**Abstract**

Prediction of human pigmentation traits, one of the most differentiable externally visible characteristics among individuals, from biological samples represents a useful tool in the field of forensic DNA phenotyping. In spite of freckling being a relatively common pigmentation characteristic in Europeans, little is known about the genetic basis of this largely genetically determined phenotype in southern European populations. In this work, we explored the predictive capacity of eight freckle and sunlight sensitivity-related genes in 458 individuals (266 non-freckled controls and 192 freckled cases) from Spain. Four loci were associated with freckling (MC1R, IRF4, ASIP, and BNC2), and female sex was also found to be a predictive factor for having a freckling phenotype in our population. After identifying the most informative genetic variants responsible for human ephelides occurrence in our sample set, we developed a DNA-based freckle prediction model using a multivariate regression approach. Once developed, the capabilities of the prediction model were tested by a repeated 10-fold cross-validation approach. The proportion of correctly predicted individuals using the DNA-based freckle prediction model was 74.13%. The implementation of sex into the DNA-based freckle prediction model slightly improved the overall prediction accuracy by 2.19% (76.32%). Further evaluation of the newly-generated prediction model was performed by assessing the model’s performance in a new cohort of 212 Spanish individuals, reaching a classification success rate of 74.61%. Validation of this prediction model may be carried out in larger populations, including samples from different European populations. Further research to validate and improve this newly-generated freckle prediction model will be needed before its forensic application. Together with DNA tests already validated for eye and hair colour prediction, this freckle prediction model may lead to a substantially more detailed physical description of unknown individuals from DNA found at the crime scene.

**Keywords:** Freckles; Ephelides; Externally visible traits; DNA-based prediction; Forensic science

### 1 Introduction

Identifying predictive biomarkers of human appearance traits is being systematically investigated by the forensic community with the purpose of individual identification merely from a biological sample [1]. Human pigmentation traits are some of the most differentiable externally visible characteristics among individuals. For this reason, researchers have been focused in the design of genetic prediction tests for eye, skin and hair colour variation [2–7]. However, DNA-based prediction of other human pigmentation traits under a strong genetic control, such as ephelides occurrence, has not been generated yet.

Ephelides (also known as freckles) are small, flat, pale-brown spots commonly observed in fair-skinned and/or red-haired individuals. Ephelides typically appear early in childhood, may increase in size, number and intensity during adolescence and partly disappear during the young adulthood period [8]. Although the development of these hyperpigmented spots may be triggered by exposure to sunlight, the occurrence of ephelides is largely genetically
The melanocortin-1 receptor (MC1R) gene seems to be the major contributor to the formation of freckles in European-origin individuals, independent of skin type and hair colour [10, 11]. From all non-synonymous allelic variants found in the MC1R gene, six have been traditionally associated with a more severe phenotype, characterised by fair skin, red hair and freckling (known as the RHC phenotype): D84E, R142H, R151C, I155T, R160W and D294H [11, 12]. Functional analyses have demonstrated that these MC1R genetic variants severely affect receptor function reducing stimulation of the pigmentation pathway. These six variants as well as other rare alleles that completely hamper MC1R function are known as ‘R’ alleles. Alternatively, weaker variants with lower penetrance are classified as ‘r’ alleles, and other rare non-synonymous variants that do not seem to have a noticeable effect in receptor function are defined as pseudoalleles [13].

The presence of alleles with impaired function ultimately results in an increased synthesis of phaeomelanin (instead of eumelanin) in melanocytes [13-15]. Nevertheless, it is thought that other genes must contribute to freckling, since a significant percentage of the individuals with freckles do not harbour mutations in the MC1R gene. Accordingly, other genes have also been associated with ephelides occurrence via genome-wide association studies, including IRF4, ASIP, TYR and BNC2 [16-18].

In this work, we analysed the role of eight genes previously associated with sunlight sensitivity in an ephelides case-control study. As far as we know, this study tackles for the first time the genetic basis of freckles in a southern European population (Spain), where the freckling genotype presents a reduced frequency compared to northern Europe. After performing an association study, we developed a multivariate regression approach where only the most informative loci responsible for ephelides were included, in order to predict human ephelides occurrence. To test the power of the newly-generated freckle prediction model in future forensic applications, we evaluated the model’s prediction performance in terms of accuracy, sensitivity and specificity by means of a cross-validation approach as well as an external validation using an independent sample.

2 Materials and methods
2.1 Study subjects and data collection

2.1.1 Original population

A total of 458 individuals (266 non-freckled controls and 192 freckled cases) were included in this study. Initially, unrelated participants were randomly selected, the percentage of the freckling phenotype being 21.86% in our population. These participants were recruited among the students and staff of the Jaume I University of Castellon. With the aim of performing a case-control association study, additional consecutive freckled volunteers were included in our study population. All individuals were Europeans of Spanish origin. Written informed consent was provided by all participants, and the study was approved by the Ethics Committee of the Jaume I University of Castellon (Castellon, Spain).

Under the supervision of a professional, each participant completed a standardised questionnaire to collect information on sex, age, pigmentation traits, history of childhood sunburns, Fitzpatrick’s skin type classification, and sun exposure habits.

Details of ephelides occurrence both during the infancy or adolescence periods and in adulthood were collected in the questionnaire (an illustration is shown in Fig. S1 in Supplementary material in online version at DOI: 10.1016/j.fsigen.2017.11.013).

2.1.2 Independent validation population

A second phase of the study was composed of 212 unrelated individuals (109 non-freckled controls and 103 freckled cases) of Spanish origin, also recruited among the students and staff of the Jaume I University of Castellon. All individuals gave a written informed consent and completed the standardised questionnaire used to collect phenotypic information.

2.2 DNA extraction

Genomic DNA was obtained from buccal swabs that were stored at ~20 °C until sample processing. DNA was isolated using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer’s protocol. After DNA extraction, all samples were diluted to a concentration of 5 ng/μl in order to prepare them for PCR amplification.

