Full Length Research Article
Advancements in Life Sciences – International Quarterly Journal of Biological Sciences

ARTICLE INFO

Date Received: 14/01/2020;
Date Revised: 22/04/2020;
Date Published Online: 25/05/2020;

Authors' Affiliation:
1. Department of Genetics, Hazara University, Mansehra - Pakistan
2. Department of Forensic Sciences, University of Swat - Pakistan

***Corresponding Author:**
Muhammad Israr
Email: afficer@gmail.com

How to Cite:
Rahat MA, Khan H, Hassan I, Haris M, Israr M (2020). DNA-based Eye Color Prediction of Pakhtun Population Living in District Swat KP Pakistan. Adv. Life Sci. 7(3): 157-163.

Keywords:
Eye colour, IrisPlex, SNPs, Multiplex genotyping, DNA, District Swat

Open Access

DNA-based Eye Color Prediction of Pakhtun Population Living in District Swat KP Pakistan

Murad Ali Rahat[1], Hakim Khan[1], Ishtiaq Hassan[1], Muhammad Haris[1], Muhammad Israr[2]*

Abstract

Background: Forensic DNA Phenotyping (FDP) or the prediction of Externally Visible Characteristics (EVCs) from a DNA sample has gained importance in the last decade or so in the forensic community. If and when the traditional forensic DNA typing via Short Tandem Repeats (STR) fails due to the absence of a reference sample, an individual can be traced by a DNA sample using FDP. Amongst the many available EVCs, eye color is one such character that can be predicted by employing previously developed IrisPlex system using Single Nucleotide Polymorphism (SNP) assay. In this study, we applied the IrisPlex system to samples collected from population of District Swat for prediction of eye colours from DNA.

Method: Eye colour digital photographs and buccal swab samples were collected from 267 Pakhtun individuals of District Swat. Any person with eye disease was excluded from the study. Genomic DNA was extracted through Phenol-Chloroform extraction method. The amplified SNPs were typed using Multiplexed Single Base Extension (SBE). The genotypes were checked for eye color phenotypes through IrisPlex online tool and correlation were checked between SNPs, Gender, pie score and eye color.

Result: Brown eye color was found prevalent as compared to intermediate and blue. Females have highly brown eye color compared to males while males have intermediate and blue. Three SNPs rs12913832 (in the *HERC2*), rs1393350 (*TYR* gene), rs1800407 (*OCA2* gene) were strongly significant to eye color. Pie score was also significant to eye color and rs12913832 SNP. IrisPlex analysis in 20 individuals of District Swat was performed. The prediction accuracy of IrisPlex for blue or brown was 100% in the studied individuals. However, the IrisPlex tool predicted the intermediate phenotype incorrectly as brown or blue.

Conclusion: It is concluded from the data that intermediate eye colour was not predicted accurately, therefore, inclusion of more SNPs in the IrisPlex system is needed to predict intermediate eye colour accurately.

SCAN ME

Introduction

Eye and hair colour are two main features in determining an individual's appearance within a population. Eye and hair colour are extremely heritable. Heritability for eye colour ranges from 61 to 100 percent and for hair, similar estimates are obtained [1-3]. Both eye color and hair color shows a great variation due to type and amount of melanin pigment as well as responsiveness to UV radiations within as well as amongst populations [4,5]. Human colouration of skin, eye and hair are one of the most changeable and having some characteristics, mostly influenced by the combination of hormonal and genetic factors, and also concerned with the environment, drugs, age, and contact with UV [6,7].

Melanin is one of the proteins mostly responsible for pigmentation of eye, hair and skin. Melanin production occurs in melanocytes and can further synthesize two types; eumelanin and pheomelanin. Eumelanin is concerned with brown and black colour while pheomelanin with yellow and red [8]. *SLC24A5* and *ASIP* genes play key role in the process of melanogenesis [9,10].

