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Forensic DNA phenotyping: Prediction of eye and hair colour and allelic frequency estimation in the Italian population for the development of a reference dataset☆

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ABSTRACT

In recent years, Forensic DNA Phenotyping (FDP) has emerged as an innovative "DNA intelligence approach" aimed at predicting externally visible individual characteristics from biological traces collected at crime scenes or from biological samples of unidentified corpses or skeletal remains.

This study aims to analyse the allele frequency distribution of genetic markers involved in the prediction of eye and hair colour in an Italian population sample, to enhance the existing European dataset. A large cohort of Italian individuals with at least three generations of ancestry was recruited, ensuring representation of phenotypic variability. The 24 SNP markers included in the HIrisPlex panel were analysed using SNaPshot sequencing technique, and the genotypic data were uploaded to the FDP web tool validated for forensic use by the international VISAGE Consortium's. This tool provides prediction probabilities for three eye colour categories (blue, brown, and intermediate) and four hair colour categories (black, blonde, brown, and red).

The results confirmed a high predictive accuracy for the Italian population as well; however, the complex genetic structure of intermediate phenotypic traits highlights the need for novel prediction models that include genetic markers associated with complex phenotypes. The strong north–south European gradient in eye colour, along with the hypothesis that variation in eye colour is associated with skin pigmentation and UV environmental adaptation, underscores the importance for further research. These should focus on novel genetic markers to refine the European dataset, in which the Italian population may be included to enhance the accuracy of predictive models.

1. Introduction

In forensic DNA profiling for human identification, the analysis of short tandem repeats (STRs) represents the gold standard analysis. However, it is uninformative in criminal cases where the genetic profile fails to match any entries in existing DNA databases, or when the donor of the biological material is unknown to law enforcement, thereby precluding comparative STR analysis [1,2]. In such instances, alternative investigative approaches become essential, particularly in contexts involving the reconstruction of an individual's physical characteristics from skeletal remains, as seen in missing person cases or mass disaster victim identification (DVI) efforts. This is especially critical when remains are severely decomposed or lack traditional evidence relevant to identification [3,4]. A recent advancement in forensic genetic investigation is Forensic DNA Phenotyping (FDP), which provides information that can serve as "biological witnesses" [5] aiding in the

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identification of both victims and suspects. FDP specifically refers to the prediction of externally visible characteristics (EVCs) – including physical appearance, biogeographical ancestry, and age – from DNA extracted from human biological samples collected at crime scenes [1,6]. Among the appearance-related EVCs, the most commonly predicted traits are eye, hair, and skin colour, as these are among the most visible and informative features in forensic investigations [7].

These pigmentation traits show significant variation among individuals of European or nearby ancestry, such as those from the Middle East and parts of Western Asia. Eye colour typically ranges from blue to green to brown, while hair colour includes blonde, brown, red, and black. A similar gradient is observed in skin pigmentation [8,9]. This chromatic diversity arises from complex evolutionary processes, including population migration histories, mate selection [10] and adaptation to environmental stimuli [11]. Collectively, these factors have contributed to the development of lighter pigmentation patterns relative to the darker traits that remain predominant in non-European populations, particularly those without European or nearby genetic admixture, who more uniformly display black hair and brown eyes [8].

The HIrisPlex system is one of the primary tools currently employed for the prediction of appearance traits, such as eye and hair colour. It is based on a single multiplex genotyping assay that targets 24 DNA variants. These genetic markers were identified through studies on European populations as being highly informative for predicting pigmentation traits, thereby reflecting the system's optimization for individuals of European ancestry [4,8,12].

Notably, as reported in the study by Walsh et al. [4], the reference dataset used for the development and validation of the eye colour prediction model comprised approximately 9188 European individuals, of whom only around 500 were of Italian origin. Given the low number of Italian samples included, there is a potential limitation in the representation of the Italian population, which may affect the predictive accuracy of the system in specific European subpopulations. Similarly, although the hair colour prediction dataset included 1551 individuals from selected regions of Europe and Japan, the representation of Italians remains limited and not clearly defined. Therefore, some of the genetic markers selected as predictive for specific trait categories, such as eye or hair colour, may not retain the same predictive value across all populations, due to differing allele frequencies and genetic backgrounds.

