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# High Y-chromosomal differentiation among ethnic groups in Dir and Swat districts, Pakistan

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**Summary**

The ethnic groups that inhabit the mountainous Dir and Swat districts of northern Pakistan are marked by high levels of cultural and phenotypic diversity. To obtain knowledge of the genetic diversity in this region, we investigated the Y-chromosomal diversity in five population samples representing the three main ethnic groups residing within these districts, including Gujar, Pashtun and Kohistani. A total of 27 Y-chromosomal short tandem repeats (Y-STRs) and 331 Y-chromosomal single nucleotide polymorphisms (Y-SNPs) were investigated. In the Y-STRs we observed very high and significant levels of genetic differentiation in nine of the 10 pairwise between-group comparisons ($R_{ST}$ 0.179 - 0.746) and the differences were mirrored in the Y-haplogroup frequency distribution. No genetic differences were found between the two Pashtun sub-ethnic groups Tarklani and Yusafzai ($R_{ST}$ = 0.000).Utmankhels, also considered Pashtuns culturally, were not closely related to any of the other population samples ($R_{ST}$ 0.451 - 0.746). Thus, our findings provide examples of both associations and dissociations between cultural and genetic legacies. When analyzed within a larger continental-scale context, these five ethnic groups tribes fall mostly outside the previously characterized Y-chromosomal gene pools of the Indo-Pakistani sub-continent. Male founder effects, coupled with culturally and topographically based constraints upon marriage and movement, is likely responsible for the high degree of genetic structure in this region.

## Introduction

Pakistan is home to over 180 million people and at least 18 ethnic groups who speak more than 60 different local languages assigned to a wide array of linguistic stocks, including, but not limited to Indo-Iranian, Indo-Aryan, Tibeto-Burman, and Dravidian (Grimes & Grimes, 2000, Newcomb, 1986). Geographically, Pakistan is situated at the crossroad linking Western and Central Asia to South Asia. Historically, Pakistanwas rpart of the British Indian Empirewhich, following the independence in 1947, was subdivided into the independent countries and kingdoms that today makes up the Indo-Pakistani sub-continent.

Despite being a country inhabited by a population of considerable ethnic diversity, the genetic legacy of many of the ethnic groups in Pakistan has remained largely unstudied. For example, the gene pools of the ethnic groups residing in the mountainous terrain ofnorthern Pakistan and northeastern Afghanistan (Fig. 1) remain poorly understood. The ethnic and cultural diversity in this geographic region has been attributed to a dynamic history of repeated invasions by Aryans (Bernhard, 1983, Parpola, 1995, Parpola, 2009), Indo-Iranians (Jettmar, 1967, Jettmar, 1996), Macedonians (Birdwood, 1959), Arabs, and Mongols (Lapidus, 2002). It is also believed that the southern coast of the Persian Gulf, the Makran Coast of Pakistan, and the territory of present-day Afghanistan likely served as passages for human dispersal in prehistoric times (Derenko et al., 2013), thereby providing a deep temporal dimension to the population dynamics within the region. Furthermore, the Hindu Kush, Hindu Raj, Karakoram and Himalayan highlands are believed to have served as physical barriers that channeled causeways of trade and communication along the Silk Route that linked the Mediterranean Basin and West Asia to Central Asia, South Asia and China for more than 16 centuries (Quintana-Murci et al., 1999, Petraglia et al., 2012, Vadime, 2001, Kuz′mina & Mair, 2008,

Hemphill & Mallory, 2004). It is therefore possible that the extant populations of the Hindu Kush and Hindu Raj highlands conserve traces of historic, and possibly even prehistoric, gene flow from geographically distant human populations (Hemphill, 2009, Hemphill, 2013a, Hemphill et al., 2013, Hemphill, 2013b).

Dir and Swat districts are located within the Khyber Pakhtunkhwa Province of northernPakistan (Fig. 1). Both districts are divided into southern (or "lower") and northern (or "upper") regions, with the former including the foothills between the northern reaches of the Indus Valley to the south and the latter including the Hindu Raj range of the greater Hindu Kush (Fig. 1). Altogether, Dir and Swat districts encompass a total of 5,284 and 6,226 $km^2$, respectively (Ali & Qaiser, 1986, Ahmad & Sirajuddin, 1996, Hazrat et al., 2007). The major ethnic groups found in Dir and Swat districts are: (i) Pashtuns (also known as Pathans), a Pashto speaking (Eastern Iranian language) agriculturist ethnic group consisting of four widely recognized patrilineally-based social groups (Bettani, Ghurghakhti, Karlani and Sarbani) which can be further subdivided into a number of sub-tribes known as khels or zais (Table S1) (Nüsser & Dickoré, 2002, Coningham & Young, 2015, Böhner & Lucarini, 2015, Caroe, 1992, Khan, 2008); (ii) Gujars, who speak Gojri (a lowland Indo-Aryan language) an agro- pastoral group with widespread clans residing in all parts of both districts who speak Gujari (a lowland Indo-Aryan language), and and (iii) Kohistanis, speakers of an array of Dardic languages, who practice a wide range of agricultural and transhumant herding subsistence strategies (Barth, 1956, Bangash, 2012). The Kohistanis are commonly thought to be descendants of ancient nomadic herders who were forced into the mountainous highlands from the low-lying fertile plains by Pashtun-speaking agriculturalists from the west during the 16$^{th}$ century (Barth, 1956, Rome, 2008, Shah, 2013). According to Barth (1956), there is little reported intermarriage between

Pashtuns, Gujars and Kohistanis because they tend to live in isolation and discourage intermarriages with members of other ethnic groups. As a result, previous researchers have described the local populations of these ethnic groups as genetically isolated and marked by high levels of inbreeding (Caroe, 1992, Glatzer, 2002, Mehdi et al., 1999, Siddique, 2014). However, these studies have not studied the genetic relationships among the populations residing within and immediately adjacent to the Hindu Raj highlands in any detail.

Analyses of genetic variants of the human Y-chromosome are useful for inferring patterns of current and past gene flow between human populations (reviewed in Oppenheimer, 2012). Due to the exclusively paternal non recombined inheritance pattern of the human Y-chromosome, the paternal line is easily traced using Y-chromosomal genetic variants, such as short tandem repeats (Y-STRs) and single nucleotide polymorphisms (Y-SNPs) (Oppenheimer, 2012). Y-STR analyses can be used to resolve the genetic relationship and paternal gene flow between current human populations, whereas the slower mutating Y-SNPs can provide information on more ancient prehistoric or historic demographic events (Karafet et al., 2008, Roewer, 2009, Larmuseau et al., 2015).

In this study we present information on 27 Y-STR and 331 Y-SNP loci for five ethnically distinct groups from Dir and Swat districts in Pakistan. We apply a series of genetic analyses in order to investigate the genetic relationships among these groups. The ethnic groups included in this study are characterized by having different lifestyles; low elevation valley agriculturists (Pashtuns), mountainous nomadic herders (Gujars), and transhumant herders (Kohistanis). Gujar and Kohistani individuals, as well as members of the three patrilineally-based Pashtun subethnic groups (Tarklanis, Utmankhels and Yusafzais), were sampled (see Table S1 for ethnic divisions among

Pashtuns/Pathans). We characterize Y-STR genetic diversity within and among these ethnically distinct groups, thereby uncovering a relatively unexplored part of the modern human gene pool. Although some of the major population groups from this region are included in the Human Genome Diversity Panel (HGDP) and have therefore been included in worldwide genetic studies (e.g., Shi et al., 2010, Cann et al., 2002), few studies have looked at the micro-geographic patterns of genetic diversity within population groups of northwestern Pakistan. We investigate whether current information about common history, culture, and language is reflected in the genetic relationships among the populations residing within or adjacent to the Hindu Raj highlands. As such, our data offers an excellent opportunity to test the nature and extent of the relationship between genetic and cultural affinity. We hypothesize that ethnicity has exerted a greater effect on the genetic associations present among the human populations residing within Dir and Swat districts than simple geographic propinquity.