2.3 SNP selection and genotyping

Previous literature was used to select our candidate gene list. We selected genes previously associated with sensitivity to sunlight and/or freckling [8, 11, 16-20]. Eventually, eight SNPs located in seven pigmentation-related genes were selected and genotyped: rs4911442 located in the ASIP gene [8, 16, 20], rs2153271 in the BNC2 gene [16], rs128896399 in SLC24A4 [18], rs16891982 in SLC45A2 [19], rs1393350 and rs1042602 in the TYR gene [18], rs12203592 in IRF4 [18-20], and rs12821256 in the KITLG gene [18]. Other candidate SNPs mentioned in the cited literature were excluded due to SNP redundancy or to extremely low frequencies in the Spanish population. SNP codes, locations, ancestral and derived alleles and their frequencies were obtained from the Ensembl Variation database (http://www.ensembl.org/info/genome/variation/index.html).

Genotyping assays were performed by using KASP Genotyping Chemistry (LGC, Hoddesdon, United Kingdom). For SLC45A2 rs16891982, TaqMan technology was used (Applied Biosystems, Foster City, USA). Genotyping analyses were carried out in a StepOnePlusTM Real-Time PCR System, with varying PCR conditions depending on the requirements of each probe. The genotype of each sample was determined by measuring allele-specific
fluorescence, using SDS v2.3 software for allelic discrimination (Applied Biosystems, Foster City, USA). For quality control, we included a negative control and a trio of samples with known genotype (major allele homozygous, heterozygous and rare allele homozygous) in each 96-well plate.

2.4 Sequencing of MC1R coding region

All DNA samples were analysed for the coding sequence of the MC1R gene by direct automated DNA sequencing, as previously described [21]. The primers used to amplify the MC1R coding region were: MC1R-F (5′-CAGCACCATGAACTAAGCAGGACACCTG-3′) and MC1R-R (5′-AAGGGTCCGCGCTTCAACACTTTCAGAG-3′). Amplification was carried out by using Type-it™ Microsatellite PCR Kit (Qiagen, Hilden, Germany), following the manufacturer’s instructions. PCR products were purified with EnzSAP (EdgeBio, Gaithersburg, USA) and subsequently sequenced by direct gene sequencing with the Sanger method. A sample with known MC1R genotype per 96-well plate was added for quality control. All sequencing results were analysed using SeqScape v2.5 software to align and detect all nucleotide changes. All detected sequence changes were confirmed manually.

Non-synonymous MC1R mutations were then defined as ‘R’, ‘r’ or ‘p’ (pseudoallele) alleles according to their impact on protein function (Table 1). R alleles included genetic variants that have been associated with the red hair colour (RHC) phenotype [22,23], and have been shown to cause a significant impairment of receptor function in previous functional in vitro or in silico analysis [21,24–29]. Genetic variants that have not been associated with the RHC phenotype [23,30,31], and have been shown to display partial loss of function or present a possibly damaging effect based on prediction analysis [25,27,32,33], were defined as r alleles. Variants in which receptor function is similar to the wild-type form were catalogued as p alleles. Individuals were classified based on the number of R and/or r alleles carried.

| Variant | Nucleotide change | Functional analysis | Prediction analysis | Polyphen score | Reference |
|---------|-------------------|---------------------|--------------------|---------------|-----------|
| R alleles |                  |                     |                    |               |           |
| D84E    | c.252C > A        | Yes                 | –                  | –             | Beaumont et al. [23] |
| R142H   | c.425G > A        | Yes                 | –                  | –             | Beaumont et al. [23] |
| R151C   | c.451C > T        | Yes                 | –                  | –             | Beaumont et al. [22] |
| I155T   | c.464T > C        | Yes                 | –                  | –             | Beaumont et al. [23] |
| R160W   | c.478C > T        | Yes                 | –                  | –             | Beaumont et al. [22] |
| D294H   | c.880G > C        | Yes                 | –                  | –             | Beaumont et al. (2006) [22] |
| Q30X    | c.88C > T         | No                  | No                 | –             | Guan et al. [24] |
| C35Y    | c.104G > A        | Yes                 | –                  | –             | Fargnoli et al. [32] and Fernandez et al. [29] |
| S41F    | c.122C > T        | Yes                 | –                  | –             | Pérez Oliva et al. [28] |
| S83P    | c.247T > C        | No                  | Yes                | –             | Kanetsky et al. [26] and Ibarrola-Villava et al. (2013) [36] |
| S83L    | c.248C > T        | Yes                 | –                  | –             | Ozola et al. [27] |
| G89R    | c.265G > C        | Yes                 | –                  | –             | Ozola et al. [27] |
| M128T   | c.383T > C        | Yes                 | –                  | –             | Pérez Oliva et al. [28] |
| L135R   | c.404T > G        | No                  | No                 | 1.000         | NEW. Never reported/not found in SNPs databases |
| Y152X   | c.456C > A        | Yes                 | –                  | –             | Fargnoli et al. [32] |
| Q233X   | c.697C > T        | No                  | No                 | –             | Martinez-Cadenas et al. [35] |
| P256S   | c.766C > T        | No                  | Yes                | –             | Hu et al. [2014] | Kanetsky et al. [26] |
Only variants with protein sequence alterations are shown. Previous in vitro or in silico functional analysis were used to classify MC1R variants into three categories. In the absence of previous published data, impact on receptor function was predicted by the PolyPhen programme. Genetic variants with PolyPhen scores lower than 0.50 were considered as p alleles (pseudoalleles with similar function compared to wild-type), while variants with scores from 0.50 to 0.95 were classified as r alleles (possibly damaging).

