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Forensic and population genetic characteristics of 62 X chromosome SNPs revealed by multiplex PCR and MALDI-TOF mass spectrometry genotyping in 4 North Eurasian populations

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ABSTRACT

X chromosome genetic markers are widely used in basic population genetic research as well as in forensic genetics. In this paper we analyze the genetic diversity of 62 X chromosome SNPs in 4 populations using multiplex genotyping based on multi-locus PCR and MALDI-TOF mass spectrometry, and report forensic and population genetic features of the panel of X-linked SNPs (XSNPid). Studied populations represent Siberian (Buryat and Khakas), North Asian (Khanty) and Central Asian (Kazakh) native people. Khanty, Khakas and Kazakh population demonstrate average gene diversity over 0.45. Only East Siberian Buryat population is characterized by lower average heterozygosity (0.436). AMOVA analysis of genetic structure reveals a relatively low but significant level of genetic differentiation in a group of 4 population studied (F_{ST} = 0.023, p = 0.0000). The XSNPid panel provides a very high discriminating power in each population. The combined probability of discrimination in females (PDf) for XSNPid panel ranged between populations from 0.99999999999999999999999982 in Khakas to 0.9999999999999999999999963 in Buryats. The combined discriminating power in males (PDm) varies from 0.999999999999999792 to 0.999999999999999819. The developed multiplex set of X chromosome SNPs can be a useful tool for population genetic studies and for forensic identity and kinship testing.

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1. Introduction

X chromosome markers provide an useful tool for the purposes of forensic genetics, as well as for basic population genetic research. Population genetic features of X-linked polymorphisms combine particular advantages of autosomal and linearly inherited (mitochondrial and Y-chromosomal) DNA markers. X chromosome, as autosomes, is characterized by enormous genetic variability of different types. At the same time, lower effective size, compared to the autosomes, results in greater inter-population variability of the X-chromosome genetic diversity, and inheritance through the male hemizygous state allows direct haplotypes determination.

In forensic applications X chromosome loci are used mainly for resolving complicated kinship cases, such as establishing the relationship between distant relatives or paternity testing in closely related males [1]. Usefulness of X-chromosomal markers in personal DNA identification is limited by lower discriminating power of X-linked loci in males compared to autosomal ones. But modern high-throughput sequencing and genotyping technologies may overwhelm this restriction by means of simultaneous analysis of large number of genetic loci.

Recent advances in the developments of highly informative panels for forensic genetics are based on single nucleotide polymorphisms (SNPs) instead of, or in addition to traditional short tandem repeats (STRs) [2–4]. SNP markers, being much more common in the genome, and easier in genotyping and multiplexing, have obvious technical advantageous over STRs. To yield the same power in kinship testing as standard sets of STRs, comparatively small number of SNPs (3–4 times larger than number of STRs) is required. [5]. Previously reported sets of X-chromosomal SNPs were either insufficient in number of loci, or characterized by complexity in multiplexing [6–9].

In this paper we analyze the genetic diversity of 62 X chromosome SNPs in 4 North Eurasian populations using multiplex

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genotyping based on multi-locus PCR and MALDI-TOF mass spectrometry, and report forensic and population genetic features of the panel of X-linked SNPs.

2. Materials and methods

2.1. Populations and samples

A total of 475 male samples from 4 populations representing Siberia (Buryat and Khakas), North Asia (Khanty) and Central Asia (Kazakh) were examined. Geographic locations of populations are shown in Fig. 1. Buryat is Siberian native population speaking the language belonging to Mongolian branch of Altaic language family and living in the area east to Baikal Lake. Buryats ($N = 120$) were collected in Kurumkan village, Buryat Republic, Russia and in Aginskoe village, Chita Oblast, Russia. Khakas are native southern Siberian people speaking the language of Turkic branch of Altaic family. Population sample of Khakas ($N = 120$) was collected in Askiz town in Khakas Republic, Russia. Khanty sample ($N = 115$) representing native North Asian population, speaking the language of Uralic language family and living in the upper reaches of the Ob River, was collected in Kazym and Russkinskoe villages, Khanty-Mansi Autonomous Okrug, Russia. Central Asian Turkic-speaking Kazakhs ($N = 120$) were collected in Almaty, Republic of Kazakhstan. This study was approved by the Ethical Review Board at the Research Institute for Medical Genetics, Tomsk, Russia.