## Materials and Methods

### Sampling and DNA extraction

A total of 100 saliva samples from males of five ethnically distinct population samples (Tarklanis, Yusafzais, Kohistanis, Gujars, and Utmankhels) were collected from individuals residing in Swat and Dir districts of northern Pakistan (Fig. 1). Members of three of these population samples (Tarklanis, Yusafzais, and Utmankhels) are commonlyrecognized as patrilineally-based sub-groups within Pashtuns (Pathans) ethnic group. Ethnicity was self-declared and all participants gave their informed written consent after the aims and procedures of the study were explained to them. Great care was taken to avoid sampling related individuals. First and foremost, 4-5 visits to the communities were initially undertaken to carefully select individuals and

record their names and ethnic relationships. At the day of sampling all the volunteers were informed to meet at their hujra (meeting place) under the guidance of a malak (a local counsellor among the elders). Before sampling, the elders and the volunteers were again interviewed to exclude closely related individuals, especially first degree paternal relatives.

Genomic DNA was isolated using a modified phenol:chloroform method as previously described (Ralser et al., 2006) and DNA concentrations were determined on a Qubit flourometer (Invitrogen, life technology, cat. Number Q32857) using the Qubit dsDNA HR Assay Kit (Invitrogen, cat. Number Q32854).

Y-STR and Y-SNP datasets

A total of 27 Y-STR loci were amplified with the Yfiler®Plus PCR Amplification kit (ThermoFisher Scientific, Cat. No. 4484678) and PCR products were separated and evaluated according to manufacturer's protocols with the modifications described by Olofsson et al. (2015a). All samples were genotyped in duplicates in the ISO17025-certified forensic genetics laboratory at the Department of Forensic Medicine, Section of Forensic Genetics, University of Copenhagen, Denmark, and concordant results were obtained between the first and second typing of all the samples. All haplotypes were reported to the Y-chromosomal haplotype reference database (YHRD) (Willuweit & Roewer, 2015) under the accession numbers YA004265 to YA004269 and are presented in Table S2.

Initial assignment of Y-chromosomal haplogroups was carried out using genotypes of Y-SNPs included on the Infinium®OmniExpressExome-8 v.1.3 BeadChip array. A total of 1,641 Y-SNPs are included on the array, of which 1,226 passed genotyping filters

(call rate ≥ 90%) among the individuals included in the study. The Y-SNPs that passed the genotyping filters were intersected with the ISOGG Y-DNA SNP index (http://isogg.org/tree/index.html, version 10.103), resulting in a final set of 331 haplogroup-defining Y-SNPs. Individual haplogroups were assigned as the most derived haplogroup where the individual's genotype matched the derived allele. The shorthand version of the ISOGG nomenclature was used, where the main haplogroup, or sub-haplogroup, is followed by the most derived Y-SNP to which the Y-chromosome could be typed (Table S3). Markers in parenthesis followed by an "x" indicate downstream markers for which the samples were typed but were found to be in the ancestral state.

Analyses

Population genetic parameters were estimated for the five ethnically distinct Pakistani population samples and for the meta-population of Dir and Swat districts, combining all of the individuals included in the study,using a framework previously described (Olofsson et al., 2015a). Genetic distances between population samples were evaluated as pairwise $R_{ST}$ distances calculated in Arlequin v. 3.5.1.2 (10,000 permutations; Excoffier & Lischer, 2010) and visualized through nonmetric multidimensional scaling (MDS) in the statistical software R v. 3.2.1 using the isoMDS function of the MASS package. Median joining networks of haplotypes were constructed in the program Network v. 5.0.0.0 (http://www.fluxus-engineering.com) and weights (1-5) were given to the included loci based on the inverted diversities (1: DYS449, DYS458, DYS481, DYS518, DYS576, DYS627; 2:DYS19, DYS389B, DYS390, DYS392, DYS393, DYS437, DYS448, DYS533, DYS570, DYS635; 3:DYS438, DYS439, DYS456; 4: DYS389I; 5: DYS391, DYS460, YGATAH4)(Olofsson et al., 2015a). The multi-copy loci in this kit, DYS385 and DYF387S1 were excluded for estimations of genetic

distances ($R_{ST}$) and construction of median joining networks as is common practice, resulting in 23 Y-STRs for these analyses. Furthermore, individuals with haplotypes displaying duplication events, null or intermediate alleles were excluded in the network analyses but for genetic distances null and intermediate alleles were counted as missing data. As is standard for Y-STR analyses, the alleles of the DYS389II locus were converted to the DYS389B nomenclature by subtracting the repeat number of DYS389I from that of DYS389II.

To place the diversity observed for the patrilineal gene pool of Swat and Dir districts in a greater geographic and ethnic context we constructed two datasets based upon previously published Y-STR data. One dataset encompassed 27 population samples (including the five from this study) from the Indian sub-continent and Southwest Asia (Roewer et al., 2009, Haber et al., 2012, Perveen et al., 2014, Lee et al., 2014, Qamar et al., 2002, Tabassum et al., 2017) (Table S4). The other dataset encompassed 53 worldwide population samples (including the five from this study and the Yusafzais population from Tabassum et al., 2017)from the HGDP panel (Cann et al., 2002, Rosenberg, 2006, Vermeulen et al., 2009), with the criterion that the populatio was represented by at least five males genotyped for both Y-STRs and Y-SNPs (Table S4). Tobe able to merge the different data sets, the data were limited to 15(DYS19, DYS389I, DYS389B, DYS390, DYS391, DYS392, DYS393, DYS437, DYS438, DYS439, DYS448, DYS456, DYS458, DYS635, and YGATAH4) of the 23Y-STRsloci. Only studies and loci typed with the commercial kits AmpFLSTR®Yfiler®PCR Amplification kit or Yfiler®Plus PCR Amplification kit (both ThermoFisher Scientific) were included in the comparisons (Table S4). This conservative approach ensures that there is no difference in nomenclature of the alleles between the different studies. $R_{ST}$ values between all groups were calculated in Arlequin

v. 3.5.1.2. The same package was used for the analyses of molecular variance (AMOVA) between all groups and between groupings based on country of origin and reported ethnicity (Table S5, S6). The text associated with Table S5 outlines the rationale behind these ethnicity aggregates. MDS plots were constructed based on the pairwise $R_{ST}$ genetic distance matrix with R software package v. 3.2.1 as described above.

