Forensic features and genetic legacy of the Baloch population of Pakistan and the Hazara population across Durand-line revealed by Y chromosomal STRs

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ABSTRACT:

Hazara population across Durand- line has experienced extensive interaction with Central Asian and East Asian populations. Hazara individuals have typical Mongolian facial appearances and they called themselves descendants of Genghis Khan's army. The people who speak the Balochi language are called Baloch. Previously, a worldwide analysis of Y-chromosomal haplotype diversity for rapidly mutating (RM) Y-STRs and with PowerPlex Y23 System (Promega Corporation Madison, USA) kit was created with collaborative efforts, but Baloch and Hazara population from Pakistan and Hazara population from Afghanistan were missing. A limited data with limited number of markers and samples is available which poorly define these populations. So, in the current study, Yfiler Plus PCR Amplification Kit loci were examined in 260 unrelated Hazara individuals from Afghanistan, 153 Hazara individuals, and 111 Balochi individuals from Baluchistan Pakistan. For the Hazara population from Afghanistan and Pakistan overall, 380 different haplotypes were observed on these 27 Y-STR loci, gene diversities ranged from 0.51288 (DYS389I) to 0.9257 (DYF387S1) and haplotype diversity was 0.9992 +/- 0.0004. For the Baloch population, every individual was unique at 27 Y-STR loci, gene diversity ranged from 0.5718 (DYS460) to 0.9371(DYF387S1). Twelve haplotypes shared between 178 individuals while only two haplotypes among these twelve were shared between 87 individuals in Hazara populations. Rst and Fst pairwise genetic distance analyses, multidimensional scaling (MDS) plot, Neighbor-joining (NJ) tree, linear discriminatory analysis (LDA), and median-joining network (MJNs) were performed, which shed light on the history of Hazara and Baloch populations. Interestingly null alleles were observed at DYS448 with specific mutation patterns in Hazara populations. The results of our study showed that the Yfiler Plus PCR Amplification Kit marker set provided substantially stronger discriminatory power in the Baloch population of Pakistan and the Hazara population across the Durand-line.

Keywords: Hazara; Pakistan; Afganistan; Baloch; Population history; Forensic Genetics

INTRODUCTION

The variation pattern in Human DNA usually provides a balance between natural selection and neutral processes. Y chromosomal variant analysis for determining the patterns of present and past flow of genes between populations is very helpful¹. Y-chromosome short tandem repeats (YSTRs) plays an important role in forensic molecular biology²⁻⁵. Usually, Y-STRs are used for (i) decidedly determine the male component of DNA mixtures under the presence of a high female DNA background as typically confronted with materials from sexual assault cases⁶, (ii) to test for paternal relationships between male individuals particularly in deficiency paternity cases with the mother not being available⁷, or (iii) for special cases in missing-person or (iv) disaster-victim identification involving males⁸, or (v) for evolutionary purposes because male family members share same haplotype distribution which may be different from individual to individual within a population group, or (vi) different geographic regions or in different ethnic groups. Normally, more paternal lineages can be differentiated with an increased number of Y-STRs⁹, such as the Powerplex Y Kit (Promega) containing 12 Y-STRs¹⁰, the AmpFISTR Y-filer PCR Amplification Kit (Life Technologies) (subsequently referred to as Y-filer) containing 17 Y-STRs¹¹ or Powerplex Y23 Kit (Promega) containing 23 Y-STRs¹², relative to the initially proposed 9-loci haplotype¹³. So, Applied Biosystems have developed Yfiler Plus PCR Amplification Kit¹⁴. The Yfiler Plus kit provides enhanced discrimination power because it includes the Yfiler loci and 10 additional STRs in which 6 are rapidly mutating (RM) Y STRs. These rapidly mutating Y STRs showed a higher mutation rate of about a few mutations every 100 generations per locus ($\mu > 10^{-2}$) compared with all other commonly used Y-STRs. Molecular biological and cytogenetical studies give us an insight into the presence of many structural variants within the human Y chromosome, which might be deletions¹⁵⁻¹⁷, duplications¹⁸⁻²⁰, and

inversions¹⁹⁻²³. Null alleles or allele drop-out are well-established factors that can occur with any PCR-based STR typing system. The reason could be the primer binding site problem or deletions within the target region^{24,25}. DYS448 lied in the proximal part of the azoospermia factor c (AZFc) region, which is considered important in spermatogenesis and made up of “ampliconic” repeats which act as substrates for nonallelic homologous recombination (NAHR). NAHR could delete larger blocks of the Y chromosome which included DYS448²⁶. This null alleles or allelic drop-out phenomenon is more commonly observed in Central Asian and East Asian populations but in the Hazara population of Pakistan, its occurrence was >16%²⁷.