Data on 6 unmixed population of Eurasian origin from the HapMap project (http://hapmap.ncbi.nlm.nih.gov/) were used in comparative analyses: CEU, Utah residents with Northern and Western European ancestry; CHB, Han Chinese in Beijing, China; CHD, Chinese in Metropolitan Denver, Colorado; GIH, Gujarati Indians in Houston, Texas; JPT, Japanese in Tokyo, Japan; TSI, Toscani in Italia.

2.2. Selection of SNPs

X chromosome SNPs (XSNP) were selected from the NCBI database focusing on the uniform distribution along the chromosome, minimal distance between adjacent markers of 1 mln bp, absence of linkage disequilibrium in HapMap populations, and gene diversity higher than 0.4. About 140 SNPs selected from the database were subjected to multiplex primer design using Sequenom Assay Design software available on-line from www.sequenom.com. Final marker set (XSNPid panel) consisted of 66 XSNP in 2 multiplexes with the maximal multiplex capacities available for the set of 140 initially selected markers. The composition of multiplex 1 (W1) included 36 SNPs, while the multiplex 2 (W2) consisted of 30 SNPs. The composition of multiplexes is shown in Table 1. Primer sequences for PCR and iPLEX reaction are given in the Supplementary table.

2.3. Genotyping

Samples were genotyped by multiplex PCR and matrix-assisted laser desorption/ionization mass spectrometry with time of flight measurement (MALDI-TOF) using Sequenom MassARRAY 4 analyzer.

In brief, multi-locus PCR was performed separately for each multiplex in 5 μl reaction volume containing 5 ng of genomic DNA template and primer mix for the corresponding multiplex. The cycle conditions used were 3 min of initial denaturation at 94°, then 42 cycles of 94° for 20 s, 56° for 40 s, and 72° for 60 s, and a final extension step of 5 min at 72°.

Subsequent steps of the experiment – SAP-reaction, iPLEX-reaction, transfer of the samples to spectro-chip, sample ionization and analysis of the mass spectra was carried out as previously described [10]. Samples transfer to the spectro-chip and ionization of samples was carried out on Sequenom MassARRAY 4 analyzer. Real-time analysis of mass spectra, as well as primary processing and documentation of the results were done with the MassARRAY TYPER 4.0 software (Agena Bioscience, San Diego, USA).

For several samples per each SNP verification of genotypes was performed by Sanger sequencing with fluorescently labeled terminators using capillary gel electrophoresis genetic analyzer ABI PRISM 3730 (Life Technologies). No discrepancy between MALDI-TOF and direct sequencing genotypes was found.

In order to evaluate the effectiveness of the panel in forensic tests with restricted amount of biological samples, we tested the genotyping efficiency in a series of experiments with lower amount of template using 500 pg, 1, and 3 ng of DNA in multiplex PCR reaction. PCR was performed as described above separately for each multiplex in 5 μl reaction volume containing various amount of genomic DNA.

Fig. 1. Geographic locations of populations.

Table 1
Allele frequencies and discriminating power of 62 X-chromosome SNP s in 4 North Eurasian populations.