### 2.5 Association analysis

Association analyses were performed using the R software (http://www.R-project.org). All analyses performed were two-sided, and a significance level of 0.05 was considered for rejection of the null-hypothesis. The conservative Bonferroni correction was used to adjust the significance level for multiple testing ($P$-value $< 4.54 \times 10^{-3} = 0.05/11$). Unknown and missing values were excluded at each specific analysis.

For each polymorphism studied, Fisher's exact test was used to check for deviations from Hardy-Weinberg equilibrium (HWE) among controls. Minor allele frequencies (MAFs) for freckled and non-freckled individuals were estimated from our population.

| Gene | Variant | Codon | Protein | Function | Ref. |
|------|---------|-------|---------|----------|------|
| P268R | c.803C > G | No | Yes | - | Ibarrola-Villava et al. [25] |
| V60L | c.178G > T | Yes | - | - | Beaumont et al. [23] and Herraiz et al. [30] |
| V92M | c.274G > A | Yes | - | - | Beaumont et al. [23] and Herraiz et al. [30] |
| R163Q | c.488G > A | Yes | - | - | Beaumont et al. [23] and Nakayama et al. [31] |
| L24M | c.70C > A | No | No | 0.863 | NECTAR (http://nectarmutation.org) |
| F45L | c.133T > C | No | Yes | - | Ozola et al. [27] and Ibarrola-Villava et al. [25] |
| L46V | c.136C > G | No | No | 0.741 | Ensembl (www.ensembl.org) |
| R67Q | c.200G > A | No | Yes | - | Fargnoli et al. [32] |
| T95M | c.284C > A | Yes | - | - | Ozola et al. [27] |
| I120T | c.359T > C | No | Yes | - | Fargnoli et al. [32] |
| V122M | c.364G > A | Yes | - | - | Jimenez-Cervantes et al. [33] |
| V156A | c.467T > C | No | No | 0.784 | Kanetsky et al. [26] |
| V193L | c.577G > T | No | No | 0.567 | Ensembl (www.ensembl.org) |
| N279K | c.837C > A | No | Yes | - | Fargnoli et al. [32] and Ibarrola-Villava et al. [25] |
| G32R | c.94G > A | No | No | 0.299 | NEW. Never reported/not found in SNPs databases |
| S47T | c.140G > C | No | No | 0.003 | Garcia-Borron et al. [57] |
| A57V | c.170C > T | No | No | 0.001 | Ensembl (www.ensembl.org) |
| G89E | c.265G > A | No | No | 0.170 | Ensembl (www.ensembl.org) |
| M128V | c.382A > G | No | No | 0.019 | NECTAR (http://nectarmutation.org) |
| A167T | c.499T > G | No | No | 0.024 | Ensembl (www.ensembl.org) |
| V208I | c.624G > A | No | No | 0.002 | Ensembl (www.ensembl.org) |
Associations between the genotyped polymorphisms and the presence of ephelides were assessed according to the co-dominant model of inheritance via binary logistic regression. Genotype-related odds ratios (ORs), their corresponding 95% confidence intervals (CIs) and associated P-values were estimated. The proportion of the total variance in freckling explained by each genetic variant was estimated using Nagelkerke pseudo-R² statistic (R²). This statistic parameter was used to rank the genetic variants included in the study based on their contribution to the freckling phenotype.

### 2.6 Prediction model

A multivariate logistic regression approach was applied to build the prediction models. The DNA-based freckle prediction model was developed using backward elimination of genetic variants based on the Akaike information criterion (AIC), being the optimal model the one with the smaller AIC value – best balance between goodness-of-fit and parsimony. For each iteration, the lowest predictor in the variable set is excluded. Then, the model is rebuilt, used to predict again freckle occurrence, and the quality of the newly generated model is re-tested. This model-building process is repeated until all remaining polymorphisms included in the prediction model have a statistically significant contribution, and the estimated information loss is minimised.

To assess the influence of sex in the freckling phenotype, the DNA-based prediction model was additionally adjusted by including sex, as well as the interaction between sex and each genetic variant as covariates in the multivariate logistic regression. As above, the optimal sex-adjusted prediction model was developed based on the AIC variable selection criteria.

Finally, statistical interactions between genetic variants were examined using the MDR software (http://www.epistasis.org/). The multifactor dimensionality reduction method is a powerful strategy for detecting and interpreting statistical locus-locus epistasis [34]. Dendrogram interaction graphs provided by MDR illustrate the presence, strength, and nature of epistatic effects. Pairwise genetic interactions were tested by including interactions into the logistic regression model.

### 2.7 Model evaluation

#### 2.7.1 Internal evaluation phase - cross-validation

In order to evaluate the predictive ability of the freckle prediction models, we applied a repeated 10-fold cross-validation approach. Briefly, individuals were randomly divided into 10 equal data subsets. For each round of cross-validation, nine data subsets were used for performing the analysis (training set) while the remaining subset was used for validating the analysis (testing set). This process was run 10 times, using a different subset as testing set each time. This entire procedure was repeated 200 times, using different random partitions of the original sample to protect against chance divisions of the dataset. Data partition was carried out using the ‘caret’ package for R software, and the repeated cross-validation approach was performed with custom programmes written in R.

Receiver operating characteristic (ROC) curve analysis was adopted to evaluate the cross-validated performance of the prediction models. The Youden Index method was used to set the optimal cut-off point – the point on the curve at which sensitivity + specificity−1) is maximised. For each prediction model, basic prediction accuracy parameters – including the area under the ROC curve (AUC), sensitivity, specificity and accuracy – were calculated from the confusion table reporting the number of true positives (TP), true negatives (TN), false positives (FP), and false negatives (FN). The final values reported were averaged from the 200 × 10 individual cross-validation curves to produce a single estimation. All calculations and plots were performed using the ‘ROCR package’ for R software.

