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Eye Color Frequency and IrisPlex Validation in Pakhtun Population Living in Shangla Valley, Pakistan

Murad Ali Rahat[1]*, Muhammad Israr[2], Hakim Khan[1], Ishtiaq Hassan[1], Mohammad Islam[1] and Muzafar Shah[3]

[1]*Department of Biotechnology and Genetic Engineering, Hazara University Mansehra, Pakistan*
[2]*Department of Forensic Sciences, University of Swat, Swat, Pakistan*
[3]*Centre for Animal Sciences and Fisheries, University of Swat, Swat, Pakistan*

ABSTRACT

DNA Intelligence is relatively new concept which relies on providing phenotypic information about unknown individuals when traditional DNA profiling is not informative. The system aids in forensic investigation by probabilistic prediction of eye, hair, skin colors as well as other phenotypic features. The Irisplex system contains a very sensitive multiplex genetic typing test that allows users to predict blue, brown and intermediate human eye color from DNA samples. To check the validity of IrisPlex system in the local Pakhtun population living in Shangla Valley of Pakistan, the current study was carried out. DNA samples as well as photographs were taken from total of 226 individuals. Exclusion and inclusion criteria were set for samples collection. The analysis showed that brown eye color individuals were greater in number than blue and intermediate eyes color individuals. Moreover, Irisplex system predicted 100% blue and brown eye color while issue in predicting in intermediate eye color. The data of the current study indicated the six Irisplex SNPs the rs16891982 and rs1800407 SNP are significant association with eye color. Conclusion: This study showed that the most prevalent human color traits among Pakhtun population of Shangla was brown eye color and the IrisPlex system accurately predicted blue and brown eye color as such as an actual eye color of the individual while intermediate was misclassified into brown eye color, so online tool of IrisPlex system needed more SNPs inclusion for the prediction of accurate intermediate eye color prediction. Therefore, the Irisplex system represents a useful tool for immediate application in accredited forensic laboratories, predicting blue and brown human eye color from DNA samples, hence, may be a useful tool in forensic investigation.

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Key words
IrisPlex, Eye color, Forensic DNA phenotyping, Externally visible characteristics

INTRODUCTION

The properties of DNA to predict certain facial features of humans are of great value for forensic investigation. This information can lead investigators on the right track after they met dead end due to lack of sufficient reference DNA material. Routine DNA-testing is based on the comparison of DNA samples recovered from a crime scene to that of a suspect or those present in a DNA database. If there is no suspect or the query DNA does not match to those in the database, it is then of little or no interest to the investigators. To overcome this limitation in forensic case work, some other properties of DNA are now used. As human appearance is based on their phenotype and the combination of proteins that they have which in turn are determined by the sequence of DNA that an individual has. Therefore, if we can determine the DNA sequence, we may predict the appearance.

Human facial morphology is a combination of many complex traits. The color of the iris as a multifactorial hereditary trait varies by race and ethnicity. Pigmentation of eye is due to a polymer known as melanin. Melanin has further two type (eumelanin and pheomelanin) that control normal pigmentation. Eumelanin is concerned with brown and black color while pheomelanin with yellow and red (Scherer and Kumar, 2010). SLC24A5 and ASIP genes play key role in the process of melanogenesis (Lamason *et al*., 2005; Liu *et al*., 2012). Genome-wide association studies (GWAS) concluded that (HERC2, IRF4, OCA2, TYR, SLC45A and SLC24A5) play important role in human coloration (Sulem *et al*., 2007). Over the last 10 years, candidate gene association studies and genome-wide association studies (GWASs), as well as subsequent prediction analyses, have established various EVC-predictive SNPs and prediction models, most notably for human pigmentation traits (Stokowski *et al*., 2007;

