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## The effect of gender on eye colour variation in European populations and an evaluation of the IrisPlex prediction model

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## Abstract

In two recent studies of Spanish individuals [1,2], gender was suggested as a factor that contributes to human eye colour variation. However, gender did not improve the predictive accuracy on blue, intermediate and brown eye colours when gender was included in the IrisPlex model [3].

In this study, we investigate the role of gender as a factor that contributes to eye colour variation and suggest that the gender effect on eye colour is population specific.

A total of 230 Italian individuals were typed for the six IrisPlex SNPs (rs12913832, rs1800407, rs12896399, rs1393350, rs16891982 and rs12203592). A quantitative eye colour score (Pixel Index of the Eye: PIE-score) was calculated based on digital eye images using the custom made DIAT software. The results were compared with those of Danish and Swedish population samples.

As expected, we found *HERC2* rs12913832 as the main predictor of human eye colour independently of ancestry. Furthermore, we found gender to be significantly associated with quantitative eye colour measurements in the Italian population sample. We found that the association was statistically significant only among Italian individuals typed as heterozygote GA for *HERC2* rs12913832. Interestingly, we did not observe the same association in the Danish and Swedish population. This indicated that the gender effect on eye colour is population specific. We estimated the effect of gender on quantitative eye colour in the Italian population sample to be 4.9%. Among gender and the IrisPlex SNPs, gender ranked as the second most important predictor of human eye colour variation in Italians after *HERC2* rs12913832. We, furthermore, tested the five lower ranked IrisPlex predictors, and evaluated all possible  $3^6$  (729) genotype combinations of the IrisPlex assay and their corresponding predictive values using the IrisPlex prediction model [4]. The results suggested that maximum three (rs12913832, rs1800407, rs16891982) of the six IrisPlex SNPs are useful in practical forensic genetic casework.

## Introduction

Investigations of pigmentary genes have gained much interest in forensic science because of the possible use of genetic markers to predict visible, physical traits of possible offenders by DNA typing of biological samples found at crime scenes. Eye colour is currently the best investigated phenotype for forensic genetic applications. SNP multiplexes dedicated to predict eye colour were published [5,4,6]. One of these, the IrisPlex assay, was validated for forensic genetic testing [7]. The IrisPlex assay consists of six SNPs (rs12913832, rs1800407, rs12203592, rs1393350, rs12896399 and rs16891982) that predict qualitative eye colour categories (blue, intermediate and brown). The IrisPlex assay predicts blue and brown eye colours with high accuracy but predict the intermediate eye colours with lower accuracy [4]. Two recent studies [1,2] suggested that gender is contributing to human eye colour variation. Males were found to be more likely to have blue eye colour compared to females, whereas females showed higher frequencies of intermediate and brown eye colour compared to those of males. Similar results were observed for already published male and female eye colour frequency data in Icelandic and Dutch populations [8] but not in Australian [9] and Polish populations [10]. Martinez-Cadenas and co-workers [1] suggested that the prediction success of the IrisPlex model and other models used to predict eye colour phenotypes from DNA genotypes may be improved by including gender as a factor. As a reply to Martinez-Cadenas and coworker [3], the inventors of the IrisPlex assay tested whether the prediction of categorical eye colour (blue, intermediate and brown) was improved when gender was considered [3]. They used two large IrisPlex datasets with a total of over 9,000 European individuals [4,11]. The results showed that gender did not improve the prediction of the eye colour categories blue, intermediate and brown and concluded that gender had minimal effect on eye colour.

In this study, we investigated if gender is associated with a quantitative eye colour measurement in an Italian population sample. We hypothesize that the effect of gender on eye colour is population specific. To test this, we compared the results obtained in the Italian population sample with those of two Northern European populations (a Danish and a Swedish population) from an already published study [12].

## Materials and methods

### **Samples and DNA purification**

Blood samples from 230 unrelated Italian individuals were collected at the Department of Forensic Medicine, University of Copenhagen, Denmark and the U.O. di Patologia Clinica, Laboratorio Analisi del Presidio Ospedaliero di Cantù-Mariano Comense, Azienda Ospedaliera Ospedale Sant'Anna di Como, Italy. The study was approved by the Danish Ethical Committee (H-3-2012-023), the Ethical Committee of Azienda Ospedaliera Ospedal Sant'Anna di Como (U.0026484.23-11-2012) and the Ethical Committee of the University of Milan-Bicocca (P.U. 0033373/12). All participants gave signed, informed consent. Samples were labelled ITA1-ITA230 and treated as anonymously donated samples.