#### 2.7.2 External evaluation phase - independent population

In order to carry out the external evaluation, only the genetic variants that were significantly associated with freckles in the first phase of the study – IRF4, ASIP, BNC2 and MC1R – were analysed in the newly collected independent samples.

This independent dataset was used to assess the performance of the newly-generated freckle prediction model regarding the correct classification of individuals as freckled or non-freckled. Subsequently, a comparison between predicted and observed data was performed, and the basic accuracy parameters (AUC, sensitivity, specificity and accuracy) were calculated from the confusion table by using the ‘pROC package’ for R software.

### 3 Results

Our original sample set comprised 266 non-freckled and 192 freckled individuals of Spanish ancestry (Table 2). The study cohort included 260 females (56.52%) and 198 males (43.04%). Interestingly, the prevalence of the freckling phenotype was remarkably higher in females than in males (68.75% vs 31.25%, P-value = 9.00 × 10⁻⁹). As expected, freckled individuals were more likely to have skin phenotypes I-II and childhood sunburns than non-freckled individuals, although no significant differences between them were found regarding sun exposure habits (Table 2).

| Table 2 | Classification of the Spanish individuals according to sex, skin phenotype and sun exposure habits. |
For each polymorphism, minor allele frequencies in freckled and non-freckled individuals from our population are detailed in Table 3. Evidence of departure from Hardy-Weinberg equilibrium among controls was observed for two SNPs: rs12896399 in SLC24A4 gene and rs16891982 in SLC45A2 – as seen in previous studies involving these human pigmentation genes [35–37]. Out of 458 individuals studied, 277 (60.48%) carried at least one non-synonymous MC1R variant, including 135 (54.43%) in a total of 248 non-freckled individuals, and 142 (75.93%) in a total of 187 freckled individuals. All genetic variants found in the MC1R gene are listed and classified in Table 1. To our knowledge, two mutations (G32R and L135R) have never been previously described, and they add up to the growing list of rare MC1R natural variants. The frequencies of R and r alleles in both freckled and non-freckled individuals are detailed in Table 3. Seventeen samples were discarded due to unsuccessful MC1R sequencing.

### Table 2

| Table 2 | Genotypic association with freckles in the Spanish population. |
|---------|---------------------------------------------------------------|
| Gene    | SNP | rs | Genotype | Non-freckled (N = 266) | Freckled (N = 192) | P-valuea |
|---------|-----|----|----------|------------------------|-------------------|----------|
| Sex     |     |    |          | N | % | N | % |         |
| Female  |     |    |          | 128 | 48.12 | 132 | 68.75 | 9.00E-06 |
| Male    |     |    |          | 138 | 51.88 | 60 | 31.25 | 0.00 |
| Unknown |     |    |          | 0 | 0.00 | 0 | 0.00 | 0.00 |
| Sun exposure | | | | | | | | |
| Never   |     |    |          | 22 | 8.27 | 17 | 8.85 | 0.438 |
| Intermittent | | | | | | | | |
| Intermitent | 134 | 50.38 | 106 | 55.21 | | | | |
| Habitual |     |    |          | 109 | 40.98 | 65 | 33.85 | 4.69 |
| Unknown |     |    |          | 1 | 0.38 | 9 | 4.69 | 4.69 |
| Skin Phototype | | | | | | | | |
| III/IV |     |    |          | 203 | 76.32 | 69 | 35.94 | 9.20E-18 |
| I/II   |     |    |          | 63 | 23.68 | 121 | 63.02 | 63.02 |
| Unknown |     |    |          | 0 | 0.00 | 2 | 1.04 | 1.04 |
| Childhood sunburns | | | | | | | | |
| No     |     |    |          | 126 | 47.37 | 37 | 19.27 | 3.52E-09 |
| Yes    |     |    |          | 138 | 51.88 | 145 | 75.52 | 75.52 |
| Unknown |     |    |          | 2 | 0.75 | 10 | 5.21 | 5.21 |

N, number of individuals; %, percentage of individuals per group among the total.

*P*-value for the Fisher's exact test. Significant results are presented in bold.

### Table 3

| Table 3 | Genotypic association with freckles in the Spanish population. |
|---------|---------------------------------------------------------------|
| Gene    | SNP | rs | Genotype | Non-freckled individuals | Freckled individuals | P-valuea | OR (95% CI) | R² (%)b | Rankc |
|---------|-----|----|----------|-------------------------|---------------------|----------|-------------|--------|-------|