* Corresponding author: Rahatmurad32@yahoo.com
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Sturm *et al*., 2008; Eiberg *et al*., 2008; Kayse *et al*., 2008; Liu *et al*., 2009, 2010, 2015; Mengel-From *et al*., 2009; Valenzuela *et al*., 2010; Quillen *et al*., 2012; Maroñas *et al*., 2014; Jacobs *et al*., 2015; Branicki *et al*., 2015; Walsh *et al*., 2017). In particular, the prediction of broad (i.e., categorical) eye color phenotypes from SNP genotypes is now achievable with practically useful accuracies, for instance by applying the IrisPlex and HIrisPlex DNA test systems that have been forensically validated (Walsh *et al*., 2014), as well as tested and applied (Kastelic *et al*., 2013; Chaitanya *et al*., 2014). Similar tools for eye color DNA prediction that largely overlap in SNP predictors with the Iris/HIrisPlex systems have also been generated and used (Valenzuela *et al*., 2010; Mushailov *et al*., 2015; Allwood and Harbison, 2013; Ruiz *et al*., 2013).

The worldwide population showed extensive variation in iris pigmentation. Eye color types mainly brown, intermediate and blue are present in the world population. All eye colors were covered by only six SNPs from six genes (Walsh *et al*., 2011). Pakistan has historically been a crossroad for a large number of populations, including Greeks, Aryans, Macedonians, Arabs and Mongols. As a result, the country is a mosaic of ethnic groups, and a large amount of distinctive facial phenotypic variation can be observed among Pakistani people.

In previous studies effects of individual SNPs that have been shown to have significant effects on color of eye in human population. In proposed study we validated the IrisPlex system and also found dominant eye color in the study Pakhtun population of District Shangla.

MATERIALS AND METHODS

Study area

The study area was Shangla District, located in Khyber Pakhtunkhwa, Pakistan. The population of Shangla district has 7,57810 individuals, consisting of different ethnic groups such as Yusufzai, Gujar, Azar, and Syed, but Pakhtun is the largest ethnic group.

Samples collection

Bioethical committee of Hazara University, Mansehra approved this research study work. Exclusion and inclusion criteria were set for samples collection. Buccal swab samples from 245 from healthy males and females individual were collected. Those having any type of eye color diseases were excluded from the study. Eye color photographs were taken with the help of Nikon D 5300 DSLR camera having manually focusses on ISO 800 with shutter 1/100 and AV 18 with Nikon AF-P 18-55 mm F/3.5-5.6 G DX.

Classification of eye into different eye color categories

Eye color categories were assigned through seven untrained individuals, into the following three eye color categories (blue, brown and intermediate). In case where problems to assign eye color categories then the categories were assigned by majority of voting system between the observers.

Extraction of genomic DNA

The top cotton portion of the Buccal swab was used for extraction of DNA through modified Phenol-Chloroform method (Green and Sambrook, 2012) in the Forensic Research Laboratory, University of Swat.

SNPs amplification and genotyping

The SNPs were amplified in 20 µl of total reaction containing each reaction of 0.25 µl of revivers and forward primers (Walsh *et al*., 2011), Thermo scientific Dream Taq Green PCR master mix (2X) (Cat NO: K1081), DNA template and water in a PCR machine name TC –XP Thermal Cycler (BIOER, CHINA) with 96 wells plate. The thermal cycle for PCR reaction cmprised initial denaturation 95˚C for 10 min followed by 33 cycles each denaturation at sec denaturation 95˚C for 30, annealing at 60˚C for 30 second, extension process was 60˚C for 5 min. The product was checked on 2% agarose gel.