Durand Line is a boundary established in the Hindu Kush around 1893 running through the tribal lands between Afghanistan and British India (modern-day Pakistan), marking their respective scopes of influence. The recognition of this line, which was named after Sir Mortimer Durand, has settled the Indo-Afghan frontier problem for the rest of the British period. Now, this is an established border between Afghanistan and Pakistan. The origin of the Hazara population is disputed. The Hazara could be of Turko-Mongol ancestry and theorized to be the descendants of an occupying army left in Afghanistan by Genghis Khan in thirteen hundred AD²⁸. The Hazara population speaks Persian with some Mongolian words. The total population of Hazaras in the world is 4.5 million. Afghanistan is considered the mainland for the Hazara population (3 million) and they are the third largest ethnic group (9%) after Tajiks (27%) and Pashtuns (42%)²⁹, while in Pakistan, Hazara is one of the distinct but small groups comprising 0.08% of the total population (<http://www.pbscensus.gov.pk>). The tribes who speak the Balochi language are called Baloch³⁰. Balochi population is 3.6% of total Pakistani population (<http://www.pbscensus.gov.pk>). They are also found in the neighboring areas of Iran and Afghanistan. Perhaps, the origin of Baloch homeland lay on the Iranian plateau. The Baloch

were mentioned in Arabic chronicles of the 10th century. The Seljuq invasion of Kermān in the 11th century started the eastward migration of the Balochi population³⁰.

In this study, we have investigated the Baloch and Hazara population from Pakistan and the Hazara population from Afghanistan using 27 Y STRs to determine their genetic history and gene diversity. This data has defined the Hazara and Baloch populations better and are supplement to the Y STR haplotype reference database (YHRD).

2. RESULTS AND DISCUSSIONS:

2.1 Allelic frequencies and Forensic parameters

We successfully obtained genotypes of 524 individuals in three ethnic groups (Balochi population, Hazara population from Afghanistan, and Pakistan) (**Supplementary Table 1**).

Allelic frequencies of Baloch ethnic group from Baluchistan, Pakistan, and Hazara ethnic groups from Pakistan and Afghanistan along with gene diversity values were shown in **Supplementary Table 2**.

DYF387S1 showed the highest gene diversity/heterozygosity in Baloch and both Hazara populations from Afghanistan and Pakistan with 0.9371, 0.9242, and 0.8792, respectively. Overall DYS570 (0.8624) showed the highest or DYS437 (0.2383) showed the lowest gene diversity/heterozygosity for single Y STR markers. Within three populations, single Y-STR markers DYS570 (0.8624), DYS449 (0.8468), DYS627 (0.7949) showed the highest gene diversity/heterozygosities while DYS460 (0.5718), DYS391 (0.3916), and DYS437 (0.2383) showed the lowest gene diversity/heterozygosities in the Baloch and both the Hazara populations from Afghanistan and Pakistan, respectively. After pooling Hazara populations together DYF387S1, DYS437 showed the highest or lowest gene diversity/heterozygosities with 0.9257

and 0.4053 respectively. The observed numbers of alleles were 222, 240, and 188 for Baloch and both the Hazara populations from Afghanistan and Pakistan, respectively on 27 Y STRs.

Allelic frequencies ranged from 0.0090 to 0.6036 in the Baloch population, 0.0038 to 0.6654 in the Hazara population from Afghanistan, and 0.0065 to 0.8627 in the Pakistani Hazara population.

We evaluated forensic parameters at seven levels (**Table 2**), the minimal 9 Y-STRs loci (DYS19, DYS389I, DYS389II, DYS390, DYS391, DYS392, DYS393, and DYS385a/b), the extended 11 Y-STRs loci (MHT+DYS438 and DYS439), PowerPlex Y12 STRs loci (extended 11 Y STRs + DYS437), Y-filer 17 STRs loci (PPY12+DYS448, DYS456, DYS458, DYS635, and Y_GATA_H4), Y21STRs loci(Y-filer + DYS481, DYS533, DYS570, and DYS576), Y27 Yfiler Plus loci (21 STRs + DYF387S1, DYS449, DYS460, DYS518, and DYS627), and 6 rapidly mutating Y STRs loci (DYS570, DYS576, DYF387S1, DYS449, DYS518, and DYS627) which are summarized in Table 2. The discrimination capacity (DC) ranged from 87.38% (the minimal 9 Y-STRs loci) to 100% (Y27 Yfiler Plus loci) with random matching probability from 0.0162 (MHT) to 0.009 (Y27 Yfiler Plus loci) and haplotype diversity (HD) ranged 0.9928 (the minimal 9 Y-STRs loci) to 1.0 (Y27 Yfiler Plus loci) in the Baloch population of Pakistan. The discrimination capacity (DC) ranged from 47.06% (the minimal 9 Y-STRs loci) to 99.35% (Y27 Yfiler Plus loci) with random matching probability from 0.0745 (MHT) to 0.0066 (Y27 Yfiler Plus loci) and haplotype diversity (HD) ranged from 0.9316 (the minimal 9 Y-STRs loci) to 0.9999 (Y27 Yfiler Plus loci) in Pakistani Hazara population while DC ranged 41.15% (the