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| Gene   | rs Number | Allele | N   | %      | MAF   | HWE P-value | N   | %      | MAF   | P-value |
|--------|-----------|--------|-----|--------|-------|-------------|-----|--------|-------|---------|
| ASIP   | rs4911442 | AA     | 233 | 87.59  | 0.062 | 0.264       | 153 | 79.69  | 0.109 | **0.044**|
|        |           | AG     | 29  | 10.90  | 0.007 | 0.190       | 36  | 18.75  | 1.89  | (1.11−3.21) |
|        |           | GG     | 2   | 0.75   | 0.001 | 0.011       | 3   | 1.56   | 2.28  | (0.38−13.83) |
|        |           | ND     | 2   | 0.75   | 0.00  | 0.00        | 0   | 0.00   |       |         |
| BNC2   | rs2153271 | TT     | 68  | 25.56  | 0.476 | 0.222       | 61  | 31.77  | 0.406 | 0.067   |
|        |           | CT     | 143 | 53.76  | 0.210 | 0.552       | 106 | 55.21  | 0.83  | (0.54−1.27) |
|        |           | CC     | 55  | 20.68  | 0.007 | 0.122       | 25  | 13.02  | 0.51  | (0.28−0.91) |
|        |           | ND     | 0   | 0.00   | 0.00  | 0.00        | 0   | 0.00   |       |         |
| IRF4   | rs12203592| CC     | 207 | 77.82  | 0.117 | 1.000       | 100 | 52.08  | 0.275 | **1.81E−05** |
|        |           | CT     | 56  | 21.05  | 0.420 | 0.741       | 77  | 40.10  | 2.49  | (1.57−3.94) |
|        |           | TT     | 3   | 1.13   |       | 0.00        | 14  | 7.29   | 6.56  | (1.68−25.54) |
|        |           | ND     | 0   | 0.00   | 0.00  | 0.00        | 1   | 0.52   |       |         |
| KITLG  | rs12821256| TT     | 243 | 91.35  | 0.041 | 1.000       | 175 | 91.15  | 0.034 | 0.417   |
|        |           | CT     | 21  | 7.89   | 0.007 | 0.012       | 11  | 5.73   | 0.73  | (0.34−1.56) |
|        |           | CC     | 0   | 0.00   | 0.00  | 0.00        | 0   | 0.00   |       |         |
|        |           | ND     | 2   | 0.75   | 0.00  | 0.00        | 6   | 3.13   |       |         |
| MC1R   | rs alleles| 0/0    | 214 | 80.45  |       |       | 110 | 57.29  | 2.49  | (1.57−3.94) |
|        |           | 0/1    | 38  | 14.29  | 0.420 | 0.741       | 64  | 33.33  | 3.26  | (2.05−5.18) |
|        |           | 1/1    | 2   | 0.75   | 0.007 | 0.122       | 13  | 6.77   | 11.61 | (2.55−52.83) |
|        |           | ND     | 12  | 4.51   | 0.00  | 0.00        | 5   | 2.60   |       |         |
| MC1R   | r alleles | 0/0    | 154 | 57.89  |       |       92  | 47.92  | 2.15  | (1.02−4.53) |
|        |           | 0/1    | 86  | 32.33  | 0.420 | 0.741       | 77  | 40.10  | 1.49  | (1.01−2.24) |
|        |           | 1/1    | 14  | 5.26   | 0.007 | 0.122       | 18  | 9.38   | 3.26  | (2.05−5.18) |
|        |           | ND     | 12  | 4.51   | 0.00  | 0.00        | 5   | 2.60   |       |         |
| SLC24A4| rs12896399| TT     | 92  | 34.59  | 0.439 | 0.033       | 58  | 30.21  | 0.479 | **0.039** |
|        |           | GT     | 113 | 42.48  | 0.476 | 0.222       | 85  | 44.27  | 1.20  | (0.77−1.85) |
|        |           | GG     | 60  | 22.56  | 0.001 | 0.004       | 49  | 25.52  | 1.33  | (0.80−2.19) |
|        |           | ND     | 1   | 0.38   | 0.00  | 0.00        | 0   | 0.00   |       |         |
| SLC45A2| rs16891982| GG     | 209 | 78.57  | 0.133 | 0.002       | 154 | 80.21  | 0.130 | 0.690   |
|        |           | CG     | 43  | 16.17  | 0.007 | 0.122       | 26  | 13.54  | 0.82  | (0.48−1.39) |
|        |           | CC     | 14  | 5.26   | 0.00  | 0.00        | 12  | 6.25   | 1.16  | (0.52−2.59) |
SNP, single nucleotide polymorphism; N, number of individuals; %, percentage of individuals per group among the total; OR, odds ratio per minor allele; CI, confidence interval; $R^2$, Nagelkerke pseudo-$R^2$ statistic.

* Indicates significant results at Bonferroni threshold of 4.50E-03.

* $P$-value for the binary logistic regression association analysis according to the co-dominant model of inheritance. Bold indicates statistically significant results.

b The proportion of total variance in freckling phenotype explained by the genetic variants was estimated using $R^2$.

c The genetic variants were ranked according to their importance in freckling phenotype.

Univariate association analyses were performed to assess the independent effects of each genetic variant on freckling occurrence in childhood (Table 3). R variants of the $MC1R$ gene were the alleles most strongly associated with freckling ($P$-value = 7.05 × 10$^{-8}$), explaining 6.91% of the variance. The rs12203592 polymorphism in the $IRF4$ gene presented the second strongest association with the presence of ephelides ($P$-value = 1.81 × 10$^{-5}$), explaining 5.92% of the variance. Weaker significant associations were also observed for r variants ($P$-value = 0.039) and rs4911442 in $ASIP$ ($P$-value = 0.044), explaining around 1% of the variance ($R^2 = 1.09\%$ and $R^2 = 1.01\%$, respectively). No associations were found for the remaining six polymorphisms, although rs2153271 in $BNC2$ showed a marginal significant association with freckling ($P$-value = 0.067).

A prediction model was then constructed based on multinomial logistic regression. To design the optimal model, we performed a step-wise analysis by iteratively excluding the lowest predictor from the multinomial logistic regression model. The genetic variants were ranked according to their impact on freckling. The DNA-based prediction model included four loci ($MC1R$, $IRF4$, $ASIP$, and $BNC2$), which together explained about 30% of the freckling phenotype variance in our population (Table 4). A repeated 10-fold cross-validation approach was used to perform an internal validation of the DNA-based prediction model by ROC analysis, which tests the power of the prediction model [38]. The prediction accuracy of the tested DNA-based prediction model was 74.13% in the population, with an AUC of 0.771, a specificity of 82.00%, and a sensitivity of 63.51% (Fig. 1A).