Multiplexed SNaPshot (Life Technologies Inc, USA) SBE chemistry was used for SNP typing. PCR product was treated with ExoSAP-IT (USB1 Corporation) at 37 °C for 15 min to eliminate unused dNTPs or PCR primers. It was followed by 85 °C for 15 min to stop the enzyme activity. Then 1.5 µl of treated PCR product was added to 2.5 µl of SNaPshot prepared reaction mix and 1.5 µl of extension primer mix. Conditions for SNPs amplification were 30 cycles as: 96°C for 10 sec, 50°C for 5 sec and 60°C for 30 sec. To clean up, the extension reaction products were treated with 1 µl of SAP at 37 °C for 80 min. Again to stop enzymatic activity, the mixture was treated at 85°C for 15 min and The purified SBE products were used to obtain electropherograms from the capillary electrophoresis that were used to genotype the samples. Added 1 µl sample, 0.1 µl of GeneScan Size Standard LIZ (Fisher Scientific International Inc.,) Formamide 8.9 µl volume (Fisher Scientific International Inc.,) per plate well of ABI Prism 3730xl Genetic Analyzer (Life Technologies Inc, USA). All these electropherograms samples were visualized by using Gene mapper software version 4.0. Each peak in the electropherograms represents allele of the SNP in the form of these possible bases (A or T or C or G) labeled with a various dyes. For the labeling of genotypes in the analysis software, bins and panels were used. On the basis of the SNPs, bins designated the bp range within which each

peak falls. When a peak falls in a specific bin, the software makes a corresponding allele call. Single peak was found in the case of homozygous genotypes while two peaks of different color were found in the case of heterozygous genotypes.

Eye color prediction model

The goal of DNA phenotyping was to determine a genotype that can accurately infer the phenotype of the individual. Model building methods can be used to find the best fitting model to describe the relationship between the outcome and predictor variables. The IrisPlex (http://hirisplex.erasmusmc.nl) eye color prediction with three outcomes (blue, intermediate and brown) based on multinomial logistic regression higher probability value denoted the specific eye color of the individuals was used.

Data analysis

The genotyping result were analyzed through online and offline software's such as Gene Mapper version of 4.0 (Applied Biosystems), used Microsoft excel 2013 and the IBM SPSS Statistics v. 23 (IBM Inc. USA).

RESULTS

Demographic data

The data analysis of the current study indicated that brown-colored eyes were even more prevalent in the Shangla population, where they accounted for 87.16% of the sample, intermediate colored eyes accounted for (11.51%), while blue-colored eyes were quite rare (1.33%). Our study also showed that out of 226 samples female samples were 81 and male samples were 145. Females were found higher in brown eye color (91.35%) blue was (1.23%) and intermediate was (7.42%). Brown eye color in males sample were (84.92%), blue was (1.47%) and intermediate was (13.61). The collated samples were also divided into four different age grouped and the analysis showed that group 1st that started from 11-20 year of age consisted of brown eye color was (27.91%), intermediate was eye was (26.92%) and blue was (33.33%), similar in the age group started 21-30 having brown eye color (23.35%), blue (33.33) and intermediate (23.07%), Similar to age group 31-40 the brown eye color was (26.90%), intermediate (23.07%) and blue was (33.33%). In the age group of 41-50 the percentage of blue eye color (21.84%), brown eye color and intermediate eye were (26.92%).

Genotypes distribution in the study population

SNPs genotypes were amplified (Fig. 1). In rs12913832 were three types of genotypes in the population of Shangla District the CT (55%) genotype was found in most individuals, followed by TT (35%) and CC (10%). Two types of genotypes GG (70.0%) and GA (30%) were observed in rs1800407 SNP. Two types of genotypes in rs12896399 SNP were reported CC (70%) and CA was (30%). Three types of genotypes were present in rs16891982 GC (25%), GG (45%) and CC was (30%). Shangla population showed two types of genotypes GG (95%) and GA (5%) in rs1393350 SNP. Only CC genotype was observed in rs12203592 SNP (Fig. 2).