## Results

Genetic diversity

The 100 individuals in this study self-identify as members of one of three major ethnic groups: Pashtuns (Pathans), Kohistanis, or Gujars. Pashtuns are further represented by individuals from three widely recognized patrilineally-based divisions, Tarklanis, Utmankhels, and Yusafzais (Table 1). Analyses of the 27 Y-STRloci resulted in the identification of a total of 82 haplotypes of which 75 were unique (Table 1). The percentage of unique haplotypes within each of the five population samples varied from 100% (20 out of 20) among Kohistanis to 45% (9 out of 20) among Utmankhels (Table 1). Seven haplotypes were shared between two to six individuals within the meta-population of Dir and Swat district and all but two haplotypes were population-specific (Table 1). The non-population-specific haplotypes were shared between four and five individuals within the meta-population of Dir and Swat districts, respectively. These include a haplotype shared by three Yusafzai individuals and one Tarklani individual and a haplotype shared by four Gujars and one Kohistani individual. As a result of the differences in unique haplotype frequencies, haplotype diversity also varied between population samples, ranging from 1.00 among Kohistanis to 0.93 among Utmankhels (Table 1). The overall power of discrimination was relatively high (0.82) for the

combined dataset but varied widely low (0.60) in Utmankhels to high (1.00) in Kohistanis, when the five ethnic groups were considered separate populations (Table 1).

Information on Y-SNPs was used to assign a Y-chromosomal haplogroup (Karafet et al., 2008, Larmuseau et al., 2015)to each individual. A relatively large number of haplogroups was observed(Table 1, Table S2, S3) and the spectrum of these haplogroups was consistent with previous studies (Qamar et al., 2002, Karafet et al., 2008, Sengupta et al., 2006, Lee et al., 2014, Kivisild et al., 2003, Zhao et al., 2009, Chennakrishnaiah et al., 2013). However, 85% of the studied individuals carry one of four haplogroups (H1-M69, G2b-M283, L1-M22(xM274), and R1a-M417,Page7) and there are large differences in the frequencies of these four haplogroups between the five population samples (Table 1, Table S2, S3). For example, haplogroup G2b-M283 occurs with very high frequency (0.80; 0.77-0.83) among Utmankhels, but is completely absent among members of three out of the other four population samples (Table 1, Table S2, S3). In contrast, haplogroup R1a-M417,Page7 occurs among members of all five population samples but frequencies range from high (0.80; 0.77-0.83) among Yusafzais and Tarklanis to low (0.10; 0.07-0.13) among Utmankhels (Table 1). Due to small sample sizes the 90% confidence intervals are relatively large and overlap for some haplogroups (Table 1).

Genetic differentiation

The genetic distances between the five groups, as estimated from the Y-STR markers using pairwise $R_{ST}$, are mostly very large and highly significant, ranging from 0.179 to 0.746, except for the pairwise comparison between Tarklanis andYusafzais (Table 2, Fig. S1). Despite being considered different ethnic subgroups of Pashtuns, members of

these two groups are not significantly different from each other genetically ($R_{ST}$ = 0, p = 0.604).

The genetic structure is also evident in the median joining network of Y-STR haplotypes (Fig. 2, Table 1). Members of the Tarklani and Yusafzai subgroups of Pashtuns are mostly found together, being separated by only a few mutational steps (Fig. 2). This is in contrast to the Utmankhels and Gujars, who, with the exception of some outliers, form distinct groups separated by a large number of mutational steps from the other groups. There are no shared haplotypes within the Kohistani group; hence they appear more scattered in the network. Nevertheless, the majority of haplotypes are still found close together in relative proximity to the Tarklani/Yusafzai aggregate(Fig. 2).

Genetics, ethnicity and geography

To examine the genetic variation in a broader context we included population samples from a wider geographic range. We used15 Y-STR loci for a worldwide data set and for a data set representing the Indian sub-continent and Southwest Asia (Table S4). The results are summarized in: (i) matrices of pairwise $R_{ST}$ values for both datasets (Table S7), (ii) a pair of AMOVA analyses for the 27 Indo-Pakistani sub-continent and Southwest Asia population samples (Tables S5, S6), and (iii) MDS plots for both datasets (Fig. 3 and Fig. S2). In the AMOVA analysis, c. 92% of the genetic variation occurs within the 27 population samples from the Indo-Pakistani sub-continent and Southwest Asia. When grouping these population samples by country of origin (Table S5A), the genetic variation among the countries accounts for only 2.2%, whereas 5.6% of the total variation is explained by difference between population samples within countries. However, when the 27 samples are instead grouped by ethnic relationships,

differences between the ethnic groups account for 4.1% of the total variation, while the variation between population samples within the ethnic groups accounts for 3.4% of the total variation (Table S5B). For the grouping based on ethnicity, the Utmankhels were treated as a separate group due to its profound genetic differences to all other population groups included in the AMOVA analyses. When the Utmankhels were instead considered part of the Pashtun/Pathan ethnic group, the variation among populations within ethnic groups increased to close to 5% (Table S6) indicating that this particular population accounts for a large amount of the between population variation.

Despite the inclusion of 27 population samples from the Indo-Pakistani sub-continent and Southwest Asia, most of the genetic variation in the MDS is still defined by the five population samples from Dir and Swat districts (Fig. 3). In this data set, limited to 15 STR loci, there are still large genetic differences between the samples from Dir and Swat districts (Table S7, Fig. S1, S2, and S3), but the reduction resolution implies that the differentiations between Gujars and Kohistanis and between Yusafzais and Kohistanis become non-significant (Table S7).

Several specific observations can be made. The Gujar sample and the Baluch (Balochi) ethnic groups from Afghanistan (Haber et al., 2012) are both outliers and occupy the same area in the MDS plot (Fig. 3), whereas the Baluch (Balochi) sample from Pakistan (Cann et al., 2002, Rosenberg, 2006, Vermeulen et al., 2009) occupies a more central position. However, the genetic distances between these samples are non-significant after correction for multiple testing. The Kohistanis occupy a more central position within the MDS plot adjacent to a large number of other sampled ethnic groups from the Indo-Pakistani subcontinent and Southwest Asia. Noticeably, the Utmankhel sample is separated by very large and highly significant genetic distances from all other groups

(Table S7), and within the MDS plots (Fig. 3 and S2) this sample occupies an isolated position. The Tarklanis and Yusafzais are marked by very similar genetic distances to the other groups included in this analysis (Table S7, Fig. 3). These results are generally mirrored when the MDS is constructed from the worldwide data set (Fig. S2, Table S7).Surprisingly, the three sub-ethnic groups of the Pashtuns sampled from Dir and Swat (Tarklanis, Utmankhels, Yusufzais) still represent outliers, observed far outside most of the known Y-STR genetic diversity in Indo-Pakistani sub-continent and Southwest Asia (Fig. 3,S2, and Table S7).

Detailed analysis of two Y-chromosomal haplogroups

To get a more detailed picture of the relationship between the five population samples from Dir and Swat districts we constructed haplotype (15 Y-STR loci) networks for individuals assigned to Y-SNP haplogroups (i) G-Page94 [(G2a-L30(xL14, L13,M278) and G2b-M283)], (ii) H1-M69, and (iii) L1-M22(xM274), and included previously published datasets from Pakistan and Afghanistan (Haber et al., 2012, Vermeulen et al., 2009)(Fig. 4). Most of the Utmankhels possess haplogroup G-Page94 (G2b-M283, more specifically) and they all cluster closely together (owing to highly similar Y-STR profiles) and with a couple of individuals from both Afghanistan and Pakistan (Fig. 4A). Only one Kohistani and one Gujar individual have a Y-SNP profile assigned to the G-Page94 haplogroup, and these two individuals share the same Y-STR haplotype, which is clearly separated from the haplotypes observed among the sampled Utmankhel individuals (Fig. 4A).

