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Gender Identification by Amelogenin Gene using PCR and Prediction of Blue, Brown and Intermediate Eye Colour from Human DNA

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ABSTRACT

Forensic analysis of human identification gender must be considered important for the testing of mixed male and female samples. In our experiment, the amelogenin gene is used for the investigation of human gender and the HirisPlex tool for eye colour prediction. A single primer pair was used to amplify the amelogenin gene. The effectiveness of the primer has been tested in known humans. For the PCR band pattern, the X-linked gene partially produced a 106 -bp fragment and the Y-linked gene produced a 112 -bp fragment. All human test results showed a male sample with two bands (106 -bp and 112 -bp) and a female sample with only one band (106 bp). We also find out the eye colour of individuals after gender confirmation through an online eye colour prediction tool. The HirisPlex tool accurately predicted the blue and brown eyes of Pakhtoon population living in Malakand division. These findings are reliable techniques for tracking the AMELX/Y gene as a marker for polymerase chain reaction testing for gender identity. It also suggests that the HIrisPlex tool is ideal for the prediction of blue and brown eye colours.

Keywords: Pakhtoon, DNA extraction, PCR assay, Amelogenin gene, Gender identification.

Introduction

Sex determination plays a very important role in the medicals diagnosis of sex-linked abnormalities and also in forensics discrimination. Gender identity is the first step in the criminal investigation process. Gender identity was recently performed in addition to the STR markers for personalization. The Amelogenin gene is one of the most useful genes for identifying humans. The AMEL gene has been proven in molecular sex-determining studies associated with skeletal remains products [1- 3]. The amelogenin gene is present on Y and X chromosomes, but the expression levels are not the same for both genes. The expression level of the X gene is 4-10 percent higher than the expression level of the Y gene [4-6]. The size of the AMEL gene is different in both chromosomes while the large sequence is highly conserved. The conserved region has been used to separate females from males individuals. The advantage of this gene is that a single primer set is using to amplify same targeted region on both the Y and X amelognin gene [7- 8].

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 concert with black-brownish

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colour that present in dark hairs, eyes and skin. Unlike eumelanin, pheomelanin is reddish-yellow and occurs in light hair color, eye color, and light skin color (9). Genome-wide association studies (GWAS) conclude that (HERC2, IRF4, OCA2, TYR, SLC45A and SLC24A5) play important role in human coloration (10).

Walsh et al 2014 has developed and published HIrisPlex tool that predicts eye, hair and skin colour based on high-probability DNA values [12- 22].
In our study, we used an extended amelogenin gene PCR method to identify human gender from known samples (males and females) to confirm the accuracy of amelogenin gene primer set. Amelogenin (AME; Xp22.31-p22. 1/Ypl 1.2), whose alleles are 106 bp and 112 bp, which correspond to the X and Y chromosomes, respectively [23].

Literature Review

The ability to predict EVC from DNA is a new area of forensic genetics that is superior to other methods of detecting unknown individuals, which was difficult with conventional STR method. To obtain a phenotypic characters it is a need in the current station in the field of forensic sciences to develop such a method to predict exact image of the person who left the sample.
In 1991 (Nakahori et al [24] for the first time sequence human AMEL gene in primates [25] these sequence is very conserved, the amelogenin gene is present on both Y and X chromosomes. The size and location of amelogenin gene differs between the different X chromosomes (2872 bp at p22 region) and Y (3272 bp 11p12) [25]. This gene encodes a matrix protein that is involved in the formation of tooth enamel, which consists of 90 percent organic compound [26]. The PCR product obtained from AMELY and AMELX chromosomes is distinguished from each other based on 6- bp deletion in the first intron. The sequence of amelogenin gene have been used in different molecular analysis to distinguish female from male with unclear phenotypes, or to determine gender from biological samples for a variety of purposes. Both X and Y products can be amplified in a single PCR reaction. As an internal positive control the amelogenin gene have great advantage to represent homologous X chromosome fragment which should always be present. Mostly two type of amelogenin primers set are using to amplify 212/ 218 or 106/112 bp fragment for X/Y [27-28]. Current several studies have shown that mutations in the amelogenin homologous gene are costly for male and females determination when used in criminal inquiries (forensic cases) or in identifying human bodies in large scale ruins [38].