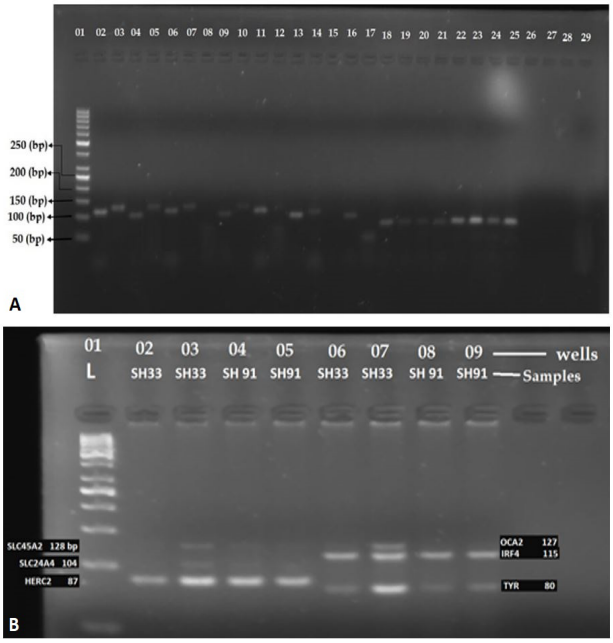

Fig. 1. Showing singleplex (A) and Multiplex-PCR amplification result (B) of six SNPs. A, Lane 1, 50 bp DNA ladder; lanes 2, 6, 10 and 14 IRF4, 3, 7, 11; Lane 15, SLC45A2; Lane 4, 8, 12 and 16 SLC24A4; Lanes 5, 9, 13 and 17 OCA2, 18, 19, 20; Lane 21 showed TYR and Lines 22, 23, 24 and 25, HERC2 SNP. In B, the 4% gel figure showed Line 1 50bp DNA marker; lines 2-5 showed the combined result of *OCA2, TYR* and *IRF4*, while lines number 6-9 showed the combined result of *HERC2, SLC24A4* and *SLC45A2*.

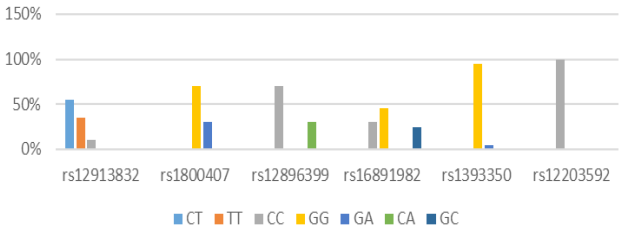

Fig. 2. Genotypes of six SNPs in the study population.

Table I. Genotypes distribution in eye color categories of Shangla population.

Eye color	SNPs												
	rs16891982			rs12896399		rs1393350		rs12913832			rs1800407		rs12203592
	GC%	GG%	CC%	CC%	CA%	GA%	GG%	CC%	CT%	TT %	GG %	GA %	CC%
Blue	00	100	0	100	00	0	100	100	0	0	100	0	100
Brown	23.53	41.17	5.30	70.69	9.41	5.88	94.12	0	58.82	41.18	44.7	55.3	100
Inter	100	0	0	0	100	0	100	0	100	0	100	0	100

Genotypes of the six SNPs in the eye color of the study population

The CT (58.82%) and TT (41.18%) genotypes of rs12913832 SNP were observed in brown eye colored individuals, intermediate was CT and CC was in blue. In this way CC (70.69%), CA (29.41%) genotypes of rs12896399 SNP were in brown eye color, Intermediate eye was CA and blue was CC genotype. GG (64.70%) and GA (35.30%) genotypes of rs1800407 SNP were observed in brown eye colored individuals, Intermediate and blue was GG genotypes. Genotypes of rs1393350 showed that GG (94.12%) and GA (5.88%) genotypes were present in brown eye color, Blue and intermediate was GG genotype. GC (23.53%), GG (41.17%) and CC (35.30%) genotypes of rs16891982 were observed in the brown eye colored individuals; intermediate was the GC and blue was GG genotype. The CC genotype of rs12203592 was seen in blue, intermediate and brown eye colored individuals (Table I).