This study aims to analyse the allele frequency distribution of genetic markers associated with externally visible characteristics (EVCs), specifically those involved in the prediction of eye and hair colour, in an Italian population sample. The analysis of 600 individuals will provide a framework for evaluating the suitability of existing predictive models for use in the Italian context.

2. Materials and methods

2.1. Ethical approval

The study has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. All analyses were performed in compliance with current legislation; all personal data collected will be processed in respect of privacy. Genetic data and biological samples are anonymized using alphanumeric codes, in accordance with ethical and legal regulations on the handling of biological samples and personal data. The project was approved by Research Ethics Committee of the University of Modena and Reggio Emilia (Protocol n. 2024-UNMRCLE-0341453, 20/12/2024) and by the Bioethical Committee of the University of Bologna (Protocol n. 0004851, 07/01/2025).

2.2. Volunteer recruitment and sample collection

The study protocol involves the recruitment of 600 male and female volunteers according to their Italian ancestry and written informed consent had been obtained from each volunteer. Inclusion criteria include being at least 18 years old and having Italian-origin parents and grandparents. To date, a total of 147 buccal swab samples has been analysed (47 females and 67 males).

Volunteers were asked to self-collect reference biological material using 4N6FLOQSwabs® Genetics (Copan) buccal swabs and to complete a questionnaire indicating their region of birth, that of their parents and grandparents and their natural eye and hair colour, including shades.

Participants were asked to report the colours that best represented their physical appearance by selecting from three predefined eye colour categories: blue, intermediate, and brown. The intermediate category included mixed shades such as blue-brown, blue-green, brown-green, green, and hazel, covering the full spectrum from deep blue to dark brown. For hair colour, participants chose from four main categories: black, brown (including both dark and light brown), blonde (including both light and dark blonde), and red.

For eye colour, participants selected from three predefined categories: blue, intermediate, and brown. The intermediate category included mixed shades such as blue-brown, blue-green, brown-green, green and hazel, covering the full spectrum from deep blue to dark brown. For hair colour, participants chose from four main categories: black, brown (including both dark and light shades), blonde (including both light and dark shades), and red.

2.3. DNA phenotyping analysis and interpretations

The DNA was extracted from the buccal swabs using ReadyAmp™ Genomic DNA Purification System and QIAamp DNA Mini QIAcube Kit (Qiagen). NanoDrop™ 2000 Spectrophotometer (ThermoFisher Scientific) was used to check the quantity of the isolated DNA.

Genotyping of the 24 SNPs targeted by the HIrisPlex system multiplex PCR was carried out using the SNaPshot™ assay (Applied Biosystems). A range of 0.5–0.7 ng/μl of DNA template was amplified using the QIAGEN Multiplex PCR Kit (Qiagen) with primer concentrations adapted according to Walsh et al. [4]. The SeqStudio Genetic Analyzer for HID (Applied Biosystems), paired with GeneMapper™ 6 software (Applied Biosystems) was used for sequencing analysis. A specific bin and panel file were generated based on the positive DNA control 9947A and the reference typing provided in the practical guide by Walsh and Kayser [12]. The resulting genotype data were uploaded to the open-source HIrisPlex Eye and Hair Colour DNA Phenotyping Webtool (https://hirisplex.erasmusmc.nl) to predict eye and hair colour, in accordance with the guidelines [13]. P-value results were interpreted using 0,7 threshold for eye colour, following Walsh et al. [14], while hair colour predictions were interpreted according to the scheme described in Walsh et al. [4].

The predicted phenotypes were compared with self-reported data from the Italian volunteer dataset to evaluate the system's precisions. The overall predictive performance of the HIrisPlex system was assessed by calculating the area under the receiver operating characteristic curve (AUC) for each colour category, using the freely available online tool SRplot [15].