Currently, numerous SNPs and various methods are being developed to determine an individual's phenotypes (gender and eye color). The identified SNPs probably differ in populations with different eye colour and gender identity. Unusually, the vast popular of iris pigmentation diversity has been recognized to a single locus, rs12913832 [29- 30]. Locus rs12913832 was also found linked with eye colour [15, 31- 32]. The rs12913832 plays preliminary role in distinguishing blue-colored irises from brown-colored irises [14]. Some studies also indicated that SNPs like rs12203592 [33, 12] associated with eye colour. While a variant have been recognised in OCA2, that has been constantly associated with variation in iris colouring [34]. In SLC24A4 gene the variant rs12896399 was found in various research groups that this SNP is associated in iris colour difference. In SLC45A2 gene marker rs16891982 have been identified that they have a key role in the development of light colour in European populations [35]. The rs1393350 and rs1126809, were found to be associated with variation in eye colour [10, 12].

Materials and methods

Samples Collection

Buccal swabs and eye colour photograph samples were collected from female and male individuals of Pakhtoon population living in Malakand division and those individuals having any kind of eye disease or heterochromia were removed from the study [36]. Stander method for DNA isolation was used to extract DNA [37].

Gender determination based on genetic marker

Gender (male, female) was determined by using a single pair of revers (5'ATCAGAGCTTAAACTGGGAAGCTG-3') and foreword primer (5'CCCTGGGCTCTGTAAAGAATAGTG- 3') Amelogenin gene (Beraud-Colomb et al., 1995). The product of amelogenin X, Y gene was amplified kept the final reaction volume 20 µl. The reaction materials were Taq Poly 0.5 µl, 2µl reaction buffer, 2µl MgCl, dNTPs was 0.4µl, 0.5 µl revers Primer, 0.5 µl forward primers, DNA 2 µl, H2O 13.5µl. The incubation processes was carry out in PCR machine (XP Thermal Cycler BIOER TECHNOLOGY). Thermo cyclic condition were kept as (1)initial denaturation was perform for 10 mint at 95°C (2) 35 cycle 95 °C for 1mint, 59°C for 1 mint, 72°C for 1 mint (3) 72°C for 10 mint. The final amplified product was visualized on 6% agarose gel. The eye colour of a specific individuals was predicted on the basis of probability value through online systems (IrisPlex)

SNP Genotyping

Six SNPs namely, TYR- rs12896399, SLC24A4- rs16891982, SLC45A2- rs1393350, OCA2- rs1800407, HERC2- rs12913832 and IFR4- rs12203592 genes were pick from the literature [14]. DNA was amplified in 20 µl volume, buffer was 2µl, MgCl2 2µl, reverse primer was 0.5µl and forward primer was 0.5µl (10µM), 0.4µl of dNTPs, 2.0 µl DNA, 0.5µl Taq polymerase and ddH2O and the reaction was carried into XP, Thermal Cycler, (Bioer technology co, ltd). PCR condition were set as: 10 minutes at 95°C was set for initial denaturation, cycling condition 35 cycles of (denaturation at 95°C for 30 sec, annealing was set at 60°C for 30 sec and extension at 60°C for 30 seconds) and extension for 5 mint at 62 °C. Multiplexed SBE chemistry was applied for SNPs amplification. After SNPs amplification the product was treated with ExoSAP- IT (USB1 Corporation) at 37 °C for 15 min to remove unwanted materials (primers/ dNTPs). To stop enzyme activity the product was incubated at 85 °C for 15 mint. From treated PCR product 1.5µl and 1.5 µl of extension primers was added with 2.5 µl of SNaPshot prepared reaction mix. To amplify the final PCR product thermal cyclic condition were set 30 cycles as: 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 30 seconds. After Amplification the product was incubated 1 µl of SAP at 37 °C for 80 minutes to clean. To stop enzymatic reaction, the product was treated at 85°C for 15 mints. Capillary electrophoresis was performed using ABI Prism 3730xl Genetic Analyzer (Life Technologies Inc, USA). GeneMapper software was for genotyping v 4.0 (Life Technologies Inc, USA).

Eye colour prediction tool

The main purpose of DNA phenotyping was to define a genotype that can exactly conclude the phenotype of the person. The HirisPlex (http://hirisplex.erasmusmc.nl) eye colour prediction with three outcomes (blue, intermediate and brown) based on multinomial logistic regression higher probability value denoted the specific eye colour of the individuals was used.