Ancestry of European people shows extensive variation in hair and iris pigmentation. Eye colour types mainly brown, intermediate and blue are present in European population, but other parts of the world have a tendency to have brown colour. It follows the theory that darker pigmentation originated in the last 2 million years after our ancestors lost their protective hair covering leading to the exposure of their lighter skin. However, they also present evidence to support the idea that the darker pigmentation in South East Asian populations may indeed result from a common African ancestor whose migration route led them out of African and into East Asia. Ultimately, it may be possible that both factors of convergent evolution and natural selection are behind the pigmentation of the skin [11]. The recent difference in eye colour is assumed to have been created through genetically mixing non-brown irises throughout primary European history [12,13]. It is also thought that the eye colour difference in Europe has been made by positive sexual selection [14,15]. On the other hand, it is suggested the eye colour difference change with skin colour and environmental adaptations [13,15]. It is also proposed that the source for European blue eye color is the southern Baltic region with reducing colour north to south [14,15]. While the particular functional outcome of several SNPs linked with eye colour is presently unknown, however, earlier a systematic study was carried out on thirty-seven SNPs from eight genes linked for eye colour in 6168 Dutch Europeans. It was found that fifteen SNPs could correctly predict brown and non-brown colours. Out of these, all eye colours were covered by only six SNPs from six genes [16]. On the basis of the previous studies, recently, IrisPlex system was developed to accurately predict non-intermediate colour. IrisPlex system consist of six SNP multiplex SNaPshot assays validated for forensics studies. Earlier, a worldwide IrisPlex study established the capability of the IrisPlex system to predict individual categorical eye colour dependably on the basis of bio-geographic ancestry thus eliminating the need for secondary data for genetics origin [17].

The aim of the instant study was to check IrisPlex system for prediction of eye colour in Pakhtun population of Swat district and compare the results with previously reported populations. We also aimed to check the accuracy of IrisPlex system on the Pakhtun population of District Swat.

Methods

Sample collection and DNA extraction

After approval of Institutional Ethical Committee, samples were collected from healthy male and female volunteers of different areas of District Swat. Individuals having heterochromia and any eye diseases were excluded. Consent form was taken from the participant mentioning written declaration by the participant, gender, eye colour, age, ethnicity, and place of birth. Buccal tissue cells were collected from the same participants by using two sterile buccal swabs for each participant. Genomic DNA was isolated through organic extraction method [18].

Digital photographs

Left Eye pictures of each participant at a distance of almost 10 cm were taken with shutter 1/100 and AV 18 by using lens 18-55 mm Nikon D5300 camera using uniform light conditions and saved in 'JPEG' format.

SNPs amplification and Genotyping

Six SNPs namely, rs12896399 (*HERC2*), rs1800407 (*OCA2*), rs16891982 (*SLC24A4*), rs1393350 (*SLC45A2*), rs12913832 (*TYR*) and rs12203592 (*IFR4*) representing the respective genes were selected. The primers used for the study were already published [16]. DNA was amplified in 20 µl reaction consisting of: 2.0 µl $MgCl_2$, 2.0 µl reaction buffer, 0.4 µl dNTPs (10µM), 0.5 µl each of forward and reverse primers (10µM), 0.5 µl *Taq* polymerase, 2.0 µl DNA and ddH_2O. The reactions were allowed to proceed in a thermal cycler (XP Thermal Cycler, BIOER TECHNOLOGY CO., LTD). Thermal cycling parameters were adjusted as; initial denaturation for 10 minutes at 95°C, followed by 35 cycles of (denaturation for 30 seconds at 95 °C, annealing and extension for 30 seconds at 60°C) followed by final extension at 62 °C for 5 minutes. 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 extension were 30 cycles as: 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 30 seconds. To clean up, the extension reaction products were treated with 1 µl of SAP at 37 °C for 80 minutes. To stop enzymatic activity, the mixture was treated at 85°C for 15 minutes. Capillary electrophoresis was performed using ABI Prism 3730xl Genetic Analyzer (Life Technologies Inc, USA).

Data Analysis

Genotype analysis was done through GeneMapper software v 4.0 (Life Technologies Inc, USA). Statistical analyses were performed through IBM SPSS Statistics 23 (IBM Inc). Correlation was checked between SNPs, eye colour pie score. Pixel Index of Eye (PIE) score was calculated for all digital eye photographs. The score ranges from -1 to 1 (from brown to blue respectively), however intermediate colour has been given a score of 0 only. The score was calculated using the following equation [19]:

$$PIE = \frac{\text{Number of pixels labelled blue} - \text{number of pixels labelled brown}}{\text{Number of pixels labelled blue} + \text{number of pixels labelled brown}}$$

Iris assessment was done through seven untrained observers. They were allowed to assess the iris colour in the digital eye colour photograph kept from them in the same distance and similar light conditions. They were asked to assign the iris colour in one of the following 3 categories, i.e. brown, intermediate, and blue. The intermediate colour was either green or hazel. In case of no consensus among observers to assess the eye colour, it was then assigned on majority-voting system between the observers.