The Y-STR network with individuals assigned to SNP-haplogroup H1-M69 is more diffuse and many individuals are separated by a larger number of mutational steps. However, most Kohistanis are found within this network, and many cluster together, sharing the same Y-STR haplotype (Fig. 4B). The network of STR-haplotypes of

individuals assigned to SNP-haplogroup L1-M22(xM274) shows at least two defined groups (Fig. 4C). All but one Gujar individual in this network share the same Y-STR haplotype, which is also shared by a single Kohistani individual (even when extended to the full 27 Y-STR loci haplotype; Table 1 and Fig. 2). Only a single Gujar individual is found in the other sub-group within the network.

## Discussion

Genetic diversity and differentiation in Dir and Swat

Our analyses of patrilineal genetic diversity among males of the five ethnic groups from Dir and Swat districts of Pakistan have yielded several insights. First, the level of Y-STR haplotype diversity within each ethnic group is generally high and comparable to average global values (Purps et al., 2014), except for the Utmankhel sample, which displays less diversity and fewer unique haplotypes (Table 1). Second, the five groups display an extreme level of genetic differentiation, both among themselves (Table 2, Fig. S1) and in relation to other groups from this geographic region (Fig. 3, Table S7). Based on the 23 single-copy Y-STR loci, the average $R_{ST}$ between these five ethnic groups is very high (0.38, Table 2), with an extreme $R_{ST}$ of 0.75 observed between Tarklanis and Utmankhels (Table 2). The middle range $R_{ST}$ values (e.g., 0.1-0.2) found between some of the ethnic groups (Gujar – Kohistani, Tarklani – Kohistani, Yusafzai – Kohistani) are comparable to genetic distances reported previously between population groups from the Indo-Pakistani sub-continent (Alam et al., 2010, Seema Nair et al., 2011, Perveen et al., 2014) and the Middle East (Triki-Fendri et al., 2015). It is intriguing that Kohistanis represent the common denominator in these middle range values, for they likely represent the indigenous population of the region with the other likely representing more recent immigrants (Barth, 1956); Tarklanis and Yusafzais

occupying the low-lying regions of southern Dir and Swat and Gujars the rugged higher-altitude Upper Swat. The extreme genetic distances we observe ($R_{ST}$> 0.4) in several of the pairwise comparisons (Gujars –Tarklanis, and the Utmankhels compared to any of the other population samples) are unusual and higher than observed between most human populations - even when occupying different continents (e.g., Purps et al., 2014). The very large genetic distances result from a number of non-overlapping, fixed, or almost fixed alleles in the five population samples (Table S2). For example in the three Pashtun population samples allele 11 is almost completely fixed for the DYS392 locus whereas a large number of alleles are found in the Gujars and the Kohistanis. Similarly the Utmankhels have allele distributions that are skewed from the mean of the whole dataset, for example showing an almost complete fixation alleles in DYS448, DYS458 and DYS635. Small sample sizes can inflate the genetic distances and with just 20 sampled individuals from each group, the $R_{ST}$ values should be interpreted with caution. However, we note that such extreme genetic distances have been observed previously between other ethnic groups living in relative geographic proximity (Zeng et al., 2014), when they have experienced prolonged and severe genetic isolation coupled with long-standing endogamy (Zeng et al., 2014, Roewer et al., 2013, Gaikwad et al., 2006). As such, it is perhaps not unexpected to observe large genetic distances between the ethnic groups of Swat and Dir districts given their isolated residential localities, their cultural preferences for endogamous marriages, as well as their differences in subsistence practices, lifestyles, and language (Barth, 1956). The high differentiation could be an effect of male founder effects (see below) and might not be mirrored in genome-wide autosomal data, but further studies are needed to clarify this. Nevertheless, our results indicate that isolated lifestyles and cultural preferences can have a very large impact on genetic distances between geographically proximate populations.

The genetic distinction between members of these ethnic groups is further underscored by differential haplogroup frequencies (Table 1). The only haplogroup shared by members of all five population samples is R1a-M417,Page7, which is not surprising as this haplogroup occurs widely throughout the Eurasian continent, especially among populations found in Central Asia and the Indo-Pakistani sub-continent (Karafet et al., 2008, Novelletto, 2007, Rosser et al., 2000, Semino et al., 2000, Sengupta et al., 2006, Underhill et al., 2010, Underhill et al., 2015, Pamjav et al., 2012).

Genetics and ethnicity

It is widely recognized that cultural factors such as language and group associations, can sometimes play a role in forming the genetic structure among human populations, especially those found in remote areas where populations are small and isolated (Gaikwad et al., 2006, Ayub & Tyler-Smith, 2009). Our AMOVA analyses confirm that this is also the case for the Indo-Pakistani sub-continent, where 4.1% of the genetic variation is explained by ethnicity whereas only 2.3% is explained by country of origin (Table S5). Hence, members of the studied ethnic groups were found to be more similar genetically to population samples assigned to their respective ethnicity than to their country of origin (Fig. 3, Table S5).

Unlike Gujars, Kohistanis, and especially Utmankhels, the Tarklanis and Yusafzais samples cannot be differentiated from each other genetically with the 23 analyzed Y-STR markers ($R_{ST}$= 0, Table 2), and the Y-SNP data show that the majority of these individuals carry variants of haplogroup R1a-M417,Page7, that are intermingled in a loosely defined group in the network (Fig. 2). Recent studies have dissected the R1a-M417,Page7 haplogroup in greater detail (Pamjav et al., 2012, Underhill et al., 2015)

and it is reasonable to hypothesize that the Pakistani individuals from this study assigned to haplogroup R1a-M417,Page7 belong to one of the sub-haplogroups of R1a-Z95, such as R1a-Z2125, R1a-M560, or R1a-M780 (Underhill et al., 2015). Although Tarklanis and Yusafzais consider themselves to be distinct subgroups of Pashtuns (Table S1), several studies have suggested that they share many cultural and linguistic characteristics (Caroe, 1992, Khan, 2008), which is clearly mirrored in our genetic data. In this particular case, our results suggest that both historic and current gene flow between members of these sub-groups (i.e., patrilineal clans) prevails despite their residence within remote areas of the Hindu Kush-Hindu Raj highlands. However, a large pool of shared common Y-chromosomal ancestry could also explain the close genetic affinity between these two subgroups. With the exception of the genetic distance to the Utmankhels, neither of these two populations was significantly different from other Pashtun (Pathan) groups from Afghanistan and Pakistan after Bonferroni correction for multiple comparisons(Fig. 3 and Table S7).