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Results

Classification of Eye Colour in Different Eye Colour Categories

The current study showed that (81.6%) of individuals having brown eye colour, (12.8%) were intermediate while (5.6%) of individuals were blue eye colour as shown figure 1.

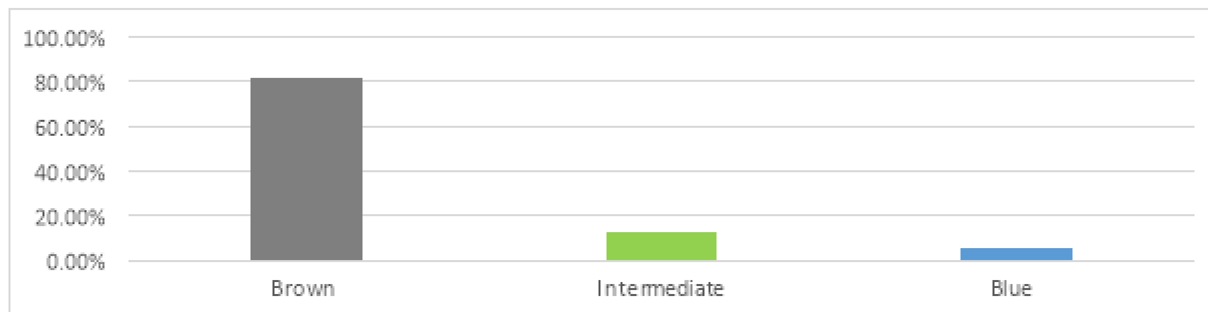

Figure 1: Percentage of brown, intermediate and blue eye colour in the study population

Molecular Analysis of the study

Quality of DNA on Agarose Gel

A total genomic DNA was extracted from buccal swab samples and were used for further experiment as shown in (figure.2) while the bad ones were reisolated.

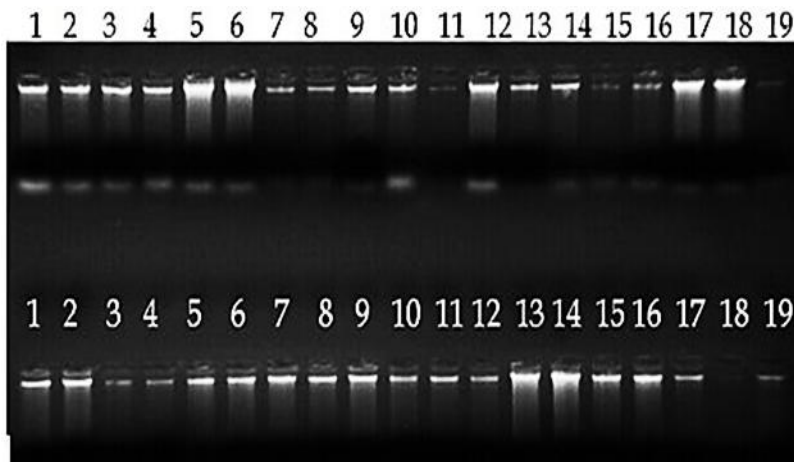

Figure. 2: Extracted DNA from buccal swab samples

Gender identification

For male and female sex determination the known male and female sample from the current study were used as a control and 30 randomly selected samples were analyzed. After PCR amplification two type of bands were obtained. Male individuals were two type of band (106 and 112-bp) while female's individual was a single 106-bp obtained. The PCR product analyzed simply by 6% agarose gel electrophoresis as shown in figure.3.