Allele frequencies were calculated using the software STATA 18 (StataCorp LLC). Differences in population genetic parameters were assessed using a Z-test in Microsoft Excel, using previously published data from European populations [16] as a reference and available on web site https://www.ensembl.org/index.html [17].

3. Results

3.1. Volunteers EVCs overview

A total of 114 volunteers were included in the preliminary study. Based on self-reported data collected via questionnaires, their geographical distribution within the Italian population was as follows: 67 from Northern Italy, 8 from Central Italy, and 39 from Southern Italy.

Regarding eye colour, volunteers self-reported their phenotypes as follows: 46 individuals reported having brown eyes and 5 hazel (n = 51), grouped under the brown category; 29 green, 9 brown/green, 3 blue/green, and 1 blue/brown (n = 42), classified as intermediate; and 21 individuals reported having blue eyes. For hair colour, 8 individuals reported black hair, 83 brown (of which 40 were dark brown and 43 light brown), 20 blonde (including both blonde and dark blonde), and 3 red.

3.2. DNA typing

DNA was successfully extracted from all samples, yielding an amount sufficient for subsequent analyses. Amplification analysis produced 86 complete SNP profiles, while the remaining 28 samples exhibited allele

Table 1
Allele frequencies in SNPs analysed. Comparison of the allele frequencies distribution between European data through a *Z-test* and the P-values are also included. P-values > 0,5 threshold are highlighted with " *". NA: statistics could not be calculated; GREEN: Higher frequencies compared to the European population; GRAY: lower frequencies compared to the European population.

SNP	Allele	Allele frequencies				
		Italian data (N=114)		European Data (N=1006)		Data Analysis
		Major allele	Minor allele	Major allele	Minor allele	Z-test *p-value*
rs1042602	G > T	0,500	0,500	0,628	0,372	0,0001*
rs1110400	T > C	1,000	0,000	0,992	0,008	0,1764
rs11547464	G > A	0,982	0,018	0,991	0,009	0,1680
rs12203592	C > T	0,899	0,101	0,884	0,116	0,4675
rs12821256	A > G	0,938	0,062	0,879	0,121	0,0061*
rs12896399	T > G	0,697	0,303	0,570	0,430	0,0001*
rs12913832	C > T	0,535	0,465	0,364	0,636	0,0000*
rs1393350	C > T	0,788	0,212	0,756	0,244	0,2732
rs16891982	G > C	0,899	0,101	0,938	0,062	0,0137*
rs1800407	G > A	0,908	0,092	0,924	0,076	0,3441
rs1805005	G > T	0,851	0,149	0,888	0,112	0,0785
rs1805006	C > A	0,991	0,009	0,990	0,010	0,8588
rs1805007	C >T	0,952	0,048	0,928	0,072	0,1718
rs1805008	C >T	0,969	0,031	0,938	0,062	0,0542
rs1805009	G > C	0,987	0,013	0,992	0,008	0,3762
rs20132689	C > A	1,000	0,000	1,000	0,000	NA
rs31226290	C > A	1,000	0,000	0,997	0,003	0,4075
rs2228479	G > A	0,921	0,079	0,931	0,069	0,5534
rs2378249	T > C	0,899	0,101	0,883	0,117	0,4410
rs2402130	A > G	0,724	0,276	0,787	0,213	0,0190*
rs28777	A > C	0,921	0,079	0,956	0,044	0,0093*
rs4959270	C > A	0,553	0,447	0,524	0,476	0,3843
rs683	T > G	0,566	0,434	0,627	0,373	0,0550
rs885479	C > T	0,991	0,009	0,930	0,070	0,0003*

dropout in one or at most two SNPs. All undetected peaks corresponded to markers within the *MC1R* gene, notably *N29insA* rs312262906, which was the most frequently affected. As a result, predictions for these 28 samples may be less accurate. Notably, although 22 of these samples exhibited locus dropout at rs312262906, only one corresponded to a red-haired individual.