Utmankhels also consider themselves to be Pashtuns (Table S1), but with $R_{ST}$ distances of 0.45-0.75 (23 loci) to the other four population samples from Dir and Swat districts (Table 2) and 0.24-0.67 (15 loci) to populations from the Indo-Pakistani sub-continent and Southwest Asia (Table S7), they are genetically different from any other sample from this geographic region included in this study (see also Fig. 3). This is also reflected in the haplogroup networks where most Utmankhels form a very distinct cluster within haplogroup G-Page94 (Figs. 2, 4, Table 1). This haplogroup is common among ethnic groups residing in the Caucasus but it is also found in medium to low frequencies among ethnic groups residing in the Middle East and southern Europe (Rootsi et al., 2012, Kivisild et al., 2003). As such, the Utmankhels may be considered a genetic outlier within the Indo-Pakistani sub-continent (Fig. S2), at least with regard to the Y-

chromosome. Such results suggest that they either have a different genetic origin than members of the other Pashtun sub-groups included in this analysis or that the Utmankhel male lineage has been subjected to severe genetic drift, perhaps due to a male founder effect or genetic bottleneck followed by isolation. The latter scenario is consistent with the lower genetic diversity observed among Utmankhels relative to that seen among members of the other sampled groups from Dir and Swat districts (Table 1). These results are intriguing given the oral tradition that members of the current Utmankhel clan are all descendants of a single adopted son of unknown origin (Barfield, 2010, Caroe, 1992). This could explain the apparent genetic isolation of the Utmankhel male lineage, although the presence of other Y-SNP haplogroups in the population sample (Table 1) indicates that least some male-mediated geneflow must have occurred in either ancient or recent times or that the bottleneck was not quite as dramatic as proposed (i.e. only one male). We note that our findings do not question the ethnic descriptions of the Utmankhels as a sub-ethnic group of the Pashtuns, but rather underline the fact that close cultural associations may arise without a closely shared genetic history. Interestingly the Utmankhels are not significantly different from a number of other populations from Eurasia, in particular many of the European populations included in the HGDP (Table S7), suggesting a closer affinity to population groups of Europe than to populations from the Indo-Pakistani sub-continent. Ancient connections with an European-derived gene pool could possibly explain why Utmankhels appear as a genetic outlier in the Indo-Pakistani sub-continent.

The Gujar population sample is also much differentiated genetically from the other populations residing in Swat and Dir districts but shares relatively close affinities to other populations from Pakistan and Afghanistan, in particular to the Baluch population samples from the region (Fig. 3, Table S7). This observation could support previously

suggested cultural connections, such as a shared transhumant lifestyle (Nijjar, 2008, Barth, 1956, Adamec, 2011) between Gujars and Baluchis despite linguistic differences (Grierson, 1903-1928, Morgenstierne, 1932, Strand, 1973). The high proportion of individuals sharing haplotype L1-M22(xM274) could again be the result of strong genetic drift. This haplogroup is today found in West Asia and the Indo-Pakistani sub-continent (Jobling & Tyler-Smith, 2003, Kivisild et al., 2003). The data could also indicate recent gene flow between Gujars and Kohistanis, since these share haplotypes within haplogroup H1-M69, G2a-L30(xL14, L13,M278), andL1-M22(xM274) (Table 1 and Fig. 4B). Haplogroup L1-M22(xM274)is found in low frequency among Kohistanis but is the most frequent haplogroup among Gujars and thus recent paternal gene flow from Gujars to Kohistanis can be speculated. The Gujars are more recent immigrants to Upper Swat and the opportunity for gene flow is therefore in place, but more data are needed to test this hypothesis.

In contrast to the other four ethnic groups included in this study, Kohistanis are more genetically diverse and not significantly different from any of the other population samples from the Indo-Pakistani sub-continent, with the exception of the Utmankhels when the data-set is restricted to 15 Y-STRs ( Figs.3, S2, Table S7). However, when all 23 single-copy Y-STR loci are considered they are indeed significantly different from all other population from Dir and Swat fistricts reflecting the ability of the rapidly mutating Y-STRs included in the YfilerPlus kit to differentiate between individuals to a higher degree. The exact relationships within haplogroup H1-M69 (the most frequent haplogroup within Kohistanis) between Kohistanis and members of other ethnic groups of Pakistan and Afghanistan are unclear (Fig. 4B). Our results could suggest that Kohistanis are more genetically admixed and have perhaps experienced less isolation than the other four ethnic groups from Dir and Swat districts included in the study.

## Conclusions

We have characterized the genetic diversity in paternal lineages of five ethnic groups residing in themountainousDir and Swat districts of the Khyber Pakhtunkhwa Province, in northern Pakistan. With the exception of Tarklanis and Yusafzais, we have documented very high levels of genetic differentiation of the male lineages between the groups. Such differences suggest either a lack of shared ancestry, perhaps due to several distinct ancient or historic migrations into this region, and/or bottlenecks and isolation events resulting in severe genetic drift in the local male gene pools. The Y-STR and Y-SNP data we present here do not offer sufficient resolution to investigate these scenarios further but the results provide a strong impetus to resolve the demographic history of this region with genome-scale analyses. Also, investigations of the maternal lineages via mitochondrial genomes should be highly informative as they may depict a different genetic history if dispersal and gene flow differ between males and females. Such a pattern has been described in geographic areas largely influenced by European settlers such as South America (Roewer et al., 2013, Fridman et al., 2014) and Greenland (Pereira et al., 2015, Helgason et al., 2006, Olofsson et al., 2015b) and it is very likely that the same pattern would be observed among the ethnic groups of the Dir and Swat districts given a common preference for patrilateral first cousin marriages coupled with post-marital virilocality (Donnan, 1988, Hussain & Bittles, 1998, Saadat & Tajbakhsh, 2013, Saify & Saadat, 2012, Wahab & Ahmad, 1996).

In concurrence with previous studies, we find that ethnicity provides a more accurate predictor of genetic associations than simple geographic propinquity. However, our data also illustrates a clear exception in that Utmankhels are not related to the other Pashtun groups genetically. Thus, their cultural association could either be a more recent phenomenon not explained by shared ancestry, or alternatively, that a founder event

such as a putative adoption among the Utmankhels, followed by strong genetic drift, have simply erased the genetic links but not the cultural connections.

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## Figure legends

**Figure 1.**Map of study area

Map of Pakistan with focus on Dir and Swat districts. Sampling localities for each of the five ethnic groups are indicated. Upper and lower dashed lines indicate the Hindu Kush and Hindu Raj ranges respectively.

**Figure 2.** Network analysis

Median joining network based on the Y-STR haplotypes (23 loci) of the five population samples. The circle sizes indicate the number of individuals with shared Y-STR haplotypes (smallest circles = one individual). The lengths of the connecting branches

indicate the number of mutational steps separating the haplotypes (shortest branch lengths = one mutational step).

**Figure 3.** Multidimentionsl scaling plot of regional populations

Multidimensional scaling (MDS) analysis of pairwise genetic distances, estimated as $R_{ST}$ (15 Y-STR loci), for 27 selected populations from the Indo-Pakistani sub-continent and neighbouring countries (stress = 0.1544333). See Table S4 for a detailed list of the included populations, number of individuals, and references.

**Figure 4.** Y-chromosome haplogroup-specific networks

Median joining network based on Y-STR haplotypes (15 loci) with individuals assigned to (A) Y-SNP haplogroups G-Page94, (B) H1-M69, and (C) Y-SNP haplogroup L1-M22(xM274). The circle sizes indicate the number of individuals that share the same Y-STR profile for these 15 loci. The smallest circles represent one individual. The lengths of the connecting branches indicate the number of mutational steps. The shortest branches represent one mutational step.

**Supplementary files, legends**

**Figure S1. MDS plot of Dir and Swat**

Multi-dimensional scaling (MDS) analysis of pairwise genetic distances, estimated as $R_{ST}$ (23 Y-STR loci), for the five population samples in this study (stress = 1.32472e-16).