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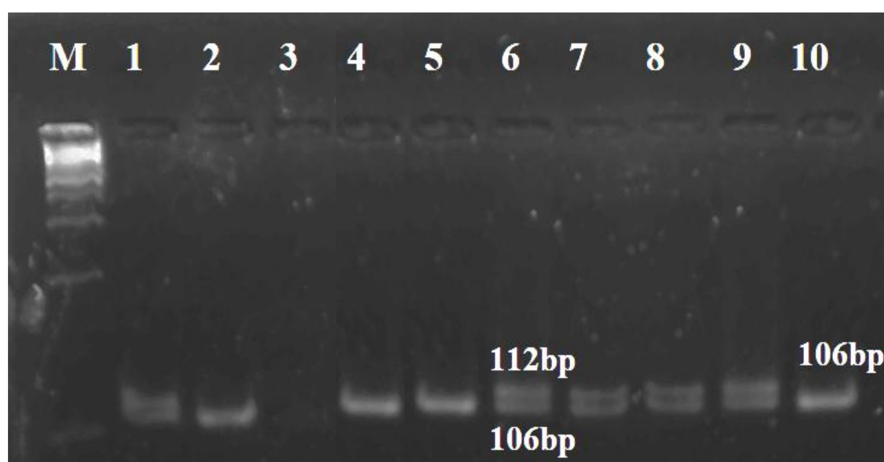

Figure. 3: PCR optimization result on 6% agarose gel. Lane M showing 100bp DNA Marker. Lane 1 male control and lane 2 Female control samples, Lane 3 indicated negative control, lane 4,5 and 10 female samples, lane 6, 7, 8 and 9 showing male samples .

Simples PCR for SNPs amplification

Figure 4 shows the amplified products of PCR for the six SNPs of TYR, SLC24A5, OCA2, HERC2, SLC45A2 and IRF4 were checked on 2% agarose gel.

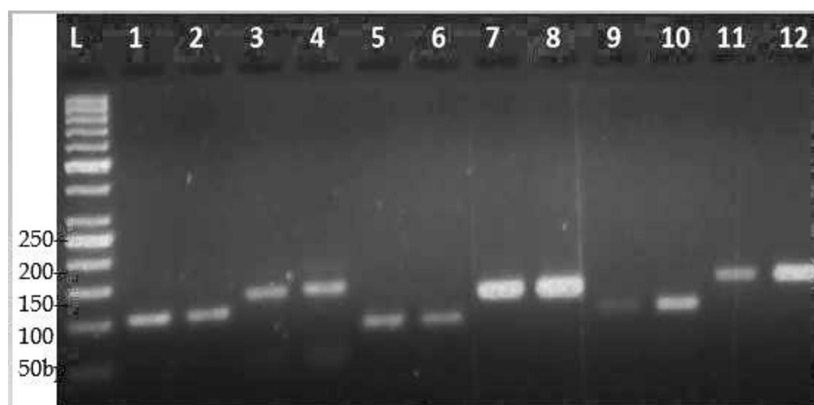

Figure. 4: figure show result of different SNPs on result on 2% agarose gel. Line L show 50bp DNA Marker. Line 1,2 indicated SLC24A5 gene SNP, 3,4 show OCA2 gene, Line 5,6 TYR, 7,8 show IRF4 gene, 9,10 indicated HERC2 gene and 11,12 show SNP of SLC45A2 gene.

Genotyping

Multiplexed SBE chemistry was applied for SNPs amplification. After amplification of SNPs the capillary electrophoresis was performed and in the electropherograms each line indicated exact SNP shown in figure 5.

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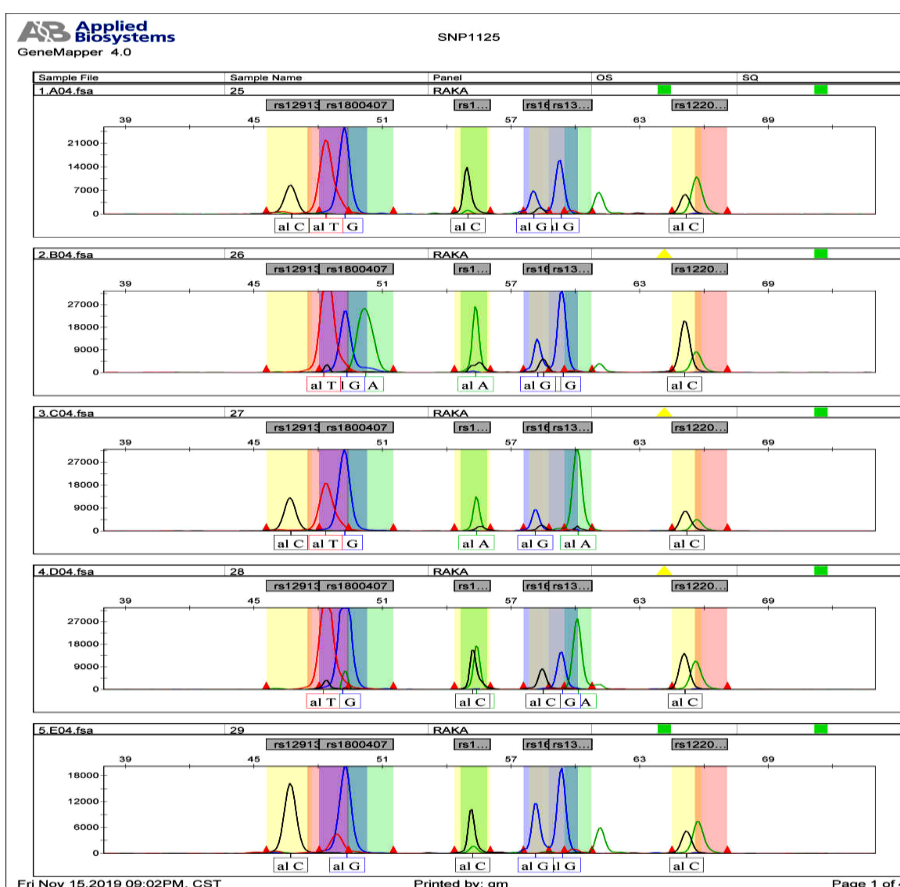