SNP	Position	Multiplex	Ancestral allele	Ancestral allele frequency			
				Buryat	Khakas	Khanty	Kazakh
rs2694742	3127322	w1	A	0.528	0.464	0.475	0.500
rs1405303	4120689	w1	C	0.716	0.623	0.309	0.591
rs4826682	5119276	w1	C	0.591	0.600	0.526	0.652
rs5962008	6325520	w1	A	0.557	0.594	0.585	0.656
rs2404797	8795378	w1	A	0.495	0.679	0.542	0.527
rs5934683	9751474	w1	T	0.767	0.595	0.606	0.667
rs7888207	11916455	w2	G	0.576	0.642	0.304	0.532
rs952076	13946956	w2	A	0.567	0.424	0.270	0.462
rs2317327	15407061	w2	A	0.566	0.346	0.702	0.576
rs4484871	22751065	w2	A	0.221	0.441	0.371	0.316
rs1351260	26948596	w1	C	0.863	0.660	0.723	0.731
rs1389433	28128729	w2	G	0.760	0.726	0.629	0.768
rs225067	29149024	w1	A	0.041	0.532	0.395	0.516
rs4454452	30985342	w2	A	0.569	0.481	0.477	0.432
rs3005641	34029930	w1	T	0.343	0.364	0.436	0.435
rs761913	37859510	w1	G	0.630	0.636	0.577	0.677
rs5963641	39216082	w2	G	0.602	0.536	0.527	0.547
rs5917990	40387891	w2	A	0.358	0.393	0.267	0.242
rs6609159	41573566	w1	T	0.278	0.333	0.215	0.308
rs205847	42791946	w1	G	0.544	0.541	0.563	0.587
rs766117	43816206	w2	G	0.432	0.417	0.568	0.432
rs5953326	49373567	w1	C	0.389	0.577	0.284	0.548
rs5915291	50379075	w2	G	0.673	0.660	0.824	0.674
rs4131729	51697194	w1	C	0.283	0.358	0.211	0.163
rs11799030	53056518	w1	C	0.299	0.350	0.253	0.344
rs4826609	54765913	w1	G	0.618	0.418	0.505	0.500
rs6624701	63749154	w1	G	0.840	0.712	0.840	0.750
rs471205	66238317	w1	A	0.340	0.255	0.196	0.261
rs5919529	67358208	w2	T	0.270	0.407	0.345	0.284
rs5937091	70745323	w1	G	0.356	0.412	0.413	0.283
rs2207739	75644692	w2	G	0.673	0.714	0.544	0.484
rs2411976	78383858	w1	A	0.475	0.613	0.602	0.663
rs5969528	81668873	w1	A	0.814	0.757	0.821	0.731
rs5922869	83049688	w2	T	0.648	0.648	0.696	0.426
rs5968597	84946832	w2	G	0.388	0.519	0.490	0.404
rs222108	86910110	w1	C	0.500	0.500	0.500	0.500
rs1474970	90394689	w2	T	0.485	0.550	0.467	0.505
rs5941047	91431385	w1	T	0.500	0.468	0.537	0.548
rs5949581	94756278	w2	G	0.612	0.632	0.386	0.656
rs5921682	100130437	w2	A	0.388	0.455	0.341	0.426
rs4898334	101387968	w1	G	0.181	0.333	0.379	0.292
rs5945770	102594936	w1	T	0.613	0.554	0.553	0.609
rs1285715	106308416	w2	T	0.552	0.616	0.355	0.568
rs5973840	107319029	w1	C	0.863	0.821	0.800	0.785
rs5974348	112218701	w1	T	0.302	0.309	0.635	0.376
rs7058109	113228559	w1	G	0.125	0.119	0.266	0.237
rs9329406	115740985	w1	G	0.547	0.425	0.458	0.473
rs217937	118511843	w1	T	0.673	0.528	0.619	0.828
rs5909923	121978166	w1	C	0.222	0.330	0.433	0.272
rs5977571	124496989	w2	A	0.457	0.574	0.393	0.505
rs916208	127941251	w2	A	0.720	0.750	0.527	0.705
rs926640	129462353	w2	G	0.552	0.595	0.652	0.484
rs2797125	130815979	w1	A	0.574	0.552	0.510	0.467
rs17391	131910697	w2	G	0.269	0.267	0.473	0.436
rs5977991	133429960	w2	A	0.417	0.505	0.418	0.379
rs5975695	135268469	w1	C	0.467	0.424	0.607	0.449
rs4825002	140430391	w2	G	0.519	0.546	0.688	0.558
rs2869922	141490609	w2	A	0.425	0.389	0.489	0.442
rs4825213	143435373	w2	T	0.573	0.536	0.494	0.537
rs1781486	144674621	w2	T	0.480	0.491	0.538	0.442
rs2504169	146358005	w2	T	0.583	0.557	0.663	0.568
rs614511	149537834	w2	G	0.495	0.657	0.584	0.474
Average gene diversity (H_e)				0.436	0.458	0.451	0.454
AMOVA F_{ST}				0.02267			
MPf				3.37×10^{-24}	1.82×10^{-25}	5.47×10^{-25}	3.40×10^{-25}
MPm				2.08×10^{-16}	1.81×10^{-17}	4.99×10^{-17}	3.30×10^{-17}

Note: chromosomal position is given according to the reference nucleotide sequence of the human genome assembly 38.2 (GRCh38.p2).