DNA was purified from 200 $\mu$ L of blood using the DNA Blood Mini Kit (Qiagen) as recommended by the manufacturer. DNA was eluted in 50 $\mu$ L AE Buffer (Qiagen).

### **Digital photographs and quantitative eye colour**

Photographs were taken at a distance of approximately 10 cm in “Raw” or “jpeg” format with a Canon EOS 5D Mark V with ISO 800, shutter 1/100 and AV 18 using a Canon EF 100mm f/2.8 L IS USM Macro Lens with manual focus. The white balance of “Raw” format photographs was changed to “Flash” using the Picture style editor software (Canon).

The iris region was automatically extracted from the digital photographs using the custom designed software DIAT and a quantitative eye colour score (Pixel Index of the Eye: PIE-score) was calculated for each individual.

The PIE-score was calculated according to the equation below as previously described [12]:

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

The PIE-score ranged from -1 to 1. The value of -1 was observed when only brown pixels were found in the iris region and the value of 1 was observed when only blue pixels were found in the iris region.

### **Qualitative categorization of eye colour**

Eye colours were assigned according to four categories (blue, light intermediate, dark intermediate and brown) by seven untrained individuals, who were placed in front of the same screen at the same distance and asked to assess the colour category of each eye image. Each eye image was assigned an eye colour category by taking the median of the assessed categories.

For a three colour categorization (blue, intermediate and brown), light intermediate and dark intermediate categories were collapsed into one category (intermediate).

### **SNP-typing**

The six IrisPlex SNPs (rs12913832, rs1800407, rs12203592, rs1393350, rs12896399 and rs16891982) were typed as part of multiplex assay with 32 pigmentary SNPs using the iPLEX<sup>®</sup> Gold Kit (Sequenom) as previously described [12].

Allele calling of the SNPs were analysed in the statistical computing software R v.2.11.0 (<http://CRAN.R-project.org/doc/FAQ/R-FAQ.html>; ISBN 3-900051-08-9). The Plate Data File with signal to noise ratios (SNR) and peak heights were exported from TYPER 4.0 (Sequenom) and imported into R. The allele balance (AB) was calculated in R as  $AB = (\text{Height of Allele 1} - \text{Height of Allele 2}) / (\text{Height of Allele 1} + \text{Height of Allele 2})$ . We defined parameters for correct allele calling; peak height > 1.0, SNR > 5 and  $|AB| > 0.8$  for homozygotes and  $|AB| < 0.2$  for heterozygotes. A value of  $|AB| > 0.8$  translates to a genotype call where the peak height of one allele was at least 9 times higher than the peak height of the other allele.  $|AB| < 0.2$  translates to a heterozygous genotype call where the peak height of one allele was maximally 1.5 times the peak height of the other allele. Modification of ABs were made for rs12913832 A homozygotes  $|AB| > 0.75$  and for heterozygotes of rs16891982,  $0.1 < |AB| < 0.3$ . All cluster plots were visually inspected. All samples were run in duplicates.

SNP types were compared between spots and duplicate typings using a custom function (PlateCompare) in R.

### **Statistical analyses**

All statistical calculations were performed using R. Bonferroni correction was applied for multiple comparisons. Independent association of gender and SNPs with the PIE-score was carried out using

the non-parametric Wilcoxon rank sum test and the Kruskal-Wallis one-way analysis of variance rank test.

The predictive analysis was performed using a multivariate linear regression model. The adjusted  $R^2$  [13] was used to select informative predictors and to analyse the prediction accuracy of the model.

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## Results

### Association between gender, the IrisPlex SNPs and eye colour

A total of 230 Italian individuals were typed for the six IrisPlex SNPs. In 95% (126 females and 92 males) of the 230 eye images, the iris region was successfully extracted and quantitative measurements of the eye colours (the PIE-score) were performed. It was not possible to calculate a PIE-score for 12 individuals due to poor quality of the eye images mainly due to reflections in the iris area. The association of gender and the six IrisPlex SNPs with the PIE-score was investigated using the non-parametric Kruskal-Wallis one-way analysis of variance rank test. After multiple comparison correction (Bonferroni), the significance threshold was  $p = 0.007$  when  $\alpha = 0.05$ . We observed a significant associations between *HERC2* rs12913832 ( $p = 2.11 \times 10^{-22}$ ), *SLC45A2* rs16891982 ( $p = 0.001$ ), gender ( $p = 1.61 \times 10^{-5}$ ) and the PIE-score. We compared the results of the Italian population sample with results from an already published study of Swedish and Danish individuals [12] (Table 1). The Danish and the Swedish individuals were typed for the IrisPlex SNPs and the PIE-score was calculated for each individual. We did not observe any significant association between gender and the PIE-score in the Danes (168 females and 183 males) ( $p = 0.502$ ) nor in the Swedes (134 females and 72 males) ( $p = 0.443$ ).