minimal 9 Y-STRs loci) to 88.46% (Y27 Yfiler Plus loci) with random matching probability from 0.0329 (MHT) to 0.0057 (Y27 Yfiler Plus loci) and HD ranged from 0.9708 (the minimal 9 Y-STRs loci) to 0.9937 (Y27 Yfiler Plus loci) for Hazara population from Afghanistan. Pooling both populations together DC ranged 40.19% (the minimal 9 Y-STRs loci) to 92% (Y27 Yfiler Plus loci) with random matching probability from 0.0334 (MHT) to 0.0032 (Y27 Yfiler Plus loci) and HD ranged from 0.9689 (the minimal 9 Y-STRs loci) to 0.9992 (Y27 Yfiler Plus loci). Interestingly six rapidly mutating Y STRs which are included in Yfiler plus kit detects high haplotype diversity (Table 2). We have observed 101 (90.99%) different haplotypes out of 111, among them, 95 (85.58%) were unique in the Baloch population and we have observed 139 (90.84%) different haplotypes out of 153, among them 131 (85.62%) were unique in Pakistani Hazara population while in Afghani Hazara population observed haplotypes were 188 (72.30%) out of 260, among them 152(58.46%) were unique. These six STRs (RM Y STRs) showed the almost same diversity, shown by PPY 23 loci. The above results are showing that Yfiler plus kit loci showed strong discrimination capacity, haplotype diversity, and random mating probabilities which provide utility for forensic identification and paternity testing in three ethnic groups (Baloch and Hazara from Pakistan while Hazara from Afghanistan).

2.2Phylogenetic analyses and Population comparisons

1 Since the anthropological or ethno-historical relationships between studied populations and
2 reference populations which are included for analysis were already known, so we used two
3 different methods on the basis of their similarity with *a priori* expectations. *Fst* is a standardized
4 variance of haplotype frequency and assumes genetic drift as being the agent that differentiates
5 populations. *Rst* is a standardized variance of haplotype size and takes into account both drift and
6 mutation as causes of population differentiation, assuming a stepwise model in which each
7 mutation creates a new allele either by adding or deleting a single repeat unit. To assess the
8 relationship between these three populations (Baloch, Hazara from Pakistan and Afghani
9 Hazaras), and the other relevant populations which are summarized in **Table 1**, pair-wise genetic
10 distances (*Rst* and *Fst*) and their corresponding p-values were calculated and were shown in
11 **Supplementary Table 3**. These *Rst* and *Fst* values were visualized using hierarchical clustering
12 heat-map (**Supplementary Figure 1 a & b**). Dendrograms give us a clear picture about the
13 organization of the data which can be compared with NJ trees or MDS plots. The utilization of
14 mean-linkage dendrograms to Y STR data gives us a consistent basis of comparison. Heat-map
15 matrix based on *Rst* values showed that Hazara from Pakistan were clustered more closely to
16 Central and East Asian (i.e. Kazakh and Mongols) populations while the Baloch population was
17 clustered with other Pakistani (i.e. Pathan and Sindhi) populations and Hazara from Afghanistan
18 were clustered with local Afghan populations. On another hand, the heat-map matrix based on
19 *Fst* values showed that the Hazara population from Pakistan was tightly clustered with local (i.e.
20 Baloch, Arain, and Pathans) populations while the Hazara population from Afghanistan was
21 clustered with Afghanistan Pathan and Northern Talysh population. The observed pattern of
22 inter-population diversity from *Rst* was in support of anthropological knowledge, while that
23 based on *Fst* revealed unexpected and unconvincing population affinities. These results are

consistent with our previous study results³¹. The pairwise *Rst* genetic distances values between Baloch and other relevant populations ranged from -0.0402 to 0.1417. According to *Rst* values, the Baloch population of Pakistan showed the closest genetic distance to Turks (-0.0402) from Ardabil, Iran while Kazakh (0.1417) from Gansu, China showed the greatest genetic distance. For the Afghan Hazara population, the Afghan population (0.0009) from Afghanistan showed the closest genetic distance and for the Pakistani Hazara group, the Afghan population (0.0381) from Afghanistan showed the closest genetic distance. To investigate the paternal relationship among these three and other reference populations, we have generated the MDS plot (figure 1) based on pairwise *Rst* matrix from supplementary table 3. In the MDS plot, we have seen that the Hazara population from Afghanistan is located closer to the Afghan population from Afghanistan and the Pathan population from northern Afghanistan which is similar to the results of another study³², while Pakistani Hazara lined closer to Kazakh and Mongolian population which is similar to our previous study's results^{27,33}.

According to *Fst* values, the Afghan Hazara population is closest to the Afghan population (0.0053) followed by the Hazara population from Balochistan, Pakistan (0.0057), and Iranian population from Mashhad, Iran (0.0077). Evolutionary relationships between the Baloch and Hazara population of Pakistan, the Hazara population from Afghanistan, and other reference populations were inferred from the Neighbor-joining tree based on *Fst* values (**Figure 2**). In neighbor-joining trees, an admixed population will always lie on the path between the source populations³⁴. In total, we have observed 14 clusters for 62 populations in NJ-tree and the Baloch population placed itself in the second cluster along with West-south Asian populations. Hazara populations from Pakistan and Afghanistan came to the fourth cluster along with the Afghani and Iranian populations. The pattern of inter-population diversity based on *Rst* was consistent with

ethnohistorical and anthropological knowledge, while that based on *Fst* shown surprising and unaccepted population affinities.