**Figure S2: Worldwide MDS plot**

Multi-dimensional scaling (MDS) analysis of pairwise genetic distances, estimated as $R_{ST}$ (15 Y-STR loci) for a) 53 population samples (from HGDP), including the five population samples from Dir and Swat as well as the Yusafzai population from Tabassum et al 2017; b) 48 populations samples from the HGDP and the Yusafzai population from Tabassum et al 2017; c-g) 49 population samples including the samples from the HGDP, the Yusafzai sample from Tabassum et al.(2017), and one of the five population samples (as indicated in sub-figures) analyzed in this study. See Table S4 for a detailed list of the included populations, number of individuals, and references. Stress values as indicated in the separate sub-figures.

**Table S1: Ethnic divisions of Pashtuns**

Major ethnic and sub-ethnic groups of Pashtuns/Pathans residing in the Khyber Pakhtunkhwa province of Pakistan.

**Table S2: Genotype data**

Haplotypes of 27 Y-STRs amplified with the Yfiler®Plus PCR amplification kit and Y-SNP haplogroups for 100 individuals from five ethnically distinct populations of the Dir and Swat district of northern Pakistan. For the Y-STR loci intermediate alles and

duplication events are highlighted. Y-SNP haplogroup names according to International Society of Genetic Genealogy (ISOGG).

**Table S3: Y-SNP calls and haplogroups**

An overview of the Y-SNP derived mutations and haplogroup assignment for each individual.

**Table S4: Population samples**

An overview of the 68 population samples included in the larger comparative analyses. Sample sizes and references to the original studies are shown. Groups marked with [a] were used both for the regional analyses (MDS, AMOVA) and the world wide analysis (MDS). Groups marked with [b] were used only for the regional analyses (MDS, AMOVA). Groups marked with [c] were used only for the world wide MDS analysis.

**Table S5. AMOVA test + description of rationale behind ethnicity aggregates**

Analyses of molecular variance (AMOVA) when population samples are grouped based on country of origin and ethnicity, respectively.

**Table S6. Alternative AMOVA test**

Analyses of molecular variance (AMOVA) when population samples are grouped based ethnicity including Utmankhels in the Pashtun ethnic group.

**Table S7A+B. Genetic distances, $R_{ST}$**

3A) Regional $R_{ST}$ analysis of population samples from the Indian subcontinent. The genetic distances, pairwise $R_{ST}$ values, below the diagonal and the corresponding p-

values above the diagonal(15 Y-STR loci), between all populations.3B) Worldwide $R_{ST}$ analysis. The genetic distances, pairwise $R_{ST}$ values, below the diagonal and the corresponding p-values above the diagonal based on the Y-chromosomal haplotype frequencies (15 Y-STR loci), between all populations.

**Table 1:** Genetic diversity

| Number of individuals sharing a Y-STR haplotype | Sub-population | | | | | Meta-population of Dir and Swat District |
|---|---|---|---|---|---|---|
| | Kohistanis | Gujars | Yusafzais | Tarklanis | Utmankhels | |
| 1 (unique) | 20[a] | 16 | 15 | 17[d] | 9 | 75 |
| 2 | | | 1 | | 1 | 2 |
| 3 | | | 1[c] | 1 | 1 | 2 |
| 4 | | 1[b] | | | | 1[e] |
| 5 | | | | | | 1[f] |
| 6 | | | | | 1 | 1 |
| Number of haplotypes | 20 | 17 | 17 | 18 | 12 | 82 |
| Sample size | 20 | 20 | 20 | 20 | 20 | 100 |
| Frequency of unique haplotypes | 1.00 | 0.80 | 0.75 | 0.85 | 0.45 | 0.75 |
| Haplotype diversity | 1.00 | 0.98 | 0.99 | 0.99 | 0.93 | 0.99 |
| Power of discrimination | 1.00 | 0.85 | 0.85 | 0.90 | 0.60 | 0.82 |

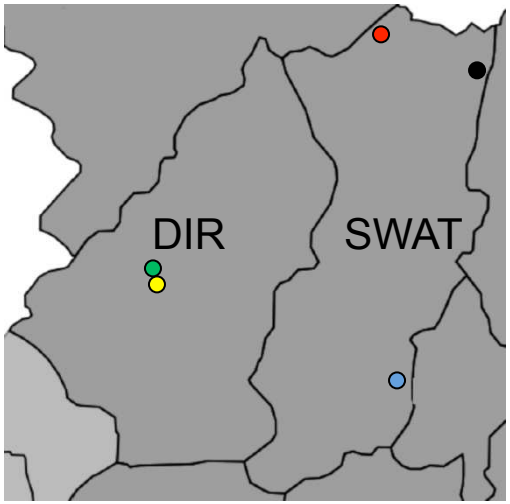
| Y-SNP haplogroup | Kohistanis | Gujars | Yusafzais | Tarklanis | Utmankhels | Combined |
|---|---|---|---|---|---|---|
| G2a-L30(xL14, L13,M278) | 1 (0.05; 0.03-0.07) | 1 (0.05; 0.03-0.07) | | | | 2 (0.02; 0.01-0.03) |
| G2b-M283 | | | | 2 (0.10; 0.07-0.13) | 16 (0.80; 0.77-0.83) | 18 (0.18; 0.17-0.19) |
| H1-M69 | 10 (0.50; 0.46-0.54) | 1 (0.05; 0.03-0.07) | | | | 11 (0.11; 0.10-0.12) |
| J2a-L25 | | | 2 (0.10; 0.07-0.13) | | | 2 (0.02; 0.01-0.03) |
| J2b-M241 | | | 1 (0.05; 0.03-0.07) | 1 (0.05; 0.03-0.07) | | 2 (0.02; 0.01-0.03) |
| L1-M22(xM274) | 1 (0.05; 0.03-0.07) | 11 (0.55; 0.51-0.59) | 1 (0.05; 0.03-0.07) | | | 13 (0.13; 0.12-0.14) |
| O2-IMS-JST0213554(xP164) | | 1 (0.05; 0.03-0.07) | | | | 1 (0.01; 0.006-0.014) |
| Q-M242(xL56, L57, L214) | 2 (0.10; 0.07-0.13) | | | | | 2 (0.02; 0.01-0.03) |
| Q-L56,L57(xL54) | | | | | 2 (0.10; 0.07-0.13) | 2 (0.02; 0.01-0.03) |
| R-M207,M734,P224,P280(xM173) | 1 (0.05; 0.03-0.07) | 2 (0.10; 0.07-0.13)) | | 1 (0.05; 0.03-0.07) | | 4 (0.04; 0.03-0.05) |
| R-M734,P224,P280(xM173) | | 1 (0.05; 0.03-0.07) | | | | 1 (0.01; 0.006-0.014) |
| R1a-M417,Page7 | 5 (0.25; 0.21-0.29) | 3 (0.15; 0.12-0.18) | 16 (0.80; 0.77-0.83) | 16 (0.80; 0.77-0.83) | 2 (0.10; 0.07-0.13) | 42 (0.42; 0.40-0.44) |

Genetic diversity in the 27 Y-STR loci and frequencies of Y-SNP hapolgroups within five ethnic groups from Dir and Swat Districts and the meta-population of Dir and Swat Districts, combining all the 100 analysed individuals in this study. The values reported for the Y-SNP haplogroups represent the observed number of individuals followed by (in brackets) the frequency, and the 90% confidence interval.
[a] One haplotype shared with four Gujar individuals; [b] Shared with one Kohistani individual; [c] Shared with one Tarklani individual; [d] One haplotype shared with three Yusafzai individuals; [e] Shared between three Yusafzai and one Tarklani individuals; [f] Shared between four Gujar and one Kohistani individuals.