We observed that *HERC2* rs12913832 showed the strongest association with the PIE-score in all three populations. This was in concordance with previous studies in which *HERC2* rs12913832 was described to be the main predictor of qualitative [14,15,16] and quantitative eye colour [17]. To assess possible confounding effects of *HERC2* rs12913832 on the gender effect, association tests of gender with the PIE-score were carried out using the non-parametric Wilcoxon-rank-sum test based on the SNP-types of *HERC2* rs12913832 (Table 2 and Figure 1). After Bonferroni correction for multiple comparisons, the significance threshold was  $p = 0.008$  when  $\alpha = 0.05$ . Gender remained significantly associated with the PIE-score ( $p = 1.05 \times 10^{-5}$ ) in the Italian individuals that were typed to be heterozygote GA for rs12913832 (Table 2). However, gender was not significantly associated with the PIE-score in individuals typed as either rs12913832 G or rs12913832 A. None of the Danish or Swedish sample groups showed significant association with gender or the PIE-score. Among Italian rs12913832 GA individuals, females had more brown eye colour (median PIE-score = -0.93) compared to the eye colour of males (median PIE-score = -0.56). Among the Italians typed as rs12913832 G, females also had a tendency to have darker eye colours than males. However, the

result was not significant ( $p = 0.045$ ) when the Bonferroni corrected threshold of  $p = 0.008$  was applied.

### **The effect of gender on eye colour in the Italian population sample**

To investigate the effect of gender on the PIE-score in the Italian population sample and hence the eye colour, we performed a multivariate linear regression analysis. We analysed the predictive values of the six IrisPlex SNPs and gender by using the adjusted  $R^2$  to select predictors for the final model. The final model included the SNPs rs12913832, rs1800407, rs16891982, rs12203592 and gender as predictors, whereas rs1393350 and rs12896399 did not show any effect on eye colour. The effect of each of the predictors was evaluated using the  $\Delta R^2$  (adjusted  $R^2$  of the model including the specific predictor minus the adjusted  $R^2$  of the model without the specific predictor but with the other predictors) (Table 3). We found the effect of gender to be 4.9 % ( $\Delta R^2 = 4.9\%$ ) in the Italian population.

## **Discussion**

### **Gender and eye colour in the Italian population sample**

Gender was previously described to be a significant predictor of quantitative eye colour when eye colour was quantified into the hue and the saturation component of the HSV colour space [17]. However, gender was ranked only no. 14 out of 14 investigated predictors and it was reported to explain only 0.04% of the hue component variation and 0.09% of the saturation component variation of the iris. Similarly, we used the PIE-score as a quantitative measurement for eye colour that essentially is based on the saturation component of the HSV colour space [12]. The PIE-score is calculated as the ratio between pixels categorized as blue and pixels categorized as brown in the iris region of a digital eye image. The categorization is based on the saturation component. We previously showed that there is a very high correlation between the PIE-score and human evaluation of the eye colour in the Danish and Swedish sample sets (Spearman correlation = -0.81) [12]. We found a high correlation in the Italian sample set (Spearman correlation = -0.85) (Supplementary Figure 1). We found the effect of gender to be 4.9 % ( $\Delta R^2 = 4.9\%$ ) in the Italian population sample. This was larger than the effect of 0.09% ( $\Delta R^2 = 0.09\%$ ) presented in a previous study [17]. The most likely explanation for this difference is that there are large gender effects on eye colours between different populations. Our effect was predicted using an Italian population collected in

Northern Italy, whereas the previous effect of 0.09% was based on individuals of Northern European descent (Dutch, British and Australian individuals).

We are aware of the fact that the number of individuals studied was relatively small and limited to only one population. However, the results presented here and by Martinez-Cadenas and co-workers [1,2] support a population specific gender effect on eye colour. To fully explore possible population specific effects, more populations need to be studied. Especially, Mediterranean populations will be interesting.