2.3 Inference of ancestry based on Y STRs

The Y haplogroups were predicted using the online Y-haplogroup predictor software (<http://www.nevgen.org/>). C2 (previously known as C3-Star cluster) was the most frequent haplogroup in Pakistani and Afghan Hazaras.

The median-joining network of haplotypes (**Figure 3**) showed a bulky central star-like cluster which represents predicated haplogroup M217 and another big cluster representing haplogroup M420 and comprises many of the identical or highly similar haplotypes. These types of features are usually inferred as past male-lineage expansions³⁵. Star-like features of haplotypes comprising haplogroup M217 (C2) have been reported previously in Hazara, Mongol, and Kazakh populations^{27,33,36}. An explanation about its origin in Mongolia was about ~1,000 years ago³⁶. The frequency of R haplogroup in the Baloch population is 36.03%, 22.22% in Pakistani Hazara, and 21.15% in Afghani Hazara. This haplogroup originated in north Asia about 27,000 years ago (<http://isogg.org/tree/index.html>). R is one of the most frequent haplogroups in Europe, with its branches reaching 80% of the population in some regions. One branch is believed to have originated in the Kurgan culture, known to be the first speakers of the Indo-European languages and responsible for the domestication of the horse³⁷. From somewhere in Central Asia, some descendants of the man carrying the M207 mutation on the Y chromosome headed south to arrive in India about 10,000 years ago³⁸. This is one of the frequent haplogroups in Pakistan and North India. In the Baloch population frequency of haplogroup L1 is 22.5% and 1.53% in Afghani Hazara. In sub-continental populations its frequency is about 7–15%^{39,40}. Genetic studies suggest that this may be one of the original haplogroups of the creators of Indus Valley

Civilization^{41,42}. The frequency of L1 is about 28% in Pakistan and Baluchistan, from where the agricultural creators of this civilization emerged⁴³. The origins of this haplogroup can be traced to the rugged and mountainous Pamir Knot region in Tajikistan³⁸.

In an earlier study³⁶, the star-cluster (C3) profile for

DYS389I-DYS389b-DYS390-DYS391-DYS392-DYS393-DYS388-DYS425-DYS426-DYS434
-DYS435-DYS436-DYS437-DYS438-DYS439 was

10-16-25-10-11-13-14-12-11-11-11-12-8-10-10. In present study mostly occurring haplotype for loci

DYS19-DYS389I-DYS389II-DYS390-DYS391-DYS392-DYS393-DYS437-DYS438-DYS439

was 15-13-29-24-10-11-13-14-11-12 which repeated itself in 43 individuals while

14-13-29-24-8-11-13-14-11-11 repeated in 9 individuals and 15-13-29-24-11-11-13-14-11-12

repeated in 8 individuals in Pakistani Hazara population while in Afghani Hazara

16-13-29-25-10-11-13-14-10-10, 15-13-29-24-10-11-13-14-11-12,

14-12-28-23-10-11-12-15-9-11, 14-13-29-24-11-13-12-15-12-12 and

15-14-32-25-11-11-13-14-9-10 haplotypes were repeated in 30, 17, 15, 12 and 11 individuals,

respectively. The occurrence of these haplotypes were previously observed in Mongols and

Kazakhs³⁵. Allelic ranges of Kazak³⁵ population from Kazakhstan Central Asia were similar

while Mongol population from Inner Mongolia were almost similar on above mentioned 10 Y

STRs. In our earlier study³¹, results showed that Hazaras have a close genetic affinity with

Turkic-speaking (Kazakh, Kyrgyz and Uyghur) and Mongolian people. Admixture and outgroup

findings further clarified that Hazara have 57.8% gene pool from Mongolians.

Here we also speculated a hypothesis that is based on hearsay that Hazaras living in Pakistan are

more conserved and they only mate with the Hazaras while across the Durand line the Hazaras

mate with other ethnic groups in Afghanistan. Results of gene diversity/heterozygosity and F-statistics tests are also supporting this hypothesis. According to results, all loci showed more diversity in the Hazara population from Afghanistan when compared with the Hazara population from Pakistan (**Figure 4**). F-statistics test within Hazara populations showed variations at four loci only (DYS393- 0.05002, DYS449- 0.01694, DYF387S1- 0.00662 and DYS385a/b- 0.00004) (**Supplementary Table 4**). These variations may be the sampling effect, population diversity, or maybe geographical boundaries. LDA is a transformation technique which is commonly used to understand genome diversity and was performed on the Hazara population, Central Asian, South Asian including the Baloch population, East Asian, and Russian population samples to explore their genetic homology. **Figure 5** shows all individual samples plotted on the two LDA factors (F1 and F2). LDA Plot showed the association of the Hazara population with East and Central Asian populations.