3.3. HIrisPlex 24 SNP allele frequencies

Allele frequencies are presented in Table 1. Some allele frequencies calculated for our Italian population differed significantly from those reported for the European reference population [16]. In particular, SNPs rs16891982, rs1042602, rs12821256, rs12896399, rs12913832, rs2402130, rs28777, and rs885479 showed statistically significant differences ($p < 0.05$) between the Italian population and the broader European populations. Among these, four SNPs exhibited a higher frequency of the mutated (*minor)* allele in the Italian population.

3.4. DNA phenotyping predictions

The predictions obtained from the HirisPlex web tool were compared with participants' self-reported data, revealing varying degrees of concordance across phenotypic categories (see Tables 2.a and 2.b). Results are presented as correct and incorrect predictions, as well as non-assigned cases (N.A.), which correspond to predictions with a probability below the established threshold. Frequency is reported for each trait.

When assessing the consistency between the self-declared and predicted eye colours, 62 phenotypes (54.3%) were found to match. In 33 cases (29%), there was no correspondence between the self-reported and predicted categories, while for 19 individuals (16.7%) no prediction was assigned by the tool. Specifically, within the blue eye colour category, predictions were correct in 85.7% of cases. In the intermediate category, no phenotypes were correctly predicted. Ten cases (23.8%) had prediction probabilities below the established threshold and were therefore classified as N.A. The incorrect predictions accounted for 76.2%, with 17 individuals predicted as blue and 15 as brown. For the brown eye colour category, the tool correctly predicted the phenotype in 86.3% of cases, and no incorrect predictions were observed. The AUC values obtained reflected the known limitations of the IrisPlex model, which performed well for blue and brown eye colours (AUC = 0.884 and 0.887, respectively) but showed substantially lower performance for the intermediate category (AUC ≈ 0.63; sensitivity < 10%), as previously reported by Walsh et al. [14] and Chaitanya et al. [6].

As for hair colour, comparison between self-reported phenotypes and the tool's predictions revealed 74 matches and 40 discrepancies. Specifically, incorrect predictions were observed in 12.5% of individuals self-reporting black hair, 36.1% with brown hair, 40.0% with blonde hair, and 33.3% with red hair, as shown in Table 2.b. It is important to note that most incorrect classifications for categories ranging from black to light brown were the result of assignments to different shades within the same colour range, rather than to distinctly different colour categories.

4. Discussion

In this study, we evaluated the performance of the HIrisPlex system for the Italian population.

Out of the 114 samples genotyped using the SNaPshot™ assay according to the conditions described by Walsh et al. [4], 25% exhibited

Table 2
Comparison between self-reported phenotypes and HIrisPlex predictions for eye colour (Table 2.a) and hair colour (Table 2.b).

a.

Eye Pigmentation Predicted	Correct	Incorrect		Not assigned	Total
Blue	18	1 Brown		2	21
Intermediate	0	Blue	Brown	10	42
		17	15		
Brown	44	0		7	51
Total	62	33		19	114

b.

Hair Pigmentation Predicted	Correct	Incorrect	Not assigned	Total
Black	7	1	0	8
Brown *(dark brown + light brown)*	53	30	0	83
Blonde *(dark blonde + light blonde)*	12	8	0	20
Red	2	1	0	3
Total	74	40	0	114

dropout in one or more *MC1R* markers, particularly at the SNP rs312262906 (*N29insA*). This finding is consistent with previously reported amplification challenges commonly observed with *INDELs*, and specifically within *MC1R* markers under low DNA input conditions [8]. Notably, for all samples showing dropout, the genotypes were included in the prediction tool, with missing data encoded as "NA", allowing predictions to be generated [13]. Although *N29insA* is a variant associated with red hair prediction when combined with other SNPs, it is relatively rare in the general population [4], and its absence does not substantially compromise the prediction of accuracy. Indeed, despite an AUC loss due to *N29insA* dropout in a red-haired sample, the prediction was nonetheless correct. Conversely, the only misclassified red-haired individual had a complete genotype profile, indicating that the misclassification was not due to a dropout.