### **Accuracy of the IrisPlex prediction model in the Italian population sample**

It is not the general assumption that women have darker eye colours than men. Thus, the results presented here and by Martinez-Cadenas and co-workers [1,2] were unexpected. It raises the question whether the gender effect is, although statistically significant in Spanish and Italian population sample sets, relevant for forensic genetic case work in specific populations. Gender was ranked 2<sup>nd</sup> among the predictors in the Spanish and Italian population samples. On the other hand, the effect may be so small that it is not relevant for the human perception of eye colour. If so, it may also raise the question whether lower ranked predictors including five of the six IrisPlex SNPs are relevant for the prediction of human perception of eye colour.

To evaluate this, we first tested the accuracy of the IrisPlex prediction model in the Italian population sample set. We used the suggested threshold probability of  $p > 0.7$  for a conclusive prediction of blue, intermediate or brown eye colour [11] and compared it to a simple prediction model with only *HERC2* rs12913832. The predictions of the simple model were calculated using the *Snipper* classifier [6] that allows prediction of the eye colour based on a subset of the IrisPlex SNPs (in this case, only *HERC2* rs12913832). The model predicted blue eye colour for rs12913832 GG individuals and brown eye colour for rs12913832 AA individuals whereas the eye colour of rs12913832 GA individuals was inconclusive (Table 4).

Table 4 shows that the simple prediction model only based on rs12913832 performed as well as the IrisPlex prediction model using all six IrisPlex SNPs in the Italian population sample. This indicated that the effect of the lower ranked predictors is too small to influence the predictive accuracy of blue, intermediate and brown eye colours in the Italian population sample.

A similar conclusion was reached for gender when the influence of gender on the predictive capability of the IrisPlex model was analyzed in two large datasets, one dataset including 5,348 Dutch Europeans and one dataset including 3,840 mixed Europeans [3].

### **The predictive properties of the IrisPlex prediction model**

To further test the predictive properties of the five lower ranked IrisPlex predictors, we evaluated all possible genotype combinations of the IrisPlex assay and their corresponding predictive values using the IrisPlex prediction model [4]. A total of  $3^6$  (729) different genotype combinations are possible with the six IrisPlex SNPs. Of these, 292 (292/729, 40.1%) were predicted as blue, 60 (60/729, 8.2%) as intermediate and 377 (377/729, 51.7%) as brown (Supplementary Table 1). If the minimum threshold of  $p > 0.7$  for a conclusive prediction was applied, all but two genotypes predicted as blue included rs12913832 GG (Table 5) and only 28 of 243 possible genotypes with rs12913832 GG were inconclusive. All except one of the inconclusive genotype combinations with rs12913832 GG included the rs16891892 CC genotype. rs16891892 is considered to be a major ancestry informative marker (AIM) and the C allele is found in high frequency in African individuals [18]. A functional role of rs16891892 in eye colour and pigmentation has not been revealed. This is contrary to rs12913832, where the allele rs12913832 G was shown to reduce the transcription of *OCA2* and hence result in a lower pigmentation level [19]. It is questionable whether rs16891892 CC can alter the eye colour phenotype determined by rs12913832 GG. Furthermore, it is highly unlikely to encounter a person with the genotype rs128913832 GG and rs16891892 CC because the rs128913832 G allele is almost exclusively found in Europeans whereas the rs16891892 C is typically found in Africans. Thus, for all practical purposes, the rs12913832 GG genotype predicts blue eye colours irrespective of the lower ranked predictors in the IrisPlex.

Among the 243 genotype combinations with rs12913832 AA, 212 reached the threshold of  $p > 0.7$  and were predicted as brown, whereas 31 genotypes were inconclusive (Table 5). Interestingly, rs1800407 AA was included in 27 of the 31 inconclusive genotype combinations with rs12913832 AA, and rs1800407 GA was included in the remaining four combinations. rs1800407 is a missense mutation in *OCA2* located 135kp downstream from rs12913832. The haplotypes of this region were previously suggested to increase eye colour prediction [6,20,5]. It was suggested [12,21] that the *OCA2* allele rs1800407 A decreased the pigmentation level in the eye when found in *cis* phase with the *HERC2* allele rs12913832 A, whereas the *OCA2* allele rs1800407 A found in

*cis* phase with the *HERC2* allele rs12913832 G will display little effect on the eye colour because the transcription of *OCA2* is reduced.

