696	0.3774
TGFb	0.3200	0.3143	0.3296	0.2891	0.3043	0.3396
LECAM	0.2200	0.1905	0.1983	0.2161	0.2101	0.1321
Insulin	0.2800	0.2381	0.1704	0.1953	0.1812	0.1792
IGF2	0.2900	0.3286	0.2877	0.2917	0.2826	0.3396
VDR	0.3700	0.4048	0.3464	0.3672	0.3116	0.4057
eNOS	0.2300	0.2095	0.1704	0.1849	0.2971	0.2170
ACE	0.4800	0.4238	0.4860	0.4583	0.3551	0.4434
ATR1	0.2100	0.1857	0.2514	0.2161	0.2319	0.2358

Hardy–Weinberg equilibrium tests demonstrated no significant deviation from expected values (after Bonferroni correction) for all of the 11 markers in each lot, or in subjects stratified according to the gender or age groups.

The observed heterozygosity was found to be in the range 0.31 (for LECAM) and 0.49 (for VDR) and differ between Bucharest and Giurgiu or Olt ($p = 0.001$) (Table 5).

Table 5. The heterozygosity values for Bucharest and Ilfov differ from the other districts (p values for "t Student" test). [a] $p < 0.05$ and [b] p (corrected for 36 tests) < 0.0013

Differences of heterozygosity values in paired districts									
Districts	Bucuresti	Calarasi	Dambovita	Dolj	Giurgiu	Ialomita	Ilfov	Olt	Teleorman
Bucuresti	-	0.0018 [a]	0.0029 [a]	0.0097 [a]	0.0008 [b]	0.0026 [a]	0.5204	0.0010 [b]	0.0018 [a]
Calarasi		-	0.7476	0.8759	0.5810	0.8953	0.0076 [a]	0.6501	0.6733
Dambovita			-	0.6803	0.8761	0.8510	0.0101 [a]	0.9385	0.9417
Dolj				-	0.5463	0.7980	0.0313 [a]	0.6009	0.6190
Giurgiu					-	0.7043	0.0030 [a]	0.9304	0.9350
Ialomita						-	0.0098 [a]	0.7705	0.7841
Ilfov							-	0.0041 [a]	0.0064 [a]
Olt								-	1.0000
Teleorman									-

The analysed polymorphisms did not statistically significantly associate (after Bonferroni correction) with blood pressure and BMI values regardless of gender.

The overall theta calculated for tested population was 0.003 and the highest theta value for the investigated markers corresponds to VDR (0.017). The polymorphism information content (PIC) values ranged from 0.2596 for MTR A2756G polymorphism to 0.3725 for ACE ID with an average PIC of 0.31. The entire dataset of observed heterozygosity (heterogeneity chi square $p = 0.99$), gene diversity or PIC ("t Student" test p values > 0.06) did not differ significantly between districts.

Delineation of a population for a genetic study based on physical characters, historic, geographical, linguistic, ethnic affiliation may be subjective and it may not reflect the natural assignment in genetic terms. Although understanding the genetic structure of population is crucial for improving the efficiency of studies on a global or regional scale, there are some ethical and economic limitations in using a large panel of genetic markers.

In the present study we investigated the distribution of eleven di-allelic markers in the Caucasian population from Bucharest and eight districts located between the Carpathian Mountains and the Danube River (previously known as Walachia region). These districts cover 15.8% of the country area and are inhabited by 24.7% of the Romanian population. The population in this region is considered to be homogenous from ethnical, linguistical and socio-cultural point of view (96.5% of the dwellers are Romanians). The samples from Bucharest city were investigated because selected subjects self-identified as dwellers of the city for at least three generations (this covers a period of time when the city was closed for registering new dwellers).

Two of the strategies used for estimating the existing genetic diversity within or between populations are testing Hardy-Weinberg equilibrium and F-statistics. The deviation from Hardy-Weinberg equilibrium represents a strong evidence for population substructure, especially if the genotyping errors are excluded. In order to check for genotyping errors, 10% of samples were blindly rescored by an independent researcher. Because no difference in genotype allocated to these samples was detected we proceed to perform statistical tests.