Table 4 Multivariate logistic regression testing freckles association with genetic variants and sex.

| Gene | SNP rs# Variable | DNA-based prediction model | Sex-adjusted prediction model |
|------|------------------|---------------------------|-------------------------------|
|      |                  | OR (95% CI)               | $P$-value*                    | OR (95% CI)               | $P$-value* |
| MC1R | R alleles        | 4.18 (2.49–7.02)          | **6.66E-08**                  | 4.21 (2.48–7.15)          | 1.02E-07  |
|      |                  | 21.51 (4.50–102.79)       | **1.20E-04**                  | 21.94 (2.48–105.16)       | 1.12E-04  |
| IRF4 | rs12203592       | 3.28 (2.05–5.26)          | **7.75E-07**                  | 3.26 (2.02–5.27)          | 1.43E-06  |
|      |                  | 15.51 (3.26–73.76)        | **5.65E-04**                  | 14.63 (3.06–70.10)        | 7.81E-04  |
| MC1R | r alleles        | 2.08 (1.30–3.32)          | **2.20E-03**                  | 2.05 (1.28–3.30)          | 2.92E-03  |
|      |                  | 3.45 (1.51–7.87)          | **3.20E-03**                  | 3.39 (1.43–7.99)          | 5.35E-03  |
|      |      | Minor allele | OR (95% CI) | Adjusted OR (95% CI) | P-value |
|------|------|--------------|-------------|----------------------|---------|
| **ASIP** | rs4911442 | AG           | 2.26 (1.22–4.17) | 9.27E-03              | 2.38 (1.28–4.45) | 6.49E-03 |
|       |      | GG           | 3.65 (0.35–38.40) | 0.281                | 5.15 (0.47–56.62) | 0.181    |
| **BNC2** | rs2153271 | CT           | 0.91 (0.55–1.49) | 0.706                | 0.98 (0.59–1.63) | 0.937    |
|       |      | CC           | 0.45 (0.23–0.90) | 0.023                | 0.48 (0.24–0.97) | 0.040    |
|       |      | Male sex     | -            | -                    | 0.49 (0.32–0.79) | 2.70E-03 |

SNP, single nucleotide polymorphism; OR, odds ratio per minor allele; CI, confidence interval.

Optimal model selection procedure was based on the Akaike information criterion (AIC).

* P-value for the multivariate logistic regression association analysis. Bold indicates significant results at Bonferroni threshold of 4.50E-03.
The inclusion of sex on the freckle prediction model appeared to increase the prediction accuracy by 2.19%, to a total of 76.32% (Fig. 1B). Slight increases in AUC (0.781), specificity (83.80%) and sensitivity (66.21%) values were observed when sex was taken into account. The results of the multivariate association analysis confirmed sex as an important variable in the estimation of the freckling phenotype ($P$-value = $2.70 \times 10^{-3}$). No significant
interaction between sex and other genetic predictors was found.

An external validation of the newly-generated prediction model, assessing the model generalisability [39], was also performed by collecting an independent cohort of 212 individuals – though only 193 individuals successfully genotyped for all loci were included in the external validation. The prediction accuracy of the freckle prediction model was 74.61% in the independent cohort, with an AUC of 0.809, a specificity of 89.12%, and a sensitivity of 59.79%. A total of 49 individuals were incorrectly predicted (misclassification rate of 25.39%) (Table 5).

**Table 5** Accuracy of the sex-adjusted freckle prediction model obtained from an independent cohort of Spanish individuals.

| Phenotype   | Predicted phenotype | Total | AUC | Sensitivity (%) | Specificity (%) | Accuracy (%) |
|-------------|---------------------|-------|-----|-----------------|-----------------|--------------|
| Freckled    | 55                  | 37    | 92  | 0.809           | 59.79           | 89.12        | 74.61        |
| Non-freckled| 12                  | 89    | 101 |                 |                 |              |              |
| Total       | 67                  | 126   | 193 |                 |                 |              |              |
| Fails (%)   | 12 (17.91)          | 37 (29.36) | 49 (25.39) | | | |

*Optimal cut-off maximising Youden Index (0.6138) was used to classify individuals.

*Genetic analyses of four loci were successfully performed in 193 out of 212 individuals (missing rate of 8.96%).

We also evaluated the prediction capacity of the sex-adjusted prediction model to determine the freckling phenotype in adulthood. Taking into account that freckles often disappear after adolescence or during young adulthood, the success rate of the prediction model was relatively good in our population (69.63%), with a high true negative rate (specificity of 80.70%) but a low true positive rate (sensitivity of 58.91%) (Fig. 2). Out of all six freckle predictors included in the model, only the r variants in *MC1R* did not show a significant contribution to freckling in adulthood (*P*-value > 0.05).
MDR analysis for locus-locus interaction detection indicated that the best freckle prediction model was composed of four genetic variables and sex (BNC2 rs2153271 was not included). The locus-locus interaction analysis showed a redundant interaction between R variants in the MCI1R gene and rs12203592 in the IRF4 gene, denoted by the blue lines connecting these two genetic determinants in the dendrogram (Fig. S2 in Supplementary material in online version at DOI: 10.1016/j.fagen.2017.11.013). However, the inclusion of this genetic interaction in our freckle prediction model did not significantly modify the prediction capacity using a repeated 10-fold cross-validation approach (AUC = 0.768, specificity = 83.20%, sensitivity = 61.27%, and accuracy = 75.45%).

### 4 Discussion

In the current study, eight freckle and sunlight sensitivity-related genes were genotyped in 458 individuals from Spain, with the intention of analysing their putative implication in the appearance of ephelides, and its potential for future forensic applications. However, only the 438 individuals successfully genotyped for all loci were included in the development and cross-validation of the prediction model. Furthermore, an external evaluation of the freckle prediction model was performed on an extra 212 sample independent dataset by genotyping the genetic variants associated with freckling in the original population.