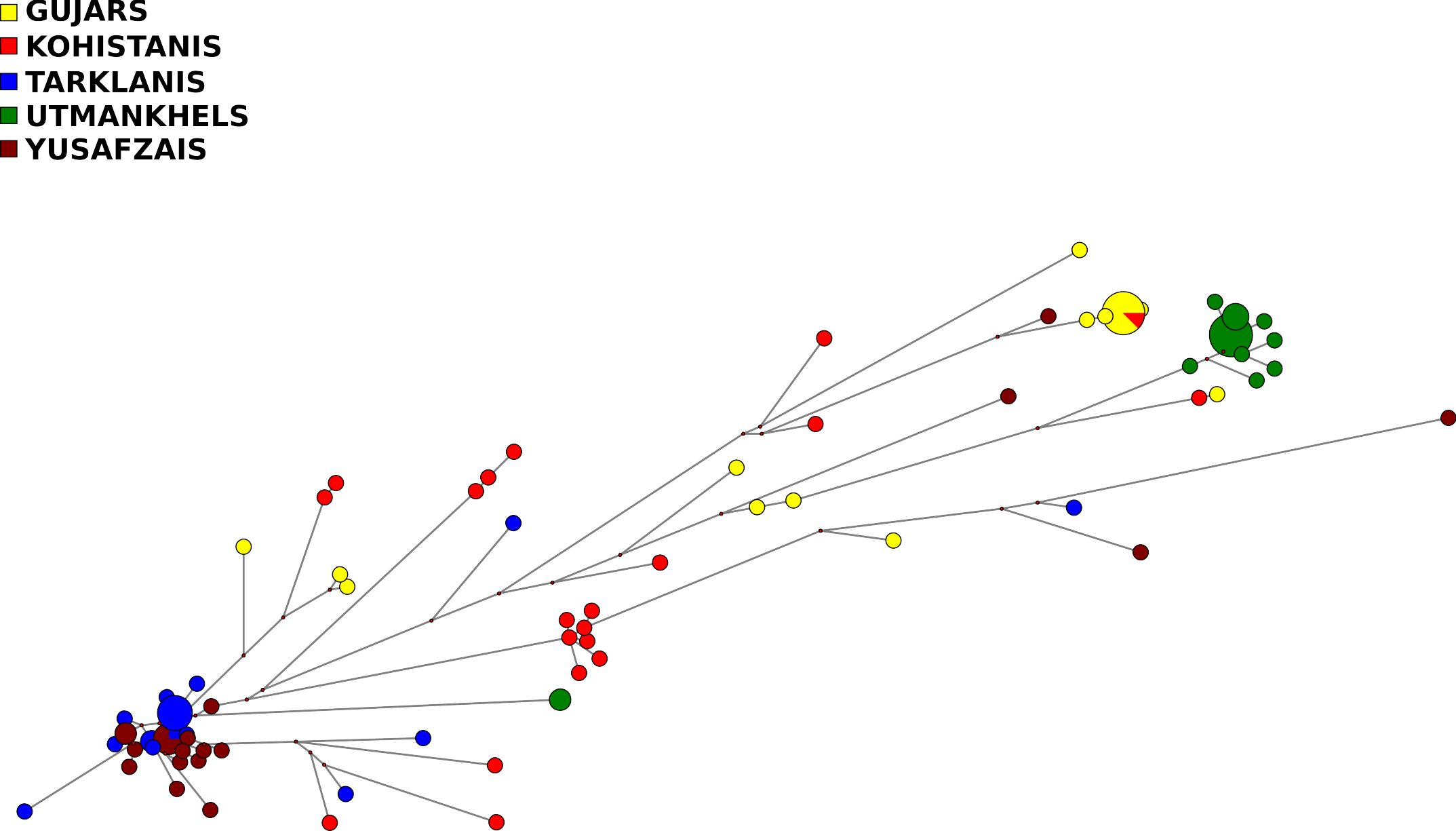
**Table 2.** Genetic differentiation

| | Gujar | Kohistani | Tarklani | Utmankhel | Yusafzai |
|---|---|---|---|---|---|
| Gujar | - | 0.003±0.0005* | 0.000±0.0005* | 0.000±0.0005* | 0.000±0.0005* |
| Kohistani | 0.179 | - | 0.000±0.0005* | 0.000±0.0005* | 0.001±0.0002* |
| Tarklani | 0.465 | 0.197 | - | 0.000±0.0005* | 0.604±0.0048 |
| Utmankhel | 0.451 | 0.517 | 0.746 | - | 0.000±0.0005* |
| Yusafzai | 0.395 | 0.154 | 0 | 0.702 | - |

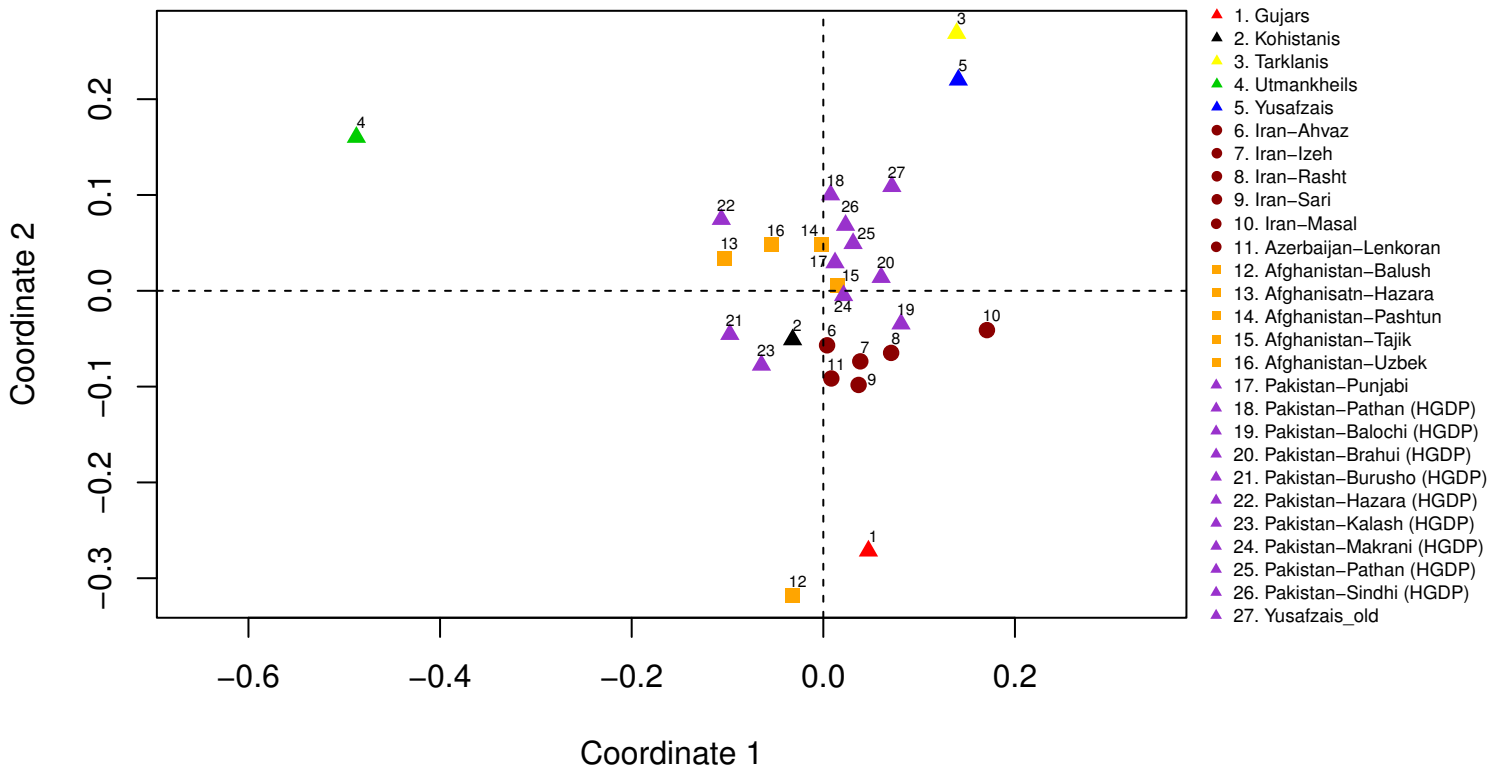
The genetic distances calculated as pairwise $R_{ST}$ values based on 23 of the 27 Y-STR loci. $R_{ST}$ values below the diagonal and the corresponding P-values above the diagonal. * Significant at 0.05 significant level with correction for multiple testing (0.05/10 = 0.005).