All the genotype data of the 20 samples present on the excel sheet were checked on online IrisPlex tool (http://hirisplex.erasmusmc.nl/) and the result was compared with phenotype data.

Results

Phenotype distribution eye colour across the study population

A Total of 267 individuals (149 males and 118 females) were selected. Brown eye colour was present in 202 (75.7%) individual's, intermediate was in 40 (15.0%) and blue eye colour was in 25 (9.4%). Brown eye colour was slightly more in females as compared to males (51.48 %, vs 48.51%), while blue (20% vs 80%) and intermediate (22.5% vs 77.5%) were less prevalent in females as compared to males.

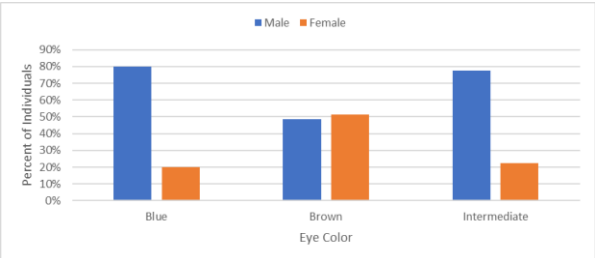

Figure 1: Gender-based eye colour distribution

SNPs amplification and Genotyping

Six SNPs included in the IrisPlex system were amplified and results are presented in (Figure 3). Product size of SNPs is from 84 bp to 128 bp.

IrisPlex SNPs genotype distributions across the study population

Result show that SNP rs12913832 having three genotypes: homozygous CC (60%), TT (30%) and

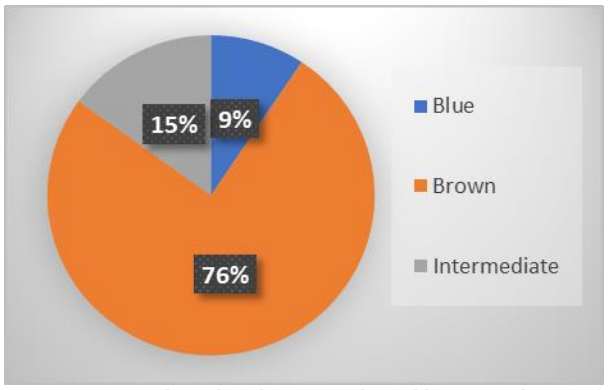

Figure 2: Eye colour distribution in the Pakhtun population of Swat

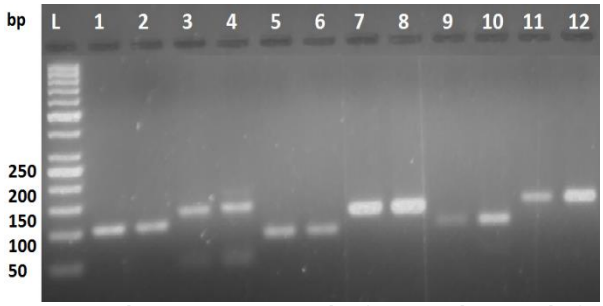

Figure 3: Gel Documentation result of PCR Product. Total of 6 markers were used. Well number 1 and 2 has result of Primer SLC24A4, well number 3 and 4 OCA2, well number 5 and 6 TYR, well number 7 and 8 IRF4, well number 9 and 10 HERC2, well number 11 and 12 SLC45A2.