It is well known that the eye colours of individuals typed as rs12913832 GA are the most difficult to predict [4]. The Italian individuals with rs12913832 GA had large variations in PIE-scores (Figure 2). Also, Danish and Swedish individuals with rs12913832 GA had eye colours ranging from blue to brown [12]. If the threshold of  $p > 0.7$  was applied to the IrisPlex model, 182 of the 243 genotypes were inconclusive, 59 genotypes were predicted as brown and only two as blue. The two genotype combinations predicted as blue both included rs1800407 AA ( $p = 0.71$  for both). Of the 59 combinations predicted as brown, 41 combinations included rs16891982 CC and 16 combinations included rs16891982 CG. The average prediction probability of the 61 genotypes with  $p > 0.7$  was only 0.82. Only 10 genotypes, all including rs16891982 CC, had  $p > 0.9$ . Thus, prediction of eye colour in the rs12913832 GA group is weak except for individuals of presumably African origin. Supplementary Table 1 lists all the IrisPlex genotype combinations, the European population frequency and the prediction probabilities for blue, intermediate and brown eye colour.

In conclusion, the analysis of the IrisPlex prediction model indicated that three of the six SNPs are important for prediction of eye colour. Of these, only rs12913832 has a known biological function in pigmentation [19]. Taking this and the similar performance of the IrisPlex prediction model and the prediction using only rs12913832 into consideration (Table 4), and following the tradition of conservative statistical weight calculations in the field of forensic genetics, it seems reasonable to restrict the prediction of eye colour to just one SNP, rs12913832. rs12913832 GG predicts blue eye colour, rs12913832 AA predicts brown eye colour and the prediction of the eye colours of individuals typed as rs12913832 GA is inconclusive. Future investigation may show whether rs1800407 should be considered since it potentially can alter the functionality of *OCA2* and hence the eye pigmentation. Also, it is still to be shown if rs16891982 is an AIM for African individuals or has a functional role in eye colour regulation.

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	Danes ( <i>N</i> =351)	<i>p</i> -value (Kruskal-Wallis)		All ( <i>N</i> =776)
		Italians ( <i>N</i> =218)	Swedes ( <i>N</i> =206)	
Gender	0.502	<b>1.61x10<sup>-5</sup></b>	0.443	0.018
rs1393350 ( <i>TYR</i> )	<b>9.90x10<sup>-5</sup></b>	0.563	0.020	<b>9.63x10<sup>-4</sup></b>
rs1800407 ( <i>OCA2</i> )	<b>2.02x10<sup>-4</sup></b>	0.316	<b>0.003</b>	<b>2.05x10<sup>-9</sup></b>
rs12203592 ( <i>IRF4</i> )	0.795	0.029	0.154	0.952
rs12896399 ( <i>SLC24A4</i> )	0.007	0.973	0.007	<b>2.62x10<sup>-9</sup></b>
rs12913832 ( <i>HERC2</i> )	<b>4.28x10<sup>-39</sup></b>	<b>2.11x10<sup>-22</sup></b>	<b>2.04x10<sup>-17</sup></b>	<b>9.57x10<sup>-108</sup></b>
rs16891982 ( <i>SLC45A2</i> )	<b>0.003</b>	<b>0.001</b>	<b>1.29x10<sup>-4</sup></b>	<b>4.17x10<sup>-14</sup></b>

Bold numbers: Significant association after multiple Bonferroni correction ( $p < 0.007$ ).

	<i>p</i> -value (Wilcoxon-rank-sum)			
	Danes	Italians	Swedes	All three
rs12913832 (G)	0.297	0.045	0.781	0.990
rs12913832 (GA)	0.381	<b>1.05x10<sup>-5</sup></b>	0.721	<b>0.002</b>
rs12913832 (A)	0.317	0.154	0.134	0.130

Bold numbers: Significant association after multiple Bonferroni correction ( $p < 0.008$ ).

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	<i>p</i> -value (ANOVA)	$\Delta R^2$ %
rs12913832 ( <i>HERC2</i> )	$2.2 \times 10^{-16}$	51.4
Gender	$1.33 \times 10^{-7}$	4.9
rs1800407 ( <i>OCA2</i> )	0.211	0.6
rs12203592 ( <i>IRF4</i> )	0.012	1
rs16891982 ( <i>SLC45A2</i> )	0.014	1.3

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		Blue	Intermediate	Brown
IrisPlex prediction model <sup>a</sup>	Sensitivity	0.96	0	1
	Specificity	0.81	1	0.55
<i>Snipper</i> (rs12913832) <sup>b</sup>	Sensitivity	1	-	1
	Specificity	0.69	-	0.70

<sup>a</sup>A total of 143 samples had a prediction probability above 0.7 and 75 samples (2 blue, 45 intermediate and 28 brown) were inconclusive ( $p < 0.7$ ).