- Utmankheils
- Tarklanis
- Yusafzais
- Kohistanis
- Gujars


GUJARS
KOHISTANIS
TARKLANIS
UTMANKHELS
YUSAFZAIS

# Fig. 3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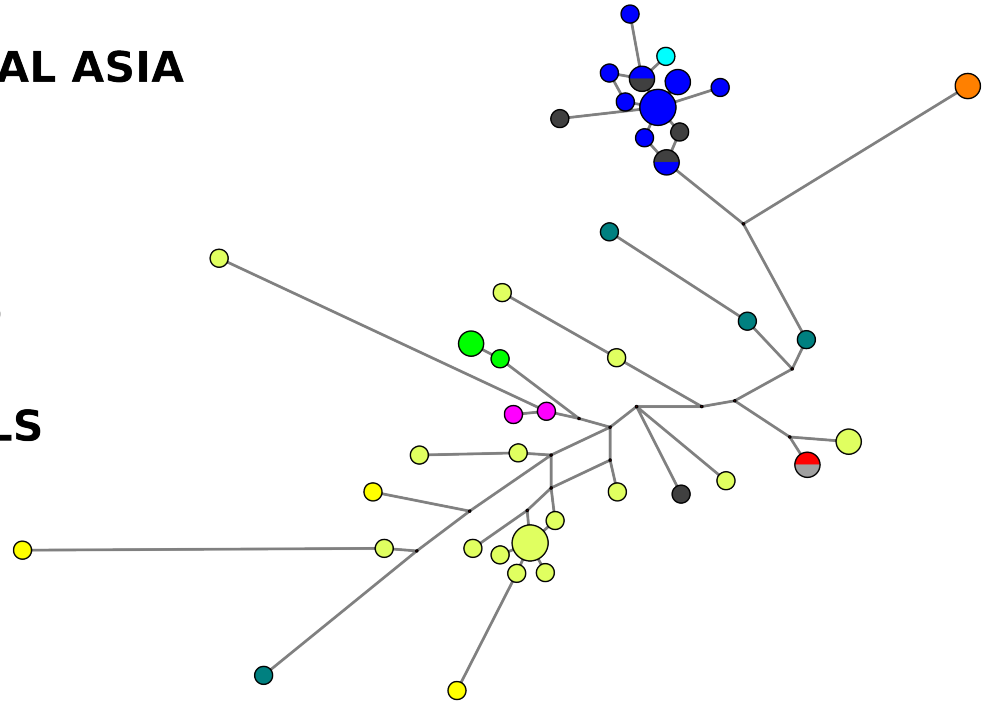
0.0
−0.1
−0.2
−0.3
−0.6
−0.4
−0.2
0.0
0.2
Coordinate 1
1. Gujars
2. Kohistanis
3. Tarklanis
4. Utmankheils
5. Yusafzais
6. Iran−Ahvaz
7. Iran−Izeh
8. Iran−Rasht
9. Iran−Sari
10. Iran−Masal
11. Azerbaijan−Lenkoran
12. Afghanistan−Balush
13. Afghanisatn−Hazara
14. Afghanistan−Pashtun
15. Afghanistan−Tajik
16. Afghanistan−Uzbek
17. Pakistan−Punjabi
18. Pakistan−Pathan (HGDP)
19. Pakistan−Balochi (HGDP)
20. Pakistan−Brahui (HGDP)
21. Pakistan−Burusho (HGDP)
22. Pakistan−Hazara (HGDP)
23. Pakistan−Kalash (HGDP)
24. Pakistan−Makrani (HGDP)
25. Pakistan−Pathan (HGDP)
26. Pakistan−Sindhi (HGDP)
27. Yusafzais_old

Fig. 4

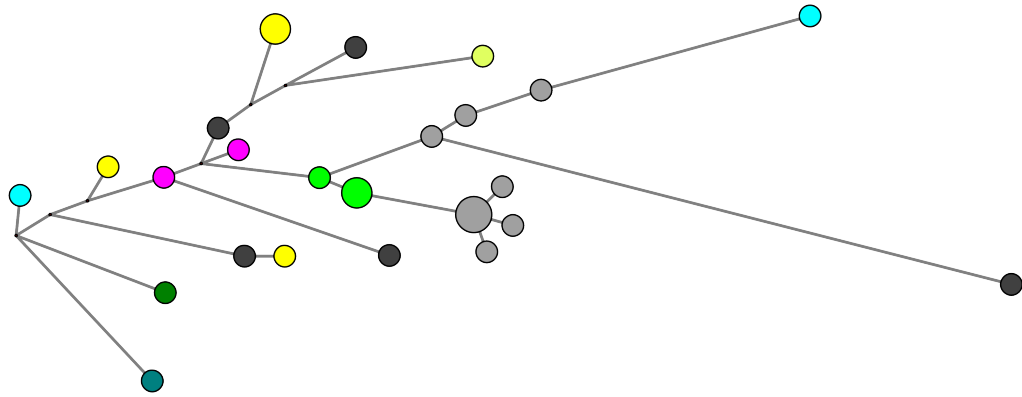
A)

- GUJARS
- NON-CENTRAL ASIA
- HAZARA
- BRAHUI
- BURUSHO
- KALASH
- PASHTUNS
- KOHISTANIS
- TARKLANIS
- TAJIK
- UTMANKHELS



B)

- NON-CENTRAL ASIA
- KOHISTANIS
- PASHTUNS
- BALUCH
- BRAHUI
- BURUSHO
- HAZARA
- KALASH
- TAJIK



C)

- BALUCH
- BRAHUI
- BURUSHO
- GUJARS
- HAZARA
- KALASH
- KOHISTANIS
- MAKRANI
- NON-CENTRAL ASIA
- PASHTUNS
- TAJIK
- UZBEK
- YUSAFZAIS