In the last few years, the genetic basis of ephelides has been adequately studied in populations of North European origin [11,16-18]. However, little is known about the genetic determinants of this pigmentation characteristic in southern European populations. Our results showed an association between ephelides and genetic variants in the IRF4, MCI1R and ASIP genes in a Mediterranean population, confirming previous studies performed in North European populations [11,17,18]. R variants of the MCI1R gene have been previously acknowledged as the most relevant locus associated with both freckling and sun sensitivity. The abnormal function of this receptor leads to a higher phaeomelanin to eumelanin ratio, commonly resulting in the well-known freckle-generating RHC phenotype. IRF4 has also been associated with freckling in several studies [8,18]. This interferon regulatory factor cooperates with MITF to activate the expression of tyrosinase in melanocytes, a function which seems to be impaired in carriers of the rs12203592*T derived allele [40]. The other main freckle-associated locus in this study, ASIP, antagonises the activation of MCI1R, leading to a down-regulation of eumelanogenesis and an up-regulation of phaeomelanogenesis. Variants in this gene, also previously linked to the RHC phenotype, may therefore have effects similar to variants of the MCI1R gene [16,17].

The impact of BNC2 rs2153271 on freckling was detected only after applying a multivariate association approach. The identification of the association between freckling and rs2153271, located in an intron of the BNC2 gene, was recently discovered in a GWAS study performed in a population of North European ancestry [16], and was additionally correlated with acquired facial pigmented spots during aging [19].

Although rs1042602 and rs1393350, in the TYR gene, have been previously associated with freckling in two European populations of Icelandic and Dutch origin [18], a lack of association between these two SNPs and ephelides occurrence was observed in our Spanish population. Accordingly, no association with freckling was previously observed for a TYR melanoma-associated genetic variant (rs1126809) in a sample set comprising three Mediterranean populations from France, Spain and Italy [36]. However, that melanoma case-control study identified a moderate association between rs16891982 in SLC45A2 and the absence of ephelides [36]. The inheritance of a set of genetic variants in SLC45A2 was also associated with freckling in a population from Brazil [41]. However, we are not able to verify this association in the current study, perhaps due to the limited number of individuals included in the sample set.

This association study allows us to develop a freckle prediction model based on five genetic predictors: R variants in MCI1R, IRF4 rs12203592, rs variants in MCI1R, ASIP rs4911442 and BNC2 rs2153271 (listed in order, from greater to lower genetic contribution). Predicting externally visible human traits from genotypes represents a potential valuable tool in forensic investigations [1]. However, DNA-based phenotyping needs a very demanding approach since externally visible characteristics are typically influenced by several genetic as well as environmental factors [42]. To date, DNA-based human eye colour prediction is the most accurate, advanced and applied test in forensic applications [3,5,43]. The IrisPlex system, a robust prediction model based on six eye colour SNP predictors, has been validated in numerous studies for forensic eye colour prediction [4,5,43]. Recently, a multiplex genotyping assay, called HirisPlex, has been developed for simultaneous hair and eye colour prediction [6], being suitable and sufficiently sensitive for forensic use approval [44]. Currently, forensic genome-wide association studies are focused on increasing the genetic determinants of different phenotypes such as body height, hair shape, baldness, and facial variation, in order to reach more detailed predictions of an unknown person’s appearance from the analysis of his/her DNA [16,45-48].

As a whole, the DNA-based freckle prediction model presents with high specificity (82.00%), but low sensitivity (63.51%). The low sensitivity levels achieved could be due to the fact that one third of the individuals carrying R variants did not display freckles, suggesting that other genetic determinants are also important in the appearance of freckles. Hopefully, future studies with increased sample sizes may add to the genetic factors influencing freckle occurrence, so that the model’s prediction potential may be significantly improved.

Interestingly, we found a much higher prevalence of ephelides in females than in males. Previous studies have also stated discrepancies in human pigmentation and sunlight sensitivity traits between sexes [19,35,49-52]. Notably, higher freckle prevalence in females was previously observed in a GWAS study performed in an northern European population [18]. Additionally, Jacobs and cols. (2015) showed that females presented a much higher occurrence of facial sun spots than males, being the total variance explained by sex higher than any of the genetic variants studied [19].
The inclusion of sex in the IrisPlex model has been proposed to improve prediction accuracy mainly for intermediate eye colours [35,50,53,54]. Adding sex as a covariate in our DNA-based freckle prediction model slightly increased the prediction performance for ephelides prevalence. In particular, it presented with both higher specificity (83.80%) and sensitivity (66.21%) if compared to the prediction model based on the five freckle genetic predictors alone. Overall, this sex-adjusted freckle prediction model could explain a high proportion of the freckling phenotypic variance ($R^2 = 32.92\%$), which is quite large compared to other human complex traits [55].

The main purpose of a prediction model is to provide correct classification for new individuals, being therefore external validation a crucial phase of the model development process. Assessing both reproducibility (internal validation) and generalisability (external validation) of the developed prediction model are necessary in order to avoid overfitted models [56]. However, application of a prediction model should only be considered after proving adequate accuracies in an independent dataset. For this reason, the discrimination ability of the newly-generated prediction model was assessed by comparing the observed and predicted outcomes in a new dataset. As usually noted in most external validations, a slight decrease of the overall prediction accuracy was observed (from 76.32% to 74.61%).

In terms of prediction power, our freckle prediction model provided a similar accuracy than the one obtained for green/hazel eye colour prediction using IrisPlex (AUC = 0.809 and AUC = 0.76, respectively), although significantly lower than the accuracy level achieved for brown (AUC = 0.93) and blue eye colour prediction (AUC = 0.91) [5]. HirisPlex also presented equivalent prediction power for hair colour prediction, with an average accuracy of 73% [44], compared to 74.61% of our freckle prediction model. Notice that our results have been reached after a validation approach with a relatively small independent sample, while both IrisPlex and HirisPlex prediction models have been validated in independent large population samples.

After our preliminary validation, the freckle prediction model developed in this study has shown to display limited success, but it also appears promising for future applications in forensics. However, further research is needed to increase the correct prediction rate (sensitivity) of the model, since the percentage of freckled individuals predicted as non-freckled is considerably high (false negative rate of 40.21%).