Validation of IrisPlex system

The six SNPs comprising this MLR-based, published eye color model (Walsh *et al*., 2011; Liu *et al*., 2009) are included in this study. The results of this calculator are prediction probabilities for blue, brown, or intermediate eye color. Following previous studies (Walsh *et al*., 2012, 2014) raw probabilities were then considered and prediction assigned to the category with the highest probability. These prediction probabilities were compiled for each individual, and compared to their reported eye. The study population showed that brown and blue eye color was predicted in all cases, while intermediate eye color in the study population 100% predicted brown eye colors (Table II). Total probability values and record of each eye sample (Table III).

Correlation and regression analysis between the study parameters with eye color

To find significant correlation between parameters with eye color the Pearson correlation test was applied to find association between study parameter and eye color. Analysis of the current study showed that rs12913832 (P- value= 0.003) and gender was significant associated with rs12203592 (P- value= 0.042) SNPs is Significant associated with eye color while the rest of SNPs were significant (Table IV). To find direct effect of parameter on eye color the liner regression analysis was applied to find association between study parameters and eye color. Analysis of the current study showed that rs12913832 (P-value= 0.006) and rs1800407 (P-value= 0.022) SNPs is significantly associated with eye color while the associations of the rest of SNPs were significant (Table V).

Table II. Validation of eye color prediction models: Prediction success (bold values) estimates from the Pakhtoon population residing within the Malakand Division.

Prediction model IrisPlex	Predicted eye color			Unde-tected	No of samples
	Blue	Brown	Intermediate		
Blue	100%	0	0	0	(3)
Brown	0	100%	0	0	(11)
Intermediate	**0**	**100%**	**0**	**0**	**(6)**

DISCUSSION

The present findings indicated that dark brown iris color was the most prevalent color trait among the Pakhtun population of Shangla District and the distribution of these color traits showed gender differences. In addition, we observed significant inter-correlations amongst the human color and Irisplex SNP. Very few studies exist on the distribution of iris color in the world population. The available studies however used different definitions and measurement techniques for iris colors, thus making comparisons with other reports are difficult. In the present study, we grouped iris colors into three categories of brown (light brown, dark brown), blue (blue/grey) and intermediate (other than blue and brown) which is similar to previous studies (Hashemi *et al*., 2010; Klein *et al*., 1998). Our data indicated that 87.16% of the study population had brown as most prevalent iris color, while intermediate was 11.51% and blue iris was observed in only 1.33% as

Table III. Irisplex 6 SNP genotypes color prediction probabilities together with samples details of Pakhtoon population. Blue color indicated Blue eye color, brown indicated Intermediate and green color indicated brown eye color.

Samples	rs1291-3832	rs180-0407	rs1289-6399	rs168-91982	rs139-3350	rs1220-3592	P blue eye	P inter eye	P brown eye	Gender	IrisPlex result	Actual eye color
1	CT	GG	CA	GC	GG	CC	0.01223	0.050034	**0.937736**	F	Brown	Inter
2	TT	GA	CC	GG	GG	CC	0.001006	0.034825	**0.964169**	F	Brown	Brown
3	CT	GG	CC	GG	GG	CC	0.050323	0.113551	**0.836126**	F	Brown	Inter
4	CT	GG	CC	CC	GG	CC	0.00276	0.020476	**0.976764**	F	Brown	Brown
5	CT	GA	CA	CC	GG	CC	0.010029	0.052298	**0.937674**	F	Brown	Brown
6	CT	GG	CA	GG	GG	CC	0.050323	0.113551	**0.836126**	M	Brown	Inter
7	CT	GA	CA	GG	GG	CC	0.001006	0.034825	**0.964169**	M	Brown	Inter
8	CT	GG	CC	GG	GG	CC	0.050323	0.113551	**0.836126**	F	Brown	Brown
9	TT	GA	CA	GC	GG	CC	0.000223	0.013989	**0.985788**	F	Brown	Brown
10	CC	GG	CC	GG	GG	CC	**0.84781**	0.087663	0.064527	M	Blue	Blue
11	TT	GA	CA	GG	GA	CC	0.001006	0.034825	**0.964169**	F	Brown	Brown
12	TT	GG	CC	GG	GG	CC	0.000272	0.01339	**0.986338**	F	Brown	Brown
13	CC	GG	CC	GG	GG	CC	**0.84781**	0.087663	0.064527	F	Blue	Blue
14	TT	GG	CC	GC	GG	CC	5.94E-05	0.005305	**0.994635**	F	Brown	Brown
15	CT	GG	CC	GG	GG	CC	0.050323	0.113551	**0.836126**	F	Brown	Inter
16	CT	GG	CC	GC	GG	CC	0.01223	0.050034	**0.937736**	F	Brown	Brown
17	CC	GG	CC	GG	GG	CC	**0.86781**	0.087663	0.064527	M	Blue	Blue
18	CT	GG	CC	GG	GG	CC	0.050323	0.113551	**0.836126**	F	Brown	Brown
19	CT	GG	CC	GG	GG	CC	0.050323	0.113551	**0.836126**	F	Brown	Inter
20	CT	GG	CC	CC	GG	CC	0.050323	0.113551	**0.836126**	F	Brown	Brown