2.4 Physical characterization of DYS448 deletions

By using the Yfiler plus kit, we have observed the null allele at DYS448 in 29 individuals in the Hazara population from Afghanistan (**Figure 6**). Certain factors can cause the phenomena of null alleles and these are deletions within the target region, primer binding sites problem that destabilize hybridization of at least one of the primers flanking the target region⁴⁴⁻⁴⁷. This phenomenon was previously reported, in which other commercial kits were used⁴⁸⁻⁵³. The current population study represents the highest frequencies of the null allele at DYS448 when compared with the previously reported population to date (**Table 3**). The core repeat motif of the DYS448 locus is the hexanucleotide repeat AGAGAT⁵⁴. DYS448 has two polymorphic domains separated by an invariant 42-bp region.

We have observed 29 null alleles among these, long deletions were covering at a minimum the N42 region and the core AGAGAT repeats downstream, and small deletions encompassing upstream repeats as well (all alignments were based on allele 20). Observed null alleles at locus DYS448 in 29 individuals from the Hazara population of Afghanistan, which were later confirmed with the GoldenEye Y20 System kit were successfully amplified using self-designed primers and sequenced (**Supplementary Table 5**) which were submitted to genbank under accession numbers MN623385 to MN623413. Overall we have observed 55 null alleles at DYS448 in the Hazara population from Pakistan and Afghanistan. Interestingly, all individuals (55) who showed deletion at DYS448 belongs to haplogroup C2 which is most frequent haplogroup in Mongol and Kazakh populations. This high frequency of allele drop-out / mutation is DYS448 in Hazara population from Pakistan and Afghanistan strongly support the evidence that they have Kazakh and Mongol origin. Whole genome or Y Chromosomal sequencing is required to get more insight of this polymorphism. The frequency of the null allele at DYS448 is more frequent in Asia more specifically in East and Central Asia when compared to the rest of the world^{26,49}. The commercial companies should pay special attention while designing DYS448 primers.

2.5 Concluding Remarks

Finally, our study demonstrates that the Yfiler plus kit detects high haplotype diversity in Baloch population from Pakistan and Hazara populations from across the Durand line (Pakistan and Afghanistan) of which two (Baloch and Afghani Hazara) were not previously studied at Yfiler plus STR loci, which in general makes it suitable for forensic casework in these groups. The recent inclusion of these data in the YHRD allows widespread use for forensic and other purposes.

3. MATERIALS AND METHODS

3.1 Samples

A total of 524 blood samples were collected, in which 111 Balochi individuals from Baluchistan Pakistan, 153 from Hazara Town Quetta, Baluchistan Pakistan (Participants were part of an earlier study²⁷ and were agreed to the secondary use of their DNA samples), and 260 from Bamyán, Afghanistan. All participants who were included in this study were unrelated individuals of at least three generations. All participants gave their informed consent either orally and with thumbprint (in case they could not write) or in writing after the study aims and procedures were carefully explained to them. This collaborative study was approved by the ethical review boards of China Medical University, Shenyang, Liaoning Province, People's Republic of China (2019/067-P), University of Health Sciences Lahore Pakistan (2017-CMU-1/14), and Ministry of Public Health, Forensic Medicine Directorate, Kabul, Afghanistan (FC-2017-02). All the experimental procedures were performed in accordance with the standards of the Declaration of Helsinki.

3.2. DNA extraction

Axygen AxyPrep Blood Genomic DNA Miniprep Kit was used to extract genomic DNA according to the manufacturer's protocol (Axygen Biosciences; CA, USA).

3.3 PCR Amplification

DNA was amplified using Yfiler Plus PCR Amplification Kit (Thermo Fisher Scientific) PCR amplification was carried out using the Applied Biosystems GeneAmp PCR System 9700 thermal cyclers. PCR amplifications were performed as recommended by the manufacturer, although using half of the recommended reaction volume (12.5 µl).

3.4. 27Y-STRs genotyping

After successful PCR amplification, The PCR products were analyzed by using an 8 capillary ABI 3500 DNA Genetic Analyzer with POP-4 polymer (Life Technologies) according to the manufacturer's protocol. GeneMapper Software version 4.0 (Life Technologies) was used for the genotype assignment. DNA typing was performed according to the manufacturer's protocol by using the locus panel and allele bins supplied by the manufacturer and allele designations corresponding with the allelic ladder supplied by the manufacturer. Genotype nomenclature was based on the recommendations of the International Society for Forensic Genetics⁵⁵.

3.5. Confirmation of Null DYS 448

For the confirmation of samples that showed no allele call at DYS448, they were re-amplified by using the Goldeneye 20Y amplification kit (Goldeneye Technology Ltd.). After confirmed with two different kits (Yfiler Plus and GoldenEye 20Y), these samples were amplified and sequenced as described elsewhere²⁷.

3.6. Quality control

Our laboratory has participated and passed the YHRD quality assurance exercise 2015. Haplotype data were already made accessible via the Y-chromosome Haplotype Reference Database (YHRD) under accession number YA004595 (Balochi) in 61st release on dated 2019 June 24, YA004312-2 (Hazara Pakistan) and YA004503 (Hazara Afghanistan) in 59th release on dated 2018 November 01. 29 sequenced samples at null allele call at DYS448 were also submitted to genbank under accession numbers MN623385 to MN623413 on dated 2019 october 28.