Figure. 5: Electropherograms of the Capillary Electrophoresis Assay

Genotypes across in the study population

Genotypes was extracted though gene mapper softer. The analysis showed that genotypes of rs12913832 SNP consisted of CT (49.4%), TT (31.6%) and CC (19.0%). rs1800407 SNP consisted of GG (82.3%) and GA (17.7%). rs12896399 were consisted of CC (58.2%), AA (6.3%) and CA (35.5%). rs16891982 were consisted of GC (32.9%), GG (51.9%) and CC (15.2%). rs1393350 SNP consisted of GA (11.4%), AA (1.3%) and GG (87.3%). While rs12203592 SNP consisted of CC genotype only as listed in Table. 1.

Table 1: Genotypes of Six SNPs present in the study population

	Genotypes							
SNPs	**CT**	**TT**	**CC**	**GG**	**GA**	**CA**	**GC**	**AA**
rs12913832	49.40%	31.60%	19.00%	-	-	-	-	-
rs1800407	-	-	-	82.30%	17.70%	-	-	--
rs12896399	-	-	58.20%	-	-	35.50%	-	6.30%
rs16891982	-	-	15.20%	51.90%	-	-	32.90%	
rs1393350	-	-	-	87.30%	11.40%	-	-	1.30%
rs12203592	-	-	100%	-	-	-	-	-

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Prediction of eye colour

The six SNPs comprising this MLR-based, published eye colour model [13] are included in this study. The results of this calculator are prediction probabilities for blue, brown, or intermediate eye colour. Following previous studies [11, 14] raw probabilities were considered and eye colour prediction assigned to the category with the highest probability. These prediction probabilities were compiled for each individual and compared to their reported eye. Total of (150) samples were checked brown eye colour individuals were (111), blue (13) and intermediate were (26) in number. In Malakand Division brown and blue eye colour was predicted in all cases, while intermediate eye colour in the population of Malakand Division 55.55% was predicted blue and the remaining 45.45% was brown eye colour as shown in Table. 2. The probability values and record of samples used in the current study is as listed in (Table.3)

Table 2: Validation of eye colour prediction model: Prediction success (bold values) estimates from the Pakhtoon population residing within the Malakand Division.

Prediction Models	**Predicted eye colour**			**Undetected**	**No of samples**
	Blue	**Brown**	**Intermediate**		
Blue	**100%**	0	0	0	**(13)**
Brown	0	**100%**	0	0	**(111)**
Intermediate	**55.55%**	**45.45%**	**0**	**0**	**(26)**