To enhance genotyping efficiency, particularly for *MC1R* SNPs with a high dropout rate, the PCR conditions will be further optimized by increasing primer concentrations and redesigning the SBE primers to improve electropherogram peak resolution [4,8].

For seven samples, additional analyses will be necessary to confirm hair colour predictions, as dropout affected SNPs potentially associated with the other three hair colour categories (black, brown, and blonde).

Before evaluating the predictive accuracy and precision of the HIrisPlex web tool, allele frequencies of the 24 SNPs were assessed in our sample of Italian volunteers and compared with those of the European reference population. Overall, allele frequencies were consistent with the European data from the 1000 Genomes Project [16], except for eight SNPs, which showed statistically significant differences (p-value < 0.05; see Table 1). These findings suggest that not all markers previously identified as predictive in the general European population may demonstrate the same predictive power within the Italian subpopulation. As this is a preliminary study based on an initial subset of 114 individuals, out of a planned total of 600 subjects, the first phase of the project focused on evaluating the allele frequencies of genetic markers most frequently reported in the literature as associated with eye and hair pigmentation. Specifically, the analysis included markers such as *HERC2* rs12913832, *SLC45A2* rs16891982, rs28777 and *KITLG* rs12821256 for hair colour [18–20], as well as *HERC2* and *SLC24A4* rs12896399 for eye colour [21,22].

In this context, the genotype considered indicative of a specific hair colour category does not show a marked trend or phenotypic specificity within the Italian sample analysed. For example, the *KITLG* gene, whose G allele is commonly associated with light hair colour in the European population, was found to have a lower frequency in the Italian population (Table 1). Moreover, the absence of significant differences in the distribution of the mutated allele across phenotypic categories (black, brown, blonde, and red hair) suggests that the predictive power of this SNP may be limited in our population.

A different pattern was observed for the *SLC45A2* and *HERC2*, for which the C and T alleles, respectively, are known from the literature to be associated with darker hair colours [20]. In our dataset, these alleles showed higher frequencies in the Italian population compared to the European reference population, and genotype analysis revealed a non-uniform distribution of the mutated allele across phenotypic categories. In particular, the mutated allele was more frequently observed among individuals with brown hair compared to other hair colour groups; however, its frequency within this group remained limited, being present in only about half of the subjects. Given that the "dark hair" category exhibited the highest number of discordant predictions compared to the actual hair colour, suggests that, although associated with the phenotype, this SNP alone may not be sufficient to ensure high predictive accuracy (Table 2).

A possible explanation for these findings lies in the complex and polygenic nature of hair colour, which is determined by the interaction of multiple genetic variants [23]. Another important factor to consider is the unbalanced distribution of individuals across phenotypic categories, which may have reduced the statistical power to detect significant associations. This variability was, however, intentionally included in the study design to represent the phenotypic diversity of the Italian population.

The challenges associated with predicting brown and blonde hair colours are further complicated by the wide range of shade variation within these categories. This phenotypic heterogeneity not only interferes with the identification of genetic markers with strong predictive value but also impacts the accuracy of self-reported hair colour by participants. As a result, discrepancies may arise between the phenotype predicted by the HIrisPlex web tool and the colour reported by volunteers, introducing an additional layer of uncertainty in the interpretation of results.

In our dataset, predictive discrepancies were observed in approximately 33% of individuals within the brown and blonde hair categories. These inconsistencies are likely due to subjective perception of hair colour shades by participants [6,8], as well as age-related hair darkening, particularly during childhood. This latter phenomenon was evident in about 4% of the samples, where the predicted hair colour appeared notably lighter than the self-reported colour [5,8,24].

To minimize inaccuracies related to self-assessment, in the next phase, researchers will assist volunteers in completing the questionnaire and in assigning the most realistic colour category.

Photos of all participants will be collected after obtaining written informed consent, to help the researchers categorise each volunteer into the appropriate hair and/or eye colour group. It should be noted, however, that even when following established criteria and relying on expert evaluation, this classification process inevitably retains a degree of subjectivity.