Table IV. Correlation analysis between the study perimeters with eye color.

		rs12913832 (n=20)	rs1800407 (n=20)	rs12896399 (n=20)	rs16891982 (n=20)	rs1393350 (n=20)	rs12203592 (n=20)	Gender (n=20)	Eye color (n=20)
rs12913832	Pearson correlation	1	.114	-.212	-.157	.154	-.189	-.149	.630**
	Sig. (2-tailed)		.632	.369	.510	.516	.426	.529	.003
rs1800407	Pearson correlation	.114	1	.286	.251	.350	-.150	-.218	-.263
	Sig. (2-tailed)	.632		.222	.286	.130	.527	.355	.263
rs12896399	Pearson correlation	-.212	.286	1	-.192	.350	.350	.055	-.088
	Sig. (2-tailed)	.369	.222		.418	.130	.130	.819	.713
rs16891982	Pearson correlation	-.157	.251	-.192	1	-.016	-.326	-.135	-.136
	Sig. (2-tailed)	.510	.286	.418		.948	.161	.570	.568
rs1393350	Pearson correlation	.154	.350	.350	-.016	1	-.053	.115	-.092
	Sig. (2-tailed)	.516	.130	.130	.948		.826	.630	.699
rs12203592	Pearson correlation	-.189	-.150	.350	-.326	-.053	1	-.459*	-.092
	Sig. (2-tailed)	.426	.527	.130	.161	.826		.042	.699
Gender	Pearson correlation	-.149	-.218	.055	-.135	.115	-.459*	1	-.201
	Sig. (2-tailed)	.529	.355	.819	.570	.630	.042		.396
Eye color	Pearson correlation	.630**	-.263	-.088	-.136	-.092	-.092	-.201	1
	Sig. (2-tailed)	.003	.263	.713	.568	.699	.699	.396	
	N	20	20	20	20	20	20	20	20

*, Correlation is significant at the 0.05 level (2-ailed); **, Correlation is significant at the 0.01 level (2-tailed).

Table V. Liner regression analysis between the study perimeters with eye color.

Parameters	Unstandardized coefficients		Standardized coefficients	T	Sig.
	B	Std. error	Beta		
(Constant)	-.475	.388		-1.223	.245
rs12913832	.620	.184	.667	3.367	.006
rs1800407	-.773	.294	-.569	-2.626	.022
rs12896399	.284	.152	.418	1.867	.086
rs16891982	.005	.173	.006	.029	.978
rs1393350	-.164	.279	-.115	-.589	.567
rs12203592	-1.121	.750	-.392	-1.494	.161
Gender	-.644	.376	-.414	-1.712	.113