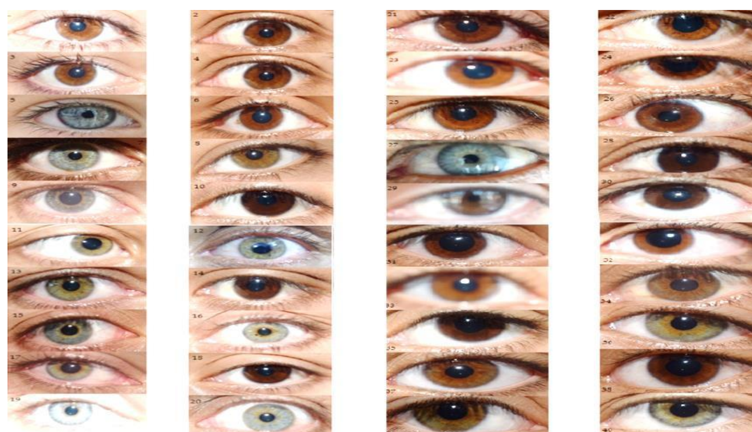

Figure 6: eye colour photographs used in study validation.

Table. 3: Irisplex 6 SNP genotypes colour prediction probabilities together with samples details of Pakhtoon population. Blue colour indicated Blue eye colour, brown indicated Intermediate and green colour indicated brown eye colour.

Samples	rs12913832	rs1800407	rs12896399	rs16891982	rs1393350	rs12203592	P Blue Eye	P Inter Eye	P Brown Eye	Gender	IrisPlex Result	Actual Eye Colour
1	CT	G	CC	GC	GG	CC	0.012	0.050	**0.9377**	M	**Brown**	**Brown**

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		G					23	034	**36**			
2	TT	GA	AA	GC	GG	CC	0.000223	0.013989	**0.985788**	M	**Brown**	**Brown**
3	CT	GG	AA	GC	AA	CC	0.01223	0.050034	**0.937736**	F	**Brown**	**Brown**
4	TT	GG	CA	CC	GA	CC	1.29E-05	0.002091	**0.997896**	F	**Brown**	**Brown**
5	CC	GG	CC	GG	GG	CC	**0.84781**	0.087663	0.064527	F	**Blue**	**Blue**
6	TT	GA	CA	CC	GG	CC	0.00705	0.005544	**0.994407**	F	**Brown**	**Brown**
7	CC	GG	CA	GC	GG	CC	**0.789894**	0.121839	0.228267	M	**Blue**	**Blue**
8	CC	GG	CC	GC	GG	CC	**0.649894**	0.121839	0.228267	F	**Blue**	**INTER**
9	CC	GG	CC	GC	GG	CC	**0.649894**	0.121839	0.228267	M	**Blue**	**INTER**
10	TT	GG	CA	GG	GG	CC	0.000272	0.01339	**0.986338**	F	**Brown**	**Brown**
11	CC	GG	AA	CC	GA	CC	0.337744	0.114804	**0.547452**	F	**Brown**	**INTER**
12	CC	GG	CA	CC	GG	CC	0.337744	0.114804	**0.547452**	M	**Brown**	**INTER**
13	CC	GG	CA	CC	GG	CC	0.337744	0.114804	**0.547452**	F	**Brown**	**INTER**
14	TT	GG	CC	GG	GG	CC	0.000272	0.01339	**0.986338**	M	**Brown**	**Brown**
15	CC	GG	CA	CC	GG	CC	0.337744	0.114804	**0.547452**	F	**Brown**	**INTER**
16	CC	GG	AA	GC	GG	CC	**0.649894**	0.121839	0.228267	M	**Blue**	**INTER**
17	CC	GG	CC	GC	GG	CC	**0.649894**	0.121839	0.228267	M	**Blue**	**INTER**
18	TT	GG	CA	GG	GG	CC	0.000272	0.01339	**0.986338**	F	**Brown**	**Brown**
19	CC	GG	CC	GG	GG	CC	**0.85781**	0.087663	0.064527	F	**Blue**	**Blue**
20	CC	GG	CC	GC	GG	CC	**0.649894**	0.121839	0.228267	M	**Blue**	**INTER**

Discussions

Amelogenin gene is present on both X and Y sex linked chromosomes. Whom PCR amplicons are of different size for male and female individuals. Because of the presence or absence of some nucleotide sequence over X-linked or Y-linked chromosomes respectively. Due to these insertions/deletions AMELX homologs in female shows single bands and AMELX/Y homologs in male shows two bands. This difference in the amelogenin gene homologs have been targeted

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by the use of single primer set in PCR. Of which Y-linked homolog always generate smaller size fragment than the X-linked homolog. Because of this differentiation in the AMELX and AMELY gene, it is used to identify the gender of almost all mammals.