3.7. Statistical analysis

Allelic and haplotype frequencies were computed by direct counting method and haplotype diversity (HD) was calculated according to:

$$HD = \frac{n}{n-1} \left(1 - \sum_i p_i^2 \right)$$

where n is the male population size and p_i is the frequency of i th haplotype. Discrimination capacity (DC) was calculated as the ratio of unique haplotypes in the samples. Match probabilities (MP) were calculated as $\sum P_i^2$, where P_i is the frequency of the i -th haplotype. Genetic distances were evaluated using the R_{st} ⁵⁶ and F_{st} ⁵⁷⁻⁵⁹ statistic, between reference populations and currently studied populations on overlapping STRs (DYS19, DYS389I, DYS389II, DYS390, DYS391, DYS392, DYS393, DYS437, DYS438, DYS439, DYS448, DYS456, DYS458, DYS635, and Y_GATA_H4) were calculated by using Arlequin Software v3.5⁶⁰. We calculated both R_{st} and F_{st} values because in the generalized stepwise mutation model, R_{st} offers relatively unbiased evaluations of migration rates and times of population divergence while on other hand F_{st} tends to show too much population similarity, predominantly when migration rates are low or divergence times are long⁵⁶. Reduced dimensionality spatial representation of the populations was performed based on R_{st} values using multi-dimensional scaling (MDS) with IBM SPSS Statistics for Windows, Version 23.0 (IBM Corp., Armonk, NY, USA). Heatmaps were generated using R_{st} and F_{st} values were generated using R program V3.4.1 platform with the help of a ggplot2 module.

Phylogenetic analysis:

A neighbor-joining phylogenetic tree was constructed for the Hazara and the reference populations based on a distance matrix of F_{st} using the Mega7 software⁶¹. We also predicted Y-SNP haplogroups in the samples from Y STR haplotypes (Yfiler STRs) using the Y-DNA

Haplogroup Predictor NEVGEN (<http://www.nevgen.org>). We have used FTDNA order for 17 Y STRs (Yfiler loci). The microvariant alleles were truncated to the next lowest integer value since values in the database were treated similarly. Any haplotypes which have null alleles or duplication variants in the Baloch or Hazara population from Pakistan or Afghanistan were excluded from the analysis. The results of NEVGEN were cross checked with Athey's Haplogroup Predictor (<http://www.hprg.com/hapest5/index.html>).

Linear discriminant analysis

R program V3.4.1 platform with the help of a ggplot2 module was used to perform linear discriminant analysis (LDA) for Hazara (Pakistan), Hazara (Afghanistan), Central Asia, East Asia, the Middle East, and Southwest Asian (Baloch) samples⁶² on overlapping (DYS19, DYS389I, DYS389II, DYS390, DYS391, DYS392, DYS393, DYS437, DYS438, DYS439, DYS448, DYS456, DYS458, DYS635, and Y_GATA_H4) STRs. The multi-copy marker like (DYS385ab) and haplotypes that have null alleles or duplication variants in the Baloch or Hazara population or any of the reference populations were excluded from the analysis. For DYS389I and DYS389II, we have subtracted DYS389I from DYS389II and used DYS389II-I for analysis.

The median-joining network

To define the genetic relationships among Balochi and Hazara individuals for 20 Y STRs (DYS19, DYS389II-I, DYS390, DYS391, DYS392, DYS393, DYS437, DYS438, DYS448, DYS456, DYS458, DYS635, Y_GATA_H4, DYS549, DYS460, DYS481, DYS533, DYS570, DYS576, DYS627), we used the stepwise mutation model and Median Joining-Maximum

Parsimony algorithm⁶³ by using the program Network 5 as described at the Fluxus Engineering website (<http://www.fluxus-engineering.com>), and the weighting criteria for Y-STRs following²⁷. Any haplotypes which have null alleles or duplication variants in the Baloch or Hazara population from Pakistan or Afghanistan were excluded from the analysis.

COMPETING FINANCIAL INTERESTS

None.

AUTHOR CONTRIBUTION

J.L. and A.A. designed this study. A.A., A.R., and S.N. and M.R., collected the samples. A.A. experimented and wrote the manuscript. A.A., J.L., A.R., S.N., R.A., S.W., and C.W., analyzed the results. A.A., and J.L., modified the manuscript. All authors reviewed the manuscript.

COMPLIANCE WITH ETHICAL STANDARDS

The study was approved (2019/067-P) by the ethical review board of China Medical University, Shenyang, Liaoning Province, People's Republic of China, and in accordance with the standards of the Declaration of Helsinki. All participants who were included in this study were unrelated individuals of at least three generations. All participants gave their informed consent either orally and with thumbprint (in case they could not write) or in writing after the study aims and procedures were carefully explained to them.

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Legends of figures and tables:

Figure 1: Two-dimensional plot from multi-dimensional scaling analysis of *Rst*-values based on Yfiler haplotypes for the Baloch population of Pakistan and Hazara populations across the Durand line with reference populations.

Figure 2: Neighbor-joining tree based on the *Fst* values between the Baloch population of Pakistan and Hazara populations across the Durand line with reference populations.

Figure 3: The median-joining network of the Baloch population of Pakistan and Hazara populations across the Durand line based on 20 Y STRs.