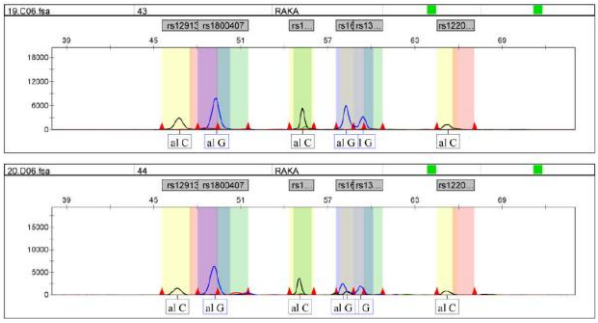

Figure 4: Electropherograms for the IrisPlex SNPs

Sample File/Marker	rs12913832	rs1800407	rs12896399	rs16891982	rs1393350	rs12203592
DNA1	CT	GG	CC	GC	GG	CC
DNA2	TT	GA	AA	GC	GG	CC
DNA3	CT	GG	AA	GC	AA	CC
DNA4	TT	GG	CA	CC	GA	CC
DNA5	CC	GG	CC	GG	GG	CC
DNA6	TT	GA	CA	CC	GG	CC
DNA7	CC	GG	CA	GC	GG	CC
DNA8	CC	GG	CC	GC	GG	CC
DNA9	CC	GG	CC	GC	GG	CC
DNA10	TT	GG	CA	GG	GG	CC
DNA11	CC	GG	AA	CC	GA	CC
DNA12	CC	GG	CA	CC	GG	CC
DNA13	CC	GG	CA	CC	GG	CC
DNA14	TT	GG	CC	GG	GG	CC
DNA15	CC	GG	CA	CC	GG	CC
DNA16	CC	GG	AA	GC	GG	CC
DNA17	CC	GG	CC	GC	GG	CC
DNA18	TT	GG	CA	GG	GG	CC
DNA19	CC	GG	CC	GG	GG	CC
DNA20	CC	GG	CC	GC	GG	CC

Table 1: SNP Genotypes of 20 samples using IrisPlex

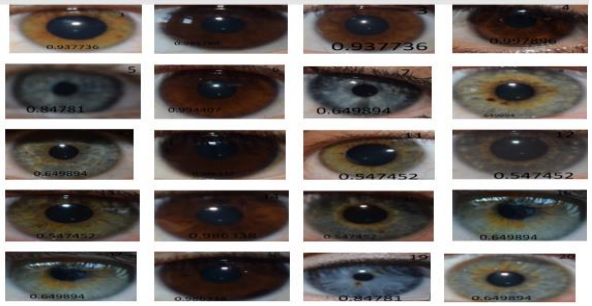

Figure 5: Eye colour Evaluation of the selected population and probabilities value of IrisPlex

heterozygous CT (10%). CC genotype occurred (100%) in blue and intermediate eye coloured individuals while in brown eyed individuals CT (25%) and TT (75%) genotype was present. SNP rs1800407 had two types of genotypes: homozygous GG (90%) and heterozygous GA (10%). GA heterozygous genotype was present only in Brown eye colour while GG homozygous genotype was present in intermediate (45%), brown (30%) and blue (15%) eye colour. SNP rs12896399 showed three types of genotype: homozygous AA (20%), CC (40%) and CA (40%) heterozygous. In three blue eye-coloured individuals, two types of genotype were present heterozygous CA (33%) and homozygous CC (66%). Brown eye-coloured individuals had three different types of genotype combinations: AA (25%), CA (50%) and CC (25%). Intermediate eye-coloured individuals also had three types of genotype combinations: AA (22%), CA (33%) and CC (44%) CC. SNP rs1691982 showed three types of genotypes: homozygous GG (25%), CC (30%) and heterozygous GC (45%). In blue eye-coloured individuals, two types of genotypes were present: heterozygous GC (66%) and homozygous GG (33%). In brown eye-coloured samples, three types of genotypes were present: homozygous CC (25%), GG (37%) and heterozygous GC (37%). Intermediate eye-coloured individuals also had three types of genotypes: homozygous CC (44%), GG (44.44%) and heterozygous GC (11%). SNP rs1393350 showed three types of genotypes: homozygous GG (85%), AA (5%) and heterozygous GA (10%). In blue eye-coloured individuals, the only genotype was homozygous GG (100%) while in intermediate coloured samples, two types were present: GA (11%) and GG (88%). Brown eye-coloured individuals had three types of genotypes: AA (12%), GA (12%) and GG (75%). SNP rs12203592 showed only one type of homozygous genotype: CC.

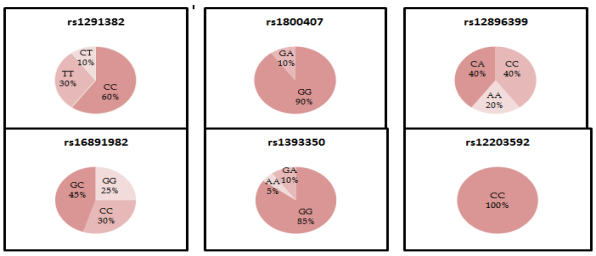

Figure 6: Genotype distribution across population of the SNPs included in IrisPlex system

Association of IrisPlex SNPs with gender and PIE score

Associations of six SNPs included in IrisPlex were checked with gender and PIE-score (Table 2). SNP rs12913832 (HERC2) showed the strongest association showing significant difference. Moreover, no significant associations with gender and PIE-score were detected. Statistical correlation found significant association of rs1291832 with eye colour phenotype.