2.4. Statistical analysis

Standard population and forensic genetic indexes (gene diversity, H_e; random genotype matching probability, MP; power of discrimination, PD) were calculated using in-house software. Exact test of linkage disequilibrium (LD) between pairs of SNPs using Markov chain random walk with 10,000 dememorization steps, as well as estimation of genetic structure by analysis of

molecular variance (AMOVA) were performed in the Arlequin software. Bonferroni correction was applied to pairwise LD *p*-values. Principal component analysis of allele frequencies was performed in Statistica 8.0 software package.

3. Results

3.1. Genotyping efficiency

Efficiency of genotyping was estimated by the percentage of read genotypes (call rate). Two out of 66 SNPs (rs2130835 in 36-plex and rs4825220 in 30-plex) demonstrated low call rate (less than 80%) was excluded from the further analysis. The rest of SNPs in the panel showed very high efficiency (call rate 99.4%) in our standard protocol with 5 ng of genomic DNA. In order to estimate forensic potential of the panel we also tested genotyping efficiency with lower DNA amount in multiplex PCR reactions. Call rate for reaction with 3 ng of genomic DNA was 97%, 89% for 1 ng of template, and 83% for reaction with 500 pg of DNA. Despite a significant drop in efficiency of genotyping at low template concentration, call rate over 80% can provide a sufficiently high discriminating power of the system.

3.2. Population genetic analysis

In the process of SNPs selection for the panel, special attention was paid to avoid the linkage disequilibrium between genetic markers. Exact test of linkage disequilibrium for genotypic data in 4 populations has confirmed the absence of significant disequilibrium in pairwise analysis. However, several pairs of loci showed the level of LD significance close to the *p*-value threshold, involving repeated SNPs. These pairs were rs4830049–rs5974708 in Khakas population, rs4830049–rs222108 in Buryats, rs4830049–rs2694742 in Khants, rs5974708–rs205847 in Kazakhs. Two adjacent markers (rs4830049 and rs5974708), at least one of which has appeared in each pair of putatively linked SNPs, were removed from the dataset. Thus, the final dataset for population and forensic genetic analysis consisted of 62 SNPs out of 66 markers included in the multiplex panel.

List of SNPs, their chromosomal position, allele frequencies and gene diversity parameters in 4 populations under study are shown in Table 1. Four North Eurasian populations demonstrate considerable genetic variability in the allele frequencies of 62 XSNPs. Differences between minimal and maximal allele frequency for the same SNP in 4 populations ranged from 0% (for rs222108) to 28.2% (for rs1405303). Three populations show average gene diversity level over 0.45. Only East Siberian Buryat population is characterized by lower average heterozygosity (0.436).

AMOVA analysis of genetic structure reveals a relatively low but significant level of genetic differentiation in a group of 4 population studied ($F_{ST} = 0.023$, $p = 0.0000$). Locus by locus AMOVA demonstrates that allele frequency of 28 out of 62 XSNPs differed significantly between populations, while 34 genetic markers are genetically homogenous. Pairwise genetic differentiation analysis reveals significant differences in all pairs of populations with the maximal genetic distance between Buryat and Khanty populations ($F_{ST} = 0.035$, $p = 0.0000$), and minimal – between Turkic-speaking Khakas and Kazakh populations ($F_{ST} = 0.011$, $p = 0.0000$).