Figure 4: Heterozygosity scattered plot for three populations

Figure 5: LDA Analysis between the Baloch population of Pakistan and Hazara populations across the Durand line, Central Asia, South Asia, Russia, and East Asian populations.

Figure 6: Electropherogram of an individual showing null type at DYS448.

Table 1: Reference Populations from Central, Eastern and South Asia populations selected as reference populations used in LDA, NJ tree and multidimensional scaling (MDS) analysis.

Table 2: Forensic parameters on 7 different levels in three ethnic groups

Table 3: Frequencies of the null allele at DYS448 in various ethnic groups across continents

Electronic Supplementary Materials (ESM):

Supplementary Figure 1: Heatmap generated using *Rst* and *Fst* values.

Supplementary Table 1: Raw genotypic data of 3 ethnic groups typed with Yfiler plus

Supplementary Table 2: Allele Frequencies and Forensic Parameters 3 ethnic groups

Supplementary Table 3: Pairwise *Rst* and *Fst* values between 3 ethnic groups and other reference populations

Supplementary Table 4: F-statistics analysis between Hazara population from Pakistan and Afghanistan

Supplementary Table 5: Sequence in the relevant flanking and repeat region of the DYS448 locus for null alleles.

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- 1 **Table 1:** Reference Populations from Central, Eastern and South Asia populations selected as
- 2 reference populations used in LDA, NJ tree and multidimensional scaling (MDS) analysis.

No	Population	Haplotypes	Country
1	Afghan	152	Afghanistan ⁶⁴
2	Pathan	125	Afghanistan ⁶⁵
3	Pathan	44	North Afghanistan ⁶⁶
4	Pathan	142	South Afghanistan ⁶⁶
5	Farsi, Arab	35	Ahvaz, Iran (accession no YA004581)
6	Farsi, Azerbaijani	34	Hamedan, Iran (accession no YA004582)
7	Han	934	Beijing, China ^{67,68}
8	Kazakh	231	Xinjiang, China ^{69,70}
9	Mongol	272	Hulun Buir, China ⁷¹
10	Mongol	454	Inner Mongolia, China ⁷²⁻⁷⁴
11	Uighur	732	Xinjiang, China ⁷⁵
12	Arab	33	Ahvaz, Iran ⁷⁶
13	Azerbaijani	39	Tabriz, Iran (accession no YA004586)
14	Azerbaijani	50	Urmia, Iran (accession no YA004587)
15	Bakhtiari	45	Izeh, Iran ⁷⁶
16	Baloch	19	Balochistan, Iran (accession no YA003794)
17	Baloch	59	Zahedan, Iran (accession no YA004238)
18	Farsi	286	Tehran, Iran (accession no YA004580)
19	Gilak	98	Gilan, Iran ⁷⁷
20	Gilaki	42	Rasht, Iran ⁷⁶
21	Iranian	27	Birjand, Iran (accession no YA003902)
22	Iranian	152	Central Iran, Iran (accession no YA003782)
23	Iranian	106	Fars, Iran (accession no YA004229)
24	Iranian	106	Golestan, Iran ⁷⁸
25	Iranian	94	Iran (accession no YA004237)
26	Iranian	161	Isfahan, Iran ⁷⁹
27	Iranian	127	Mashhad, Iran (accession no YA003903)
28	Kurd	51	Iran (accession no YA004244)
29	Kurdish	77	Kermanshah, Iran (accession no YA004584)
30	Kurdish	73	Kurdistan, Iran (accession nos YA003795 and YA004585)
31	Lor	37	Lorestan, Iran (accession nos YA003796 and YA004243)
32	Lurs	9	Kohgiluyeh-Buyer Ahmad, Iran (accession no YA003797)
33	Mazandarani	44	Sari, Iran ⁷⁶

34	Mazani	126	Mazan daran, Iran ⁷⁷
35	Parsee	17	Fars, Iran (accession no YA003798)
36	Qashqae	15	Fars, Iran (accession no YA003799)
37	Sistani	64	Zabol, Iran (accession no YA004241)
38	Talysh	15	Masal, IranSouth ⁷⁶
39	Turk	11	Ardabil, Iran (accession no YA004240)
40	Zoroastrian	6	Yazd, Iran (accession no YA003800)
41	Luri	60	Ilam, Iran Kurdish (accession no YA004583)
42	Arain	85	Punjab, Pakistan ⁸⁰
43	Baloch	98	Balochistan, Pakistan (accession no YA004595)
44	Gujjar	20	Swat and Dir District, Pakistan ⁸¹
45	Hazara	160	Balochistan, Pakistan ²⁷
46	Kashmiri	175	Azad Kashmir, Pakistan ⁸²
47	Kohistani	20	Swat and Dir District, Pakistan ⁸¹
48	Pathan	269	Pakistan ⁸³
49	Punjabi	383	Punjab, Pakistan ³
50	Roma	278	Punjab, Pakistan (accession no YA004554)
51	Saraiki	51	Southern Punjab, Pakistan (accession no YA004225)
52	Saraki	148	Punjab, Pakistan ⁸⁴
53	Sindhi	98	Sindh, Pakistan ⁸⁵
54	Yousafzai Pathan	71	KhyberPakhtunkhwa, Pakistan (accession no YA003748)
55	Tharklani, Pashtun	20	Swat and Dir District, Pakistan ⁸¹
56	Uthmankheil, Pashtun	20	Swat and Dir District, Pakistan ⁸¹
57	Yousafzai, Pashtun	20	Swat and Dir District, Pakistan ⁸¹
58	Urdu, Punjabi	241	Punjab, Pakistan (accession no YA004381)
59	Kazakh	305	Kazakhstan ⁸⁶
60	Kazakh	67	East Kazakhstan, Kazakhstan (accession no YA003700)
61	Kazakh	99	South Kazakhstan, Kazakhstan (accession no YA003729)
62	Southwest Asian	493	United Kingdom ⁸⁷
63	British Pakistani	132	United Kingdom ⁸⁸
Total haplotypes		8457	