Prediction Model accuracy

Original IrisPlex system was tested on 20 samples of unrelated individuals of Pakhtun population of District Swat. The individual eye colour was assigned by high probability value of IrisPlex system. Predicted eye colour was compared to the real eye colour. Accuracy of IrisPlex system for predicting brown eye colour was 100 percent with a high probability value of 0.994407, blue eye colour was also predicted 100 percent with a probability value of 0.84781. Intermediate eye colour was incorrectly predicted as blue (55.55%) with 0.649894 probability or brown (44.55%) with 0.547452 probability. Total probabilities and eye colour prediction result of the IrisPlex system is shown in Table 3.

Discussion

All individuals were classified in three eye colour categories: blue, brown and intermediate. The Pakhtun population of Swat district have dominantly brown eye colour followed by the intermediate and blue eye (Figure 2). Our data show almost similar results with Spanish population where brown eye colour is dominant over other colours. In Europe, blue eye colour decreases from Northern to Southern while brown colour increases, from average 17.8% in southern Europe and 81% in Northern Europe. Estonia, Norway and Northern Ireland showing the highest number (75%) of blue-eye colour. Northern Italy (29%) and France (28.7%) showed average to low blue-eye colour individuals. Greece (12.4%) and Spain (16%) showed the lowest average frequency of blue eye colour [16,17,19,20]. So, it may be inferred from the data that specificity of any eye colour is not related with any population or region of the world.

Eye colour percentage turn out to change between male and females (Figure 1). The percentage of brown eye females was higher than male while intermediate and blue eye colours were more dominant in males as compared to females. In comparison, in a study of Polish individuals, most dominant colour was blue (52.5%) followed by hazel and green (12.5% and 21.4%) while brown was comparatively low (13.7%). Blue eye males were common (58.1%) then blue eye females (42.2) [21]. Our results concords with those of Spanish population where males were found to have predominantly blue and intermediate colours while females have dominantly brown eye colours. Blue-eyed females (8.5%) were less than blue-eyed males (14.71%) whereas brown colour was more prevalent in females (78.45%) than males (71.43%) in the Spanish population [22]. Some similar results were found in other countries where blue colour was more prevalent in males than females in populations such as Iceland, Holland [23], Australia [24], Poland [25]

		rs12913832	rs1800407	rs12896399	rs16891982	rs1393350	rs12203592	Gender	Eye colour	Pie score
rs12913832	Pearson Correlation	1	-.248	-.083	.131	-.180	.[a]	-.075	.801**	.801**
	Sig. (2-tailed)		.291	.727	.582	.449	.	.754	**.000**	**.000**
	N	20	20	20	20	20	20	20	20	20
rs1800407	Pearson Correlation	-.248	1	.186	.059	-.134	.[a]	-.034	-.358	-.358
	Sig. (2-tailed)	.291		.432	.806	.574	.	.888	.121	.121
	N	20	20	20	20	20	20	20	20	20
rs12896399	Pearson Correlation	-.083	.186	1	.590**	.180	.[a]	.337	-.240	-.240
	Sig. (2-tailed)	.727	.432		.006	.449	.	.146	.308	.308
	N	20	20	20	20	20	20	20	20	20
rs16891982	Pearson Correlation	.131	.059	.590**	1	.353	.[a]	.548*	-.063	-.063
	Sig. (2-tailed)	.582	.806	.006		.127	.	.012	.792	.792
	N	20	20	20	20	20	20	20	20	20
rs1393350	Pearson Correlation	-.180	-.134	.180	.353	1	.[a]	.363	-.201	-.201
	Sig. (2-tailed)	.449	.574	.449	.127		.	.115	.395	.395
	N	20	20	20	20	20	20	20	20	20
rs12203592	Pearson Correlation	.[a]	.[a]	.[a]	.[a]	.[a]	.[a]	.[a]	.[a]	.[a]
	Sig. (2-tailed)	.	.	.	.	.		.	.	.
	N	20	20	20	20	20	20	20	20	20
Gender	Pearson Correlation	-.075	-.034	.337	.548*	.363	.[a]	1	-.036	-.036
	Sig. (2-tailed)	.754	.888	.146	.012	.115	.		.880	.880
	N	20	20	20	20	20	20	20	20	20
Eye colour	Pearson Correlation	.801**	-.358	-.240	-.063	-.201	.[a]	-.036	1	1.000**
	Sig. (2-tailed)	**.000**	.121	.308	.792	.395	.	.880		**.000**
	N	20	20	20	20	20	20	20	20	20
Pie score	Pearson Correlation	.801**	-.358	-.240	-.063	-.201	.[a]	-.036	1.000**	1
	Sig. (2-tailed)	**.000**	.121	.308	.792	.395	.	.880	**.000**	
	N	20	20	20	20	20	20	20	20	20