For practical considerations, knowing that ephelides tend to disappear during aging due to undetermined reasons, we tested the accuracy of our prediction model to determine the presence of ephelides in adulthood. The model presented an AUC of 0.756, a slightly lower value compared to the AUC value obtained for freckle prediction in childhood (0.781 for internal cross-validation and 0.809 for external validation). No significant contribution to freckling prediction in adulthood was noted for $r$ variants, suggesting that other unknown predictors could have an independent additional impact on the freckling phenotype in adulthood. Interestingly, after adjustment by IRF4 rs12203592 (the most associated genetic predictor for freckling in adulthood), the minor allele of SLC45A2 rs16891982 was weakly associated with the absence of freckles in adulthood (OR = 0.64 (0.43–0.94), $P$-value = 0.023). As revealed by the MDR analysis, the effect of this SLC45A2 variant may not be strongly influenced by a locus-locus interaction, since there is no interaction between SLC45A2 and the rest of the freckle loci (Fig. S2B in Supplementary material in online version at DOI: 10.1016/j fsigen.2017.11.013).

Since the sample set used to build and evaluate this freckle prediction model was relatively small, the ability to accurately predict the presence of ephelides may be further improved by using larger studies. It is likely that phenotype prediction accuracy may be substantially improved by including undiscovered genetic variants at novel loci or by taking into account possible gene interactions affecting the freckling phenotype. Also, DNA samples from different populations of European origin should be included in further studies in order to validate this DNA predictive test for future forensic applications.

It is also important to note that predicting complex phenotypic traits from DNA studies remains a difficult task even if all genetic loci involved – and the interactions among them – are taken into account, since different environmental factors may always have a considerable effect (for example, the effect of UV exposure on freckle occurrence).

5 Conclusions

The main genetic variants involved in freckle appearance in the Spanish population are located in the MC1R and IRF4 genes, with minor contributions from ASIP, BNC2 and perhaps other as yet unknown freckle genes. However, the influence on freckling of different MC1R variants (R or r) is substantially different. As a result, the DNA-based model for freckle prediction developed in this work considers five genetic determinants: R variants of the MC1R gene, IRF4 rs12203592, r variants of the MC1R gene, ASIP rs4911442 and BNC2 rs2153271 – in order of greatest to lowest contribution – reaching a cross-validated prediction accuracy of up to 74.13%, a more than respectable percentage. When sex is added to the model, the cross-validated prediction accuracy reached is even higher, growing up to 76.32%.

Furthermore, the newly-generated freckle model was tested in an independent cohort reaching an acceptable specificity level of 89.12%. However, the sensitivity of the model is certainly improvable, attaining a percentage of around 60%.

For a more detailed pigmentation phenotype prediction, future research may focus on designing multiplex genetic analysis for simultaneously predicting different externally visible traits. Perhaps the inclusion of two more SNPs in the HirisPlex system, BNC2 rs2153271 and ASIP rs4911442, could increase the forensic potential of this DNA-based prediction test to include freckle occurrence (as well as the current eye and hair colour prediction).

Acknowledgements
We are extremely grateful to all the volunteers for giving their consent to take part in this study, as well as to all the medical specialists for supervising phenotype collection of all samples. We also thank Rafael Velasco for providing us with the freckle photographs.

This work was supported by grant number GV2016/156 from the Education Council of the Generalitat Valenciana, and co-funded by the Jaume I University of Castellon. BH is funded by the Jaume I University of Castellon under a Predoctoral Research contract (No. 15721).

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For practical considerations, knowing that ephelides tend to disappear during aging due to undetermined reasons, we tested the accuracy of our prediction model to determine the presence of ephelides in adulthood. The model presented an AUC of 0.756, a slightly lower value compared to the AUC value obtained for freckle prediction in childhood (0.781 for internal cross-validation and 0.809 for external validation).

No significant contribution to freckling prediction in adulthood was noted for variants suggesting that other unknown predictors could have an independent additional impact on the freckling phenotype in adulthood. Interestingly, after adjustment by IRF4 rs12203592 (the most associated genetic predictor for freckling in adulthood), the minor allele of SLC45A2 rs16891982 was weakly associated with the absence of freckles in adulthood (OR = 0.64 (0.43–0.94), p-value = 0.023). As revealed by the MDR analysis, the effect of this SLC45A2 variant may not be strongly influenced by a locus-locus interaction, since there is no interaction between SLC45A2 and the rest of the freckle loci (Fig. S2B).

Under the supervision of a professional, each participant completed a standardised questionnaire to collect information on sex, age, pigmentation traits, history of childhood sunburns, Fitzpatrick's skin type classification, and sun exposure habits. Details of ephelides occurrence both during the infancy or adolescence periods and in adulthood were collected in the questionnaire (an illustration is shown in Fig. S1).

**E-extra**

**E-component**

MDR analysis for locus-locus interaction detection indicated that the best freckle prediction model was composed by four genetic variables and sex (BNC2 rs2153271 was not included). The locus-locus interaction analysis showed a redundant interaction between R variants in the MC1R gene and rs12203592 in the IRF4 gene, denoted by the blue lines connecting these two genetic determinants in the dendrogram (Fig. S2). However, the inclusion of this genetic interaction in our freckle prediction model did not significantly modify the prediction capacity using a repeated 10-fold cross-validation approach (AUC = 0.768, specificity = 83.20%, sensitivity = 61.27%, and accuracy = 75.45%).

**Highlights**

- Prediction of human appearance from DNA is a useful tool to identify unknown persons.
• Genetic variants in *MC1R*, *IRF4*, *ASIP* and *BNC2* contribute to freckling in Spain.
• A preliminary DNA-based prediction model for the presence of ephelides is developed.
• Accuracy of the newly-generated freckle prediction model is reasonably high.
• Further research is needed before practical use in forensics of the newly-generated freckle model.

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