The condition of Hardy-Weinberg equilibrium was accomplished in the investigated lots, even if the subjects from each district are stratified according to gender. A deviation from the equilibrium condition was observed in subjects grouped according to the age: in the subjects between 18-26 (e.g. HSPG, LECAM, Insulin), 35-42 (e.g. eNOS, VDR) and 51-58 (e.g. TGFb), these differences being not significant after Bonferroni correction was applied (at $p < 0.0045$).

Data regarding distribution of disease associated markers frequently come from case control studies, in which tested subjects are not necessarily representative for the population and may need correction for population stratification. Selective pressure can determine population specific allele frequency and population specific disease risk. For di-allelic markers a reasonable estimation of allele frequency in a population can be realized by genotyping at least 100-150 samples [13]. We consider that the selection criteria and the number of tested samples reduced the possibility of significant errors on estimated allele frequencies, although our multigenerational samples may contain any kin groups (e.g. cousins, half and full sibs). Allele frequencies in Romanian samples were compared to previous population studies on healthy and unrelated

individuals. Broadly, the allele frequencies were comparable with a number of studies on Caucasian populations.

From the eleven investigate markers only the VDR Fok is differently represented in Bucharest *vs.* Calarasi, Dâmbovita or Teleorman ($p < 0.00008$).

Some markers from INS-IGF2 [14-16], ACE [17-21], VDR [22-24], TGFb [25], MTR [26], MTHFR genes change the distribution according to the age and they seem to be associated with some selective advantage [27, 28], early mortality [29-30] or longevity whereas a substantial number of studies found no such effects. For example, the MTHFR 677T allele tends to become a common allele (50.5%) in new-borns from Italy [31].

When distribution of markers was investigated in age grouped subjects a progressively increase of the MTHFR 677T allele in subjects between 59-66 years old to those between 18-26 (21.7% vs 41%, $p = 0.0033$) was detected. These observations concur with previous reports in Spain [32-34], Italy (women from Sicily) [35], Swiss [36], Ashkenazi women [37] and Japan [38] which report the increase in the frequency of the MTHFR 677 TT genotype or of T allele in younger generations. These findings are in apparent contrast to results obtained in French [39] and *Swedish* [40] where only a trend towards a lower frequency of the mutation in elderly was described. MTHFR 677C/T is not an important determinant of life span in Jordanian [41] population.

During the period when subjects selected for this study were born, early folate treatment for the pregnant women in Romania was not a generalized practice. To clarify the relation between MTHFR C677T and age, our data must be reconfirmed in studies involving subjects with large age differences, for which data regarding intake of multivitamins and biomarkers of folate status are available.

Excepting MTHFR and MTR, no other changes of allele distribution according to the age were detected in our study.

F-statistics measure the amount of inbreeding-like effects within or among subpopulations and within the entire population. The F coefficients present great differences between loci under neutrality [42-44] but high values may be found at closely linked neutral loci where selection acts. The highest theta (0.017) value for the investigated markers corresponds to VDR, which is however far from the values corresponding to SNPs with high population differentiation [45]. The overall theta (0.003) estimated based on our samples indicated that 0.3% of the total genetic variation is distributed among subpopulations and 99.7% of the variation is within subpopulations.

The tested markers have a median information content (0.25-0.5) according to the classification of PIC. The values for gene diversity and PIC were similar ($p > 0.06$) in investigated districts. The distributions of genotypes and allelic variants and the values for theta, PIC and gene diversity indicate no significant differentiation of population in investigated area.

Conclusion

The distribution of markers in our districts is similar to those published for other caucasian populations. VDR Fok presents a different distribution in Bucharest and Calarași, Dâmbovița or Teleorman. The frequency of MTHFR 677T allele progressively increases from the middle age subjects to the youngest ones. The relevance of the result may be evaluated in an extended context to decipher its potential adaptive significance.

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