Table 2: SNPs associations with gender and PIE- Scores and eye colour phenotype

P Bl Eye	P Inter Eye	P Br Eye	IrisPlex Result	Eye Colour
0.012229683	0.050034333	0.937735983	Brown	Brown
0.000222877	0.01398916	0.985787963	Brown	Brown
0.012229683	0.050034333	0.937735983	Brown	Brown
1.29E-05	0.002091151	0.997895931	Brown	Brown
0.84781029	0.087662846	0.064526864	Blue	Blue
4.87E-05	0.005544217	0.994407066	Brown	Brown
0.649894489	0.121838766	0.228266745	Blue	Blue
0.649894489	0.121838766	0.228266745	Blue	INTER
0.649894489	0.121838766	0.228266745	Blue	INTER
0.000271927	0.013390292	0.986337781	Brown	Brown
0.337744385	0.114803871	0.547451744	Brown	INTER
0.337744385	0.114803871	0.547451744	Brown	INTER
0.337744385	0.114803871	0.547451744	Brown	INTER
0.000271927	0.013390292	0.986337781	Brown	Brown
0.337744385	0.114803871	0.547451744	Brown	INTER
0.649894489	0.121838766	0.228266745	Blue	INTER
0.649894489	0.121838766	0.228266745	Blue	INTER
0.000271927	0.013390292	0.986337781	Brown	Brown
0.84781029	0.087662846	0.064526864	Blue	Blue
0.649894489	0.121838766	0.228266745	Blue	INTER

Table 3: The Irisplex result of the eye color of district Swat population. Bold color represents the probability value of specific eye color

and France [26], as well as in this study (Figure 1). These data indicate darker eye colours for females and brighter eye colours for males.

Although previous studies showed significant association of IrisPlex SNPs with eye colour in Northern European populations [16,20,27,28], In this study, rs12913832 (in the *HERC2*), rs1393350 (*TYR* gene), rs1800407 (OCA2 gene) showed strong association with eye colour (Table 3). Previously, rs12913832 is shown to account for most eye colour variations in Caucasians [12,14-17,19,20,22-25,27,29], while the remaining 5 SNPs apparently have a relatively minor role [16,20,28,30].

The eye colour distribution in the current data set (n = 20) consisted of brown = 8 (40.0 %), intermediate = 9 (45.0%) and blue = 3 (5.0%) eye colour. The SNPs association test is presented in (Table 2), which shows that rs12913832 is associated most significantly with eye colour (P-value = 0.000). The allele (CC) is responsible for having non-brown eyes. All brown-eyed subjects were either homozygous or heterozygous i.e. TT or CT. The remaining non blue and non-brown were homozygous for CC genotype. rs12203592 SNP was not included in they were not polymorphic in this population (Figure 6). SNP, rs12913832 associated with eye colour was previously in rank 1 [20], shows a durable geographic design in genotypes across District Swat.

Irisplex online tool (http://hirisplex.erasmusmc.nl/) was used for the present samples. Considering all SNPs of irisplex, probabilities for eye colours were generated for the samples. The highest probability value of irixplex system was used to assign eye colour of the individual. Predicted eye colour was compared to the real eye colour. Accuracy of IrisPlex system for predictingd brown eye colour was 100 percent with a high probability value of 0.994407, blue eye colour was also predicted 100 percent with a probability value of 0.84781. Intermediate eye colour was incorrectly predicted as blue (55.55%) with 0.649894 probability or brown (44.55%) with 0.547452 probability (Table 3). In New Zealand, accuracy for blue eye colour was 89% and 94% for brown, while prediction accuracy was low (46%) for intermediate eye colour [31]. In the United States, the correct eye color prediction rate was 95% and 88% for blue and brown eye colors, respectively, the intermediate color at this threshold did not yield any true positive predictions [32]. In Slovenian population sensitivity was 93.6% for blue, 58.1% for brown, and 0% for intermediate eye color [33]. In Portuguese population the results demonstrated eye colour prediction accuracies of the IrisPlex system of 90% and 60 % for brown and blue eye color, respectively, and 77 % for intermediate eye color [34].