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1 **Table 2:** Forensic parameters on 7 different levels in three ethnic groups

Hazara Pakistan	MHT 9 Y-STRs	EHT 11 Y-STRs	PPY-12 Y-STRs	Yfiler 17 Y-STRs	PPY23 21 Y-STRs	Yfiler plus 27 Y-STRs	6 RM Y STRs
No of Samples	153	153	153	153	153	153	153
RMP	0.0745	0.0577	0.0577	0.0123	0.0084	0.0066	0.0091
HD	0.9316	0.9485	0.9485	0.9942	0.9981	0.9999	0.9974
No of haplotypes	72	81	81	117	140	152	139
NUH	54	63	63	97	132	151	131
DC	0.4705	0.5294	0.5294	0.7647	0.9150	0.9934	0.9084
% of Unique Haplotypes	0.3529	0.4176	0.4176	0.6339	0.8627	0.9869	0.8562
Hazara Afghanistan							
No of Samples	260	260	260	260	260	260	260
RMP	0.0329	0.0285	0.0272	0.0184	0.0129	0.0057	0.0101
HD	0.9708	0.9753	0.9765	0.9854	0.9909	0.9982	0.9937
No of haplotypes	107	122	124	166	190	230	188
NUH	64	81	83	132	157	207	152
DC	0.4115	0.4692	0.4769	0.6384	0.7307	0.8846	0.723
% of Unique haplotypes	0.2461	0.3115	0.3192	0.5076	0.6038	0.7961	0.5846
Pak-Afg Hazara							
No of Samples	413	413	413	413	413	413	413
RMP	0.0334	0.0268	0.0262	0.0113	0.007	0.0032	0.0058
HD	0.9689	0.9756	0.9761	0.9911	0.9954	0.9992	0.9966
No. of Haplotypes	166	191	193	273	317	380	320
NUH	109	137	139	223	274	357	274
DC	0.4019	0.4624	0.4673	0.661	0.7675	0.92	0.7748
% of Unique Haplotypes	0.2639	0.3317	0.3365	0.5399	0.6634	0.8644	0.6634
Baloch Pakistan							
No of Samples	111	111	111	111	111	111	111
RMP	0.0162	0.0136	0.0136	0.0095	0.0092	0.009	0.0114
HD	0.9928	0.9954	0.9954	0.9995	0.9998	1	0.9975
No of haplotypes	97	100	100	108	110	111	101
NUH	93	96	96	105	109	111	95
DC	0.8738	0.9009	0.9009	0.9729	0.9909	1	0.9099
% of Unique haplotypes	0.83783	0.8648	0.8648	0.9459	0.9819	1	0.8558

2 RMP= Random Matching Probability ; HD= Haplotype Diversity ; NUH= No. of unique
3 haplotypes; DC = Discrimination Capacity

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5

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Table 3: Frequencies of the null allele at DYS448 in various ethnic groups across continents

Continent	Population	Number of samples	No of del	%	Reference
Asia	Hazara (Pak & Afg)	413	55	13.31%	Current Study
	Korean	708	6	0.85%	Park et al ⁴⁹
	Kalmykia	99	7	7.07%	Roewer et al ⁵²
	Japan	1079	10	0.92%	Mizuno et al ⁸⁹
	Malaysia	980	3	0.30%	Chang et al ⁴⁸
	Nepal	769	3	0.39%	Parkin et al ⁵⁰
	Tajikistan	124	3	2.41%	Balaresque et al ²⁶
	Kyrgyzstan	87	9	10.34	Balaresque et al ²⁶
	China	130	3	2.30%	Balaresque et al ²⁶
	Asian	330	2	0.61%	AmpFISTR Yfiler™ database
Europe	Spain	247	1	0.40%	Sanchez et al ⁵³
Africa	Egypt	208	1	0.48%	Balaresque et al ²⁶
Americas	Mexico	326	1	0.30%	Gutierrez-Alarcon et al ⁹⁰
	African American	985	2	0.20%	AmpFISTR Yfiler™ database
	Caucasian (USA)	1276	2	0.16%	AmpFISTR Yfiler™ database
		7761	109	1.40%

Linear Discriminant Analysis