the least prevalent. Similar study on Pakhtun population of district Swat showed that brown eye color is prevalent in the study population followed by intermediate and blue (Rahat *et al*., 2020). Worldwide studies also indicated nearly similar results to our finding for brown eye color. The 90% prevalence of eye color (brown) categories was observed in Uzbekistan (Ulivi *et al*., 2013; Katsara and Michael, 2019) Armenia (80.15%), Azerbaijan (71.42%), Tajikistan (85.47%), Kazakhstan (85.0%), Georgia 73.68% (Ulivi *et al*., 2013; Katsara and Michael, 2019) and high brown eye color is also recorded in Korea, (Chang *et al*., 2010) and Japan (Iida *et al*., 2009). In the present study as reported in other studies we report here for the first time brown eye color as the most prevalent (87.16%) among in the Pakhtun inhabitants of the of Shangla district. This stands in contrast to various populations of Europe (Iceland, Germany and Denmark) in which previous studies report that brown eye colored individuals comprise a small proportion of the population compared to irises of other colors (Mengel-From *et al*., 2009; Katsara and Michael, 2019). In the current study intermediate eye color was found among 11.51% of sampled individuals of the study area which is similar to the work of Rahat *et al*. (2020). The intermediate eye colored individuals with almost same ratio (similar to present work) have been reported worldwide; 12.5% in Poland, 11.65% in Kazakhstan (Ulivi *et al*., 2013; Katsara and Michael, 2019), 14.15% in Iceland (Sulem *et al*., 2007), 6.02% in Uzbekistan (Katsara and Michael, 2019) and 7.67% in Tajikistan (Ulivi *et al*., 2013; Katsara and Michael, 2019). In contrast to the present finding, intermediate eye colored individuals are present much higher percentage in France (44%), Spain (55.2%) and Germany (39.6%) (Diaz *et al*., 2009; Katsara and Michael, 2019). Blue eye color is the most prevalent eye color in Iceland (73.90%), Denmark (64.84%) and Poland (52.50%) (Lock-Andersen *et al*., 1999; Sulem *et al*., 2007; Mengel-From *et al*., 2009). The present study is not in agreement with the studies described but supports the findings observed in the populations of Armenia, Azerbaijan, Tajikistan and Georgia (Ulivi *et al*., 2013; Katsara and Michael, 2019) where the lowest percentage of individual with blue colored eyes were recorded. Dark brown eyes have been shown to be dominant in humans and in many parts of the world, it is nearly the only iris color present (Eiberg and Mohr, 1996). Dark pigment of brown eyes is most common in East Asia, Southeast Asia, South Asia, West Asia, Oceania, Africa, Americas, etc. as well as parts of Europe such as Spain and Southern Italy (Sulem *et al*., 2007). Grey-blue eye color has also been shown to be the least prevalent in the Tehran eye study (Imesch *et al*., 1997) and consistent with the present study.

In contrast to iris color research there have been some attempts to understand iris structure diversity in different populations. However, all of these studies have limited their work to a particular continent. As each research group categorized iris structure variation differently we are still not able to compare the phenotypic distribution of these traits in different continental regions of the world. Further studies are needed to investigate the scenario behind the eye color prevalence.

Eye color has been tentatively associated with demographic factors, like sex. Looking at sex-based distribution of eye color it was found that brown eye color is present in a higher percentage among both males and females. Similarly, intermediate was the second most prevalent in eye color in the individuals male 13.61%, female 7.42%, followed by blue eye color males 1.47%, females 1.23%, respectively. It has been reported that in Italian and Spanish populations brown eye color is the dominant eye color in females while males are mostly blue and intermediate eye color (Martinez-Cadenas *et al*., 2013; Marano and Fridman, 2019). Similar to work of Rahat *et al*. (2020) where brown eye color is higher in females of Pakhtoon population of district Swat while in males the intermediate and blue eye colors are dominant. However, this finding was not replicated in Danish or Swedish samples (Pietroni *et al*., 2014). Consequently, from this study and previous studies, it is concluded that the association between eye color and sex may be largely restricted to some specific populations. This significant sex difference suggests that there may be an as yet unidentified sex-related factor contributing to human eye color variation. Further studies are required in order to elucidate the influence of gender into the eye color variation in humans.