PIE-score was assessed by comparing photographs with human groupings of the similar images. The PIE-score showed association with colour phenotypes (Table 2). This was predictable because the PIE-score is created on the numbers of blue and brown. Blue pixels (both light and dark) were classified as blue and brown pixels (both light and dark) were classified as brown. Variation in the PIE-score was mostly clarified by SNP rs12913832. After the association for the effect of rs12913832 influenced the PIE-score significantly, whereas rs16891982, rs12896399, rs1393350, rs1800407 and rs12203592 did not (Table 2). Our result is similar to [35] where PIE score is significant with eye colour and rs1291832 SNP.

This study shows reliability of the IrisPlex model for accurately predicting brown and blue eye colour in Pakhtun population of Swat. The IrisPlex system, with sign providing for data across Swat district, is highly informative and appropriate for FDP applications in forensic sciences. However, more detailed study is required for increasing the accuracy of prediction especially for intermediate colours. In the near future, these data along with data for prediction of hair colour, age and other such EVCs that may become available with further studies may be used for forensic case work. These types of studies may be required for police and law enforcement agencies to solve cold cases where no data are available except DNA samples without any reference to match.

Acknowledgement

This research was funded by Higher Education Commission of Pakistan (HEC) under National Research Program for Universities Grant No. NRPU # 6828.

Authors' Contribution

MAR and MH performed experimental work. HK and IH were involved in planning and data analysis. MI was the Principal Investigator.

Competing interest

All the authors declare that they have no competing interest that can affect the current study.

References

1. Bräuer G, Chopra V. Estimating the heritability of hair colour and eye colour. Journal of Human Evolution, (1980); 9(8): 625-630.
2. Lin B, Mbarek H, Willemsen G, Dolan C, Fedko I, *et al*. Heritability and genome-wide association studies for hair color in a Dutch twin family based sample. Genes, (2015); 6(3): 559-576.
3. Zhu G, Evans DM, Duffy DL, Montgomery GW, Medland SE, *et al*. A genome scan for eye color in 502 twin families: most variation is due to a QTL on chromosome 15q. Twin Research and Human Genetics, (2004); 7(2): 197-210.
4. Rees JL. The genetics of sun sensitivity in humans. The American Journal of Human Genetics, (2004); 75(5): 739-751.
5. Sturm RA. Molecular genetics of human pigmentation diversity. Human molecular genetics, (2009); 18(R1): R9-R17.
6. Sand BJ, Babich M, Haghighi AZ (2009) Transdermal drug delivery compositions and topical compositions for application on the skin. Google Patents.
7. Shapiro J Hair Disorders: Current Concepts in Pathophysiology, Diagnosis and Management, An Issue of Dermatologic Clinics. Chapter: Book Name. 2012 of publication; 31; Elsevier Health Sciences.
8. Scherer D, Kumar R. Genetics of pigmentation in skin cancer—a review. Mutation Research/Reviews in Mutation Research, (2010); 705(2): 141-153.
9. Lamason RL, Mohideen M-AP, Mest JR, Wong AC, Norton HL, *et al*. SLC24A5, a putative cation exchanger, affects pigmentation in zebrafish and humans. Science, (2005); 310(5755): 1782-1786.
10. Liu F, Van Der Lijn F, Schurmann C, Zhu G, Chakravarty MM, *et al*. A genome-wide association study identifies five loci influencing facial morphology in Europeans. PLoS genetics, (2012); 8(9).