In order to place 4 native populations under study into Eurasian genetic context we have performed principal component (PC) analysis of allele frequencies in a dataset consisted of 4 populations reported here and 6 unmixed populations of Eurasian origin from the HapMap project (see Section 2). Space of two first PCs explaining 62.2% of allele frequency variability is shown on Fig. 2. North Eurasian populations form a separate cluster located in the middle of PC1-2 space between European (CEU, TSI), Indian (GIH) and East Asian (JPT, CHB, CHD) populations.

3.3. Forensic genetic analysis

Because of the different transmission patterns of the X chromosome in men and women, identification potential of X-chromosomal markers is significantly different between sexes. Therefore the matching probability (MP) and discrimination power (PD) were calculated separately for females (MPf and PDf) and males (MPm and PDm). MPf and MPm values are shown in Table 1. In general, the panel of 62 X-linked SNP markers (XSNPid panel) exhibits a very high combining discriminating power in each population. The combined probability of discrimination in females (PDf) ranged between populations from 0.99999999999999999999999982 in Khakas to 0.9999999999999999999999963 in Buryats. PDm varies from 0.999999999999999792 to 0.9999999999999999819.

4. Discussion

Population genetic analysis of 62 X chromosome SNPs reveals the high level of genetic diversity in 4 native North Eurasian populations. North Asian Khanty, Siberian Khakas and Central Asian Kazakh population demonstrate average gene diversity over 0.45. Relatively low genetic diversity in Buryats probably reflects the higher degree of genetic isolation of this native Siberian people and was also demonstrated previously using Y chromosome haplotypes, autosomal polymorphic *Alu* insertions and X-linked STR loci [11–14].

Despite very similar levels of within-population diversity, populations under study are significantly different from each other in XSNPs allele frequencies ($F_{ST} = 0.023$). Genetic relationships between populations illustrated by principal component analysis demonstrate clustering of population according to their geographic locations. The similar pattern of genetic structure for populations under study in the context of Eurasian genetic space was found with other types of genetic markers, including autosomal STRs, Y chromosome haplotypes and various autosomal biallelic markers [11,12,15,16].

Forensic genetic features of the XSNPid panel show high discriminating power of the system. Until recently, for the purposes of forensic DNA identification only panels of microsatellite markers have been used. Current standard sets of DNA identity markers adopted in the United States (CODIS, Combined DNA Index System) and in Europe (European Standard Set) provide high and generally sufficient power of individual identification ($MP = 3–5 \times 10^{-16}$) [17]. In our previous work, it was shown that a set of 15 STR loci also provides a very high probability of discrimination of unrelated individuals in native populations of Russia ($MP = 3.04 \times 10^{-15}$–$1.56 \times 10^{-17}$) [15,18].

However, low multiplexing capacity and complexity of genotyping of STR loci, coupled with recent advances in sequencing and genotyping technologies obviously will lead to the replacement of STR markers by more simple and informative systems [2–4]. Forensic panels based on SNPs or on combination of SNPs and STRs and genotyped by massive parallel sequencing or high-throughput SNP typing are actively developed. Comparative characteristics of some panels for DNA identification based on various platforms are shown in Table 2.

The advent of relatively inexpensive massively parallel sequencing (MPS) devices has led to the development of SNP panels for DNA identification based on MPS. Two major developments utilizing MPS are HID-Ion AmpliSeq Identity Panel, focused on personal MPS sequencers produced by Life Technologies, and