The worldwide genotypic distribution of the six IrisPlex SNPs has been previously described (Walsh *et al*., 2011). Present study investigated the association of

the rs12913832 SNP in HERC2 gene with eye colors in the Pakhtun population of district Shangla. Similar to some earlier studies (Mengel-From *et al*., 2009; Ulivi *et al*., 2013; Alghamdi *et al*., 2019; Rahat *et al*., 2020) our study showed that at the individual level, the CC genotype was found linked with blue, intermediate with CC and CT genotypes, whereas TT (53.16%), CT (46.84%) genotypes were strongly linked with brown eye color. Another study described the same linkage from Pakistani Brahui, Balochi, Hazara, Makrani, Sindi, Kalash, and Burusho populations (Walsh *et al*., 2011; Freire-Aradas *et al*., 2012). A comprehensive study of 51 populations (934 individuals) worldwide showed that blue/intermediate eye color is linked with GG/CC (homozygous or heterozygous) genotypes in population of Europe and adjacent territories (West Asia and Middle East) while the rest of the world population AA/TT genotypes was reported to be linked with the brown eye color (Walsh *et al*., 2011). A recent study on the Japanese population also demonstrated the association of TT/AA genotypes with brown eye color (Walsh *et al*., 2011). The SNP rs1800407 show a significant correlation in our study. However, this SNP rs1800407 was also previously found significant with eye color (Branicki *et al*., 2011; Liu *et al*., 2009; Rahat *et al*., 2020). The rest of SNPs were not significant with the eye color of study population.

Developing a better understanding of eye color, features will contribute greatly to the fields of forensics, anthropology and public health. At present, many forensic groups are developing pigmentation predictor tools. The primary purpose of these tools is to allow crime scene investigators to predict the eye color of unidentified individuals from small samples of their DNA. Irisplex, perhaps the most well-known of these tool. A previous study in an Italian population has shown 76% accuracy of Irisplex system (Salvoro *et al*., 2019). A similar study by Dembinski and Picard (2014) found 58%, 95%, 11% accuracy on Irisplex system for brown, blue and intermediate eye color prediction, respectively. Allwood and Harbison (2013) found 89% accuracy for blue eye color and 94% for brown eye color while (Rahat *et al*., 2020) found 100% accuracy for blue and brown eye. Compared to previous studies the present study investigated that blue and brown eye color was predicted 100% while the intermediate eye color phenotypes are misclassified, predicted as brown. Issues in predicting intermediate eyes have already been shown in previous studies (Liu *et al*., 2009; Walsh *et al*., 2011b, 2012, 2014; Ruiz *et al*., 2013; Dembinski and Picard, 2014: Yun *et al*., 2014, 2015; Freire-Aradas *et al*., 2014; Salvoro *et al*., 2019; Bulbul *et al*., 2020; Carratto *et al*., 2020; Rahat *et al*., 2020).

CONCLUSION

This study concludes that phenotypically the ratio of brown eye colored individuals is high in Shangla District. Based on sex there exists a significant male-female eye color alteration in the Pakhtun population of the study population. Linear and Pearson analysis shows associations of eye color with 12913832 SNP in HERC2 gene. DNA prediction model (IrisPlex) showed higher accuracy for predicting brown and blue eye color, while uncertainty remained for intermediate eye color. Hence, we suggest that genotyping more SNPs that not included in the IrisPlex system geographical region, gender may even improve the prediction accuracy in our population. Finally, our prediction model is based on biological samples that were taken under controlled setting; this may not be the case for a forensic sample retrieved from a crime scene. Hence, further replication of the model using forensic sample is needed. We also found that the accuracy of production of blue and brown eye color is much higher than all three categories (blue, brown and intermediate) so we recommend blue and brown eye color classification instead of three eye color. The dark intermediate should be classified with brown and light intermediate be classified with blue.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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