11. Crawford NG, Kelly DE, Hansen ME, Beltrame MH, Fan S, *et al*. Loci associated with skin pigmentation identified in African populations. Science, (2017); 358(6365): eaan8433.
12. Eiberg H, Troelsen J, Nielsen M, Mikkelsen A, Mengel-From J, *et al*. Blue eye color in humans may be caused by a perfectly associated founder mutation in a regulatory element located within the HERC2 gene inhibiting OCA2 expression. Human genetics, (2008); 123(2): 177-187.
13. Posthuma D, Visscher PM, Willemsen G, Zhu G, Martin NG, *et al*. Replicated linkage for eye color on 15q using comparative ratings of sibling pairs. Behavior genetics, (2006); 36(1): 12.
14. Cavalli-Sforza LL, Cavalli-Sforza L, Menozzi P, Piazza A The history and geography of human genes. Chapter: Book Name. 1994 of publication; Princeton university press.
15. Frost P. European hair and eye color: a case of frequency-dependent sexual selection? Evolution and Human Behavior, (2006); 27(2): 85-103.
16. Walsh S, Liu F, Ballantyne KN, van Oven M, Lao O, *et al*. IrisPlex: a sensitive DNA tool for accurate prediction of blue and brown eye colour in the absence of ancestry information. Forensic Science International: Genetics, (2011); 5(3): 170-180.
17. Walsh S, Liu F, Wollstein A, Kovatsi L, Ralf A, *et al*. The HIrisPlex system for simultaneous prediction of hair and eye colour from DNA. Forensic Science International: Genetics, (2013); 7(1): 98-115.
18. Green MR, Sambrook J. Molecular cloning. A Laboratory Manual 4th, (2012).
19. Andersen JD, Johansen P, Harder S, Christoffersen SR, Delgado MC, *et al*. Genetic analyses of the human eye colours using a novel objective method for eye colour classification. Forensic Science International: Genetics, (2013); 7(5): 508-515.
20. Walsh S, Wollstein A, Liu F, Chakravarthy U, Rahu M, *et al*. DNA-based eye colour prediction across Europe with the IrisPlex system. Forensic Science International: Genetics, (2012); 6(3): 330-340.
21. Pośpiech E, Karłowska-Pik J, Ziemkiewicz B, Kukla M, Skowron M, *et al*. Further evidence for population specific differences in the effect of DNA markers and gender on eye colour prediction in forensics. International journal of legal medicine, (2016); 130(4): 923-934.
22. Martinez-Cadenas C, Peña-Chilet M, Ibarrola-Villava M, Ribas G. Gender is a major factor explaining discrepancies in eye colour prediction based on HERC2/OCA2 genotype and the IrisPlex model. Forensic Science International: Genetics, (2013); 7(4): 453-460.
23. Sulem P, Gudbjartsson DF, Stacey SN, Helgason A, Rafnar T, *et al*. Genetic determinants of hair, eye and skin pigmentation in Europeans. Nature genetics, (2007); 39(12): 1443.
24. Duffy DL, Montgomery GW, Chen W, Zhao ZZ, Le L, *et al*. A three–single-nucleotide polymorphism haplotype in intron 1 of OCA2 explains most human eye-color variation. The American Journal of Human Genetics, (2007); 80(2): 241-252.
25. Branicki W, Brudnik U, Wojas-Pelc A. Interactions between HERC2, OCA2 and MC1R may influence human pigmentation phenotype. Annals of human genetics, (2009); 73(2): 160-170.
26. Katsara M-A, Nothnagel M. True colors: A literature review on the spatial distribution of eye and hair pigmentation. Forensic Science International: Genetics, (2019); 39: 109-118.
27. Branicki W, Brudnik U, Kupiec T, Wolańska-Nowak P, Szczerbińska A, *et al*. Association of polymorphic sites in the OCA2 gene with eye colour using the tree scanning method. Annals of human genetics, (2008); 72(2): 184-192.
28. Ruiz Y, Phillips C, Gomez-Tato A, Alvarez-Dios J, De Cal MC, *et al*. Further development of forensic eye color predictive tests. Forensic Science International: Genetics, (2013); 7(1): 28-40.
29. Sturm RA, Duffy DL, Zhao ZZ, Leite FP, Stark MS, *et al*. A single SNP in an evolutionary conserved region within intron 86 of the HERC2 gene determines human blue-brown eye color. The American Journal of Human Genetics, (2008); 82(2): 424-431.
30. Mengel-From J, Børsting C, Sanchez JJ, Eiberg H, Morling N. Human eye colour and HERC2, OCA2 and MATP. Forensic Science International: Genetics, (2010); 4(5): 323-328.
31. Allwood JS, Harbison S. SNP model development for the prediction of eye colour in New Zealand. Forensic Science International: Genetics, (2013); 7(4): 444-452.
32. Dembinski GM, Picard CJ. Evaluation of the IrisPlex DNA-based eye color prediction assay in a United States population. Forensic Science International: Genetics, (2014); 9111-117.
33. Kastelic V, Pośpiech E, Draus-Barini J, Branicki W, Drobnič K. Prediction of eye color in the Slovenian population using the IrisPlex SNPs. Croatian medical journal, (2013); 54(4): 381-386.
34. Dario P, Mouriño H, Oliveira AR, Lucas I, Ribeiro T, *et al*. Assessment of IrisPlex-based multiplex for eye and skin color prediction with application to a Portuguese population. International journal of legal medicine, (2015); 129(6): 1191-1200.
35. Pietroni C, Andersen JD, Johansen P, Andersen MM, Harder S, *et al*. The effect of gender on eye colour variation in European populations and an evaluation of the IrisPlex prediction model. Forensic Science International: Genetics, (2014); 111-6.