The amelogenin gene is situated on both the Y and X chromosomes of human being. The size differences in this gene have been utilized for gender determination in the case of forensic work. In the present study we accurately determined female and male individuals on the basis of this gene as shown in (figure 3). Previously the AMEL gene has been used in various studies [1; 39, 3, 4, 40]. The structure and properties of this gene make it a very good candidate gene for sex identification from complicated forensic materials.

Eye colour show a varied global spread across North Africa and to Europe, the Central Asia, and Middle East. Eye colour showed extremely variation in the eye colour pigmentation that ranges from brown to blue and intermediate [30]. Previous studies showed that higher prevalence of eye colour (brown) categories was reported in Uzbekistan, Azerbaijan, Armenia, Tajikistan, Georgia, Kazakhstan [41- 42], Korea [43] and Japan [44]. In the present study we reported for the first time that brown eye colour is prevalent among the inhabitants of the Malakand Division as shown as (figure. 1) our study is similar to the previous mention studies. This stands in contrast to various populations of Europe (Iceland, Germany and Denmark) in which previous studies report that brown eye coloured individuals comprise but a small proportion of the population relative to those possessing irises of other colours [45, 10, 46; 34, 42]. In the present study the sampled individuals showed (12.8%) of intermediate eye colour. Similar results were present in the literature according to the present data for example, in Kazakhstan 11.65%, in Poland 12.5% and Iceland 14.15% intermediate eye colour individuals were found [10; 31, 41, 42]. The ratio of intermediate eye colour in Tajikistan is 7.67% and Uzbekistan 6.02% [41].In compared to the present result some different results were also present where the intermediate eye colour ratio is (39.6%) in Germany, (44%) in France and (55.2%) Spanish population 45]. Blue eye colour individuals are dominant in Denmark (64.84%), Poland (52.50%), and Iceland (73.90%) [46, 10, 34, 31].

The current study is not similar to the for-mention studies but supports the results recorded in the populations of Azerbaijan, Armenia, Georgia and Tajikistan Georgia [42]. A lots of studies have been performed in European, East Asian, South Asian, African and American populations [47, 49, 48; 50] generally, blue eye colour is predominant in Northern Europe whereas brown eye colour is extremely dominant in East Asia, American, Oceania, Africa and Sub- Saharan [51]. Worldwide different studies was conducted to understand the diversity in eye structure in different population but all of these research is limited categories to a particular countries so based on each study showed different structure variation so we are unable to compared our current study with previous studies. Further studies recommended to known the higher prevalence of eye colour in the world.

A better understanding of eye colour characteristics contributes significantly to the area of forensic, medicine, public health and anthropology. Today, several forensic research worker are developing pigment prediction tools. The main perseverance of these tools is to tolerate crime scene investigators to predict the eye colour of an unknown individual from a small sample of DNA. HirisPlex is probably the most famous of these tools. A previous study of the Italian population showed 76% accuracy of the Irisplex system [20]. A similar study by [16] found that the Irisplex system could predict brown, blue, and intermediate eye colours with 58%, 95%, and 11% accuracy, respectively. Allwood et al (2013) [52] found 89% accuracy in blue eye colour and 94% accuracy in brown eye colour.

Associated to earlier research, in the current study, blue and -brown eye colour is 100% predictable, the median intermediate colour phenotype is misclassified, 76.92% brown, 23.08%

was classified as blue. The medium dark phenotype was predicted to be predominantly brown, the light-eyed individuals were blue. Problems with predicting the mean eye have already been shown in previous studies.
[12- 22].

Conclusion

In our experiment, we concluded that employing amelogenin gene for the gender testing of unknown specimens through polymerase chain reaction is a reliable approach. Baccul swab is an authentic source for DNA isolation. Short fragment size is helpful in studying degraded DNA samples. Due to the difference in between the X and Y homologs of the same gene in male and female individuals was prominent and showing no ambiguity. No additional marker nor post PCR tests are required for the study validation.
We also concluded that the DNA prediction model (hirisplex) indicated high correctness in predicting brown and blue eye colours, while the uncertainties continued in the intermediate eye colour.

Limitations and Future Studies

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. Hence, we suggest that genotyping more SNPs that not included in the IrisPlex system straight increase the prediction accuracy in our study.

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