Focusing on eye colour, some discrepancies also emerged between the data from the Italian population and those reported in the European literature. In particular, the mutated allele of the *SLC24A4* gene was found to be less frequent in the Italian population compared to the European average and showed a relatively uniform distribution across different phenotypic categories (brown, blue, and intermediate eye colours), suggesting limited predictive power of this marker in distinguishing eye colour variants within our population. In contrast, the *HERC2* gene showed a pattern more consistent with expectations. Although the T allele (mutated) exhibited an overall lower frequency in the Italian population compared to the European average, it was more frequently observed (32%) among individuals with brown eyes, while the CC genotype was predominantly found among those with blue eyes (17%). These results support a genotype–phenotype association in line with the existing literature, at least for the two chromatic extremes.

Intermediate eye colours, on the other hand, remain the most challenging category to predict accurately. Individuals with the heterozygous CT genotype were often classified by the prediction tool as having brown eyes, while those with the CC genotype were classified as having blue eyes, even though they self-reported green or hazel eye colours. Consequently, approximately 97% of the observed discrepancies between predicted and self-reported eye colour fell within this intermediate category. The observed discrepancies may be attributed to the inherently complex nature of phenotypic traits such as eye and hair colour, which result from the interplay of multiple genes and regulatory variants. Additionally, these differences may reflect a distinct genetic structure of the Italian population compared to the broader European reference, likely shaped by a long history of genetic admixture involving Mediterranean, Middle Eastern, and Eastern European populations [10,25–27]. Although current predictive tools are based on statistically validated models and a panel of 24 SNPs associated with common phenotypes, they may not yet account for the full range of genetic variation involved, particularly those variants with weaker modulatory effects or those specific to certain subpopulations. Therefore, identifying additional genetic markers and expanding predictive models may be a critical step toward improving accuracy, especially for intermediate phenotypes.

5. Conclusion

This preliminary study, based on an initial sample of 114 Italian individuals, assessed the predictive performance of the HIrisPlex system for eye and hair colour by comparing predicted phenotypes with self-reported data and European reference allele frequencies. The results showed high concordance for the extreme phenotypes (e.g., blue or brown eyes, black or red hair), but revealed some limitations in predicting intermediate traits, such as blonde or brown hair and intermediate eyes. These findings highlight the need to expand current genetic panels by including additional markers with higher predictive power for intermediate phenotypes and diverse population groups. The next phases of the study will analyse the full sample size (600 individuals), extend the analysis to include additional genetic variants and evaluate gene–gene associations involved in the determination of eye and hair colour. Such efforts will contribute to refining the European reference dataset, allowing for a more accurate representation of the Italian population and, ultimately, enhancing the precision and reliability of genotype-based phenotypic prediction models to facilitate legislation and usage in forensic Italian routine investigations.

6. Declaration of generative AI in scientific writing

During the preparation of this work the authors used ChatGPT-4 (developed by OpenAI) in order to assist with language editing. After using this tool/service, the authors reviewed and edited the content as needed and takes full responsibility for the content of the publication.

Data availability

Data supporting the findings of this study are provided within the article and are available from the corresponding author upon request.

CRediT authorship contribution statement

Giulia Fazio: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Denise Gianfreda:** Conceptualization, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Sara Amurri:** Methodology, Formal analysis, Investigation, Writing – review & editing. **Beatrice Corradini:** Methodology, Writing – review & editing. **Gianmarco Ferri:** Conceptualization, Writing – review & editing. **Francesca Ferrari:** Formal analysis, Investigation, Writing – review & editing. **Ilaria Borciani:** Formal analysis, Investigation, Writing – review & editing. **Carla Bini:** Conceptualization, Methodology, Resources, Supervision, Writing – review & editing. **Rossana Cecchi:** Resources, Supervision, Writing – review & editing. **Susi Pelotti:** Conceptualization, Resources, Supervision, Writing – review & editing. **Anna Laura Santunione:** Resources, Supervision, Writing – review & editing.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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