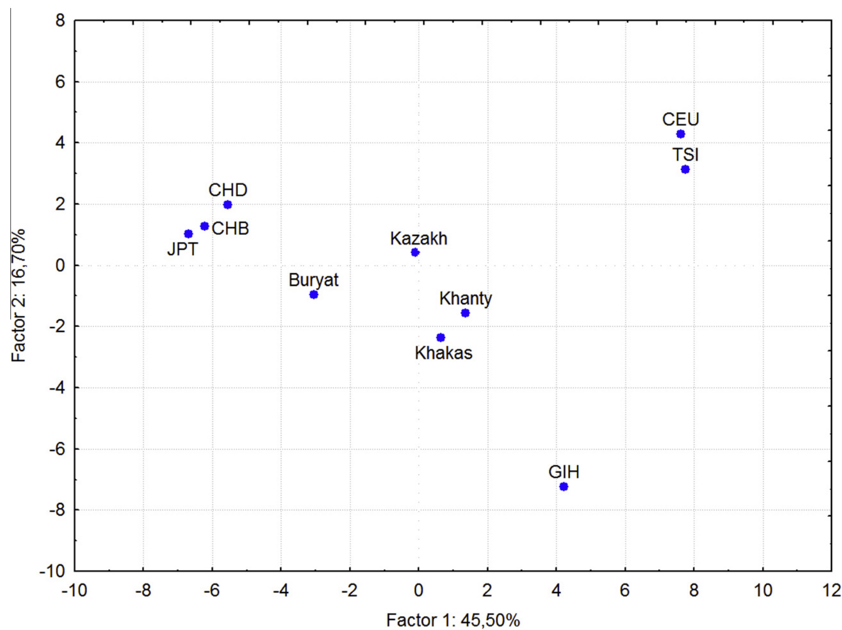

Fig. 2. Space of two first principal components of 62 XSNPs allele frequency variability.

Table 2
Comparative characteristics of some DNA identification panels.

Panel	Type of markers	Number of markers*	Platform	Probability of identity (PI)	References
CODIS	aSTR	14/13	Fragment analysis/capillary gel-electrophoresis	5.02×10^{-16}	[17]
ESS	aSTR	17/16	Fragment analysis/capillary gel-electrophoresis	3.04×10^{-16}	[17]
PowerPlex 16	aSTR	16/15	Fragment analysis/capillary gel-electrophoresis	2.81×10^{-17}	[15,17,19]
PowerPlex Fusion	aSTR	24/23	Fragment analysis/capillary gel-electrophoresis	6.58×10^{-29}	[17]
SNPforID	aSNP	52	SBE/capillary gel-electrophoresis	10^{-17}–10^{-20}	[20]
Genplex 49-plex	aSNP	49	Genplex/capillary gel-electrophoresis	10^{-17}–10^{-19}	[21]
HID-Ion AmpliSeq Identity Panel	aSNP + YSNP	124/90	MPS	1×10^{-31}–6×10^{-35}	[19]
ForenSeq DNA Signature Prep Kit	aSTR + YSTR + XSTR + SNP	230/94	MPS	n.d.	
53 XSNP	XSNP	67/53	MALDI-TOF MS	6.9×10^{-16}–1×10^{-19}	[9]
XSNPid	XSNP	66/62	MALDI-TOF MS	2.08×10^{-16}–1.82×10^{-25}	This work

Abbreviations: aSTR – autosomal short tandem repeats; MALDI-TOF MS – matrix-assisted laser desorption/ionization mass spectrometry with time of flight measurement; MPS – massive parallel sequencing; SBE – single base extension; XSTR – X chromosome STR; YSTR – Y chromosome STR; n.d. – no data.

* Total number of markers/number of markers for identity testing. Some panels also include additional markers for sex determination, phenotypic characteristics and others.

ForenSeq DNA Signature Prep Kit for Illumina personal MPS machines. The cumulative matching probability for HID-Ion AmpliSeq Identity Panel varies in different populations in the range of 1×10^{-31}–1×10^{-33} [19] (Table 2).

Despite substantial progress in research of autosomal SNPs forensic panels, the niche for X-linked markers is still open. Early XSNPs panels were based on conventional typing technologies (real-time PCR, capillary gel electrophoresis) and did not reach the level of discriminating power of standard STR sets [6–8]. Among new developments the closest analog to our panel is the set of 52 XSNP markers, proposed recently by Li et al. [9] (Table 2). This panel is also based on MALDI-TOF mass spectrometry, however, has a smaller multiplexing capacity (17–18 markers per plex) and requires four separate multiplex PCR reactions and 4 iPLEX reactions, whereas our panel is assembled in two multiplexes and has power of discrimination in several orders of magnitude higher.

In conclusion, we report a promising multiplex set of X chromosome SNPs which can be a useful tool for population genetic studies as well as for forensic identity and kinship testing.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.legalmed.2015.12.008.

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