<sup>b</sup>A total of 98 samples fulfilled the prediction criteria ( $LR > 3$ ) and 120 samples (2 blue, 61 intermediate and 57 brown) were inconclusive ( $LR < 3$ ).

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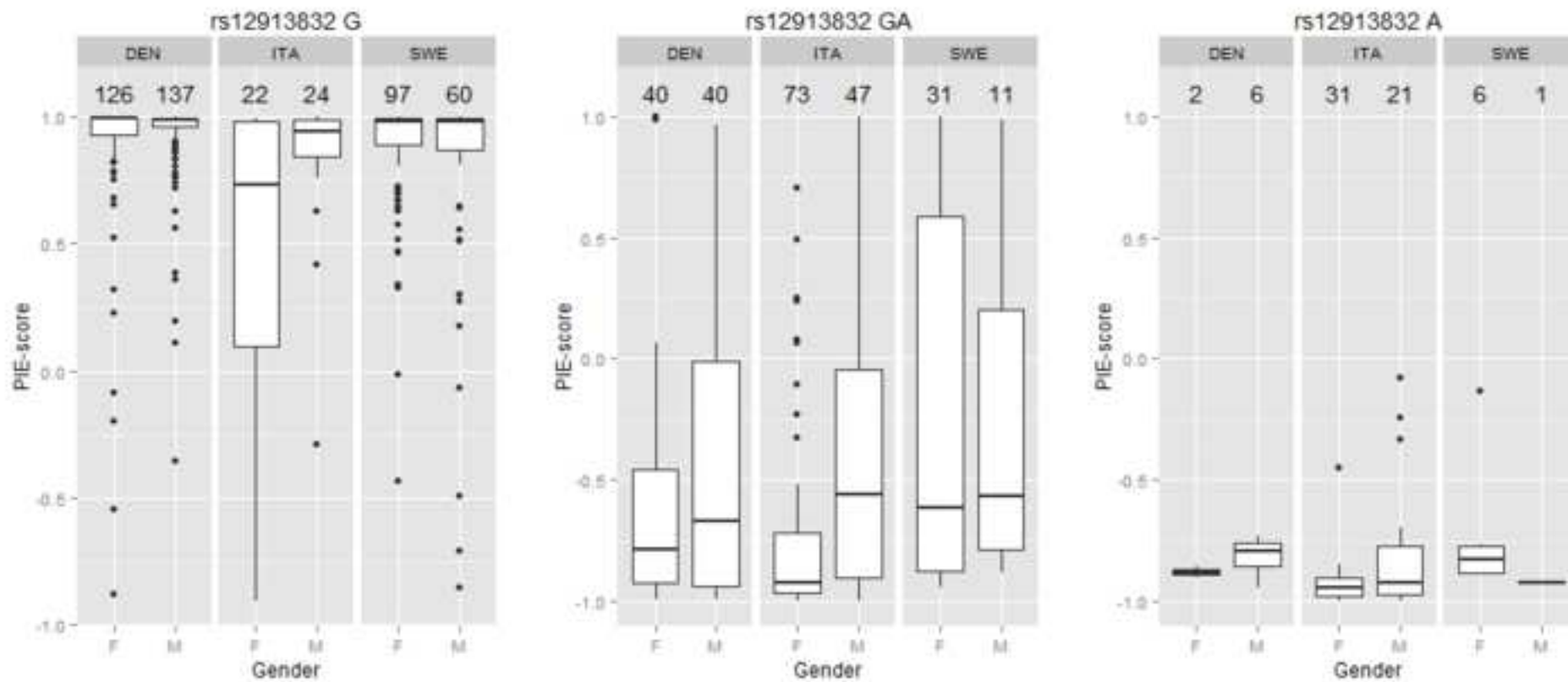
	rs12913832 GG	rs12913832 GA	rs12913832 AA
Blue	215 (242)	2 (50)	0 (0)
Intermediate	0 (0)	0 (54)	0 (6)
Brown	0 (1)	59 (139)	212 (237)
Inconclusive (p<0.7)	28	182	31

Number in parentheses: Prediction without a threshold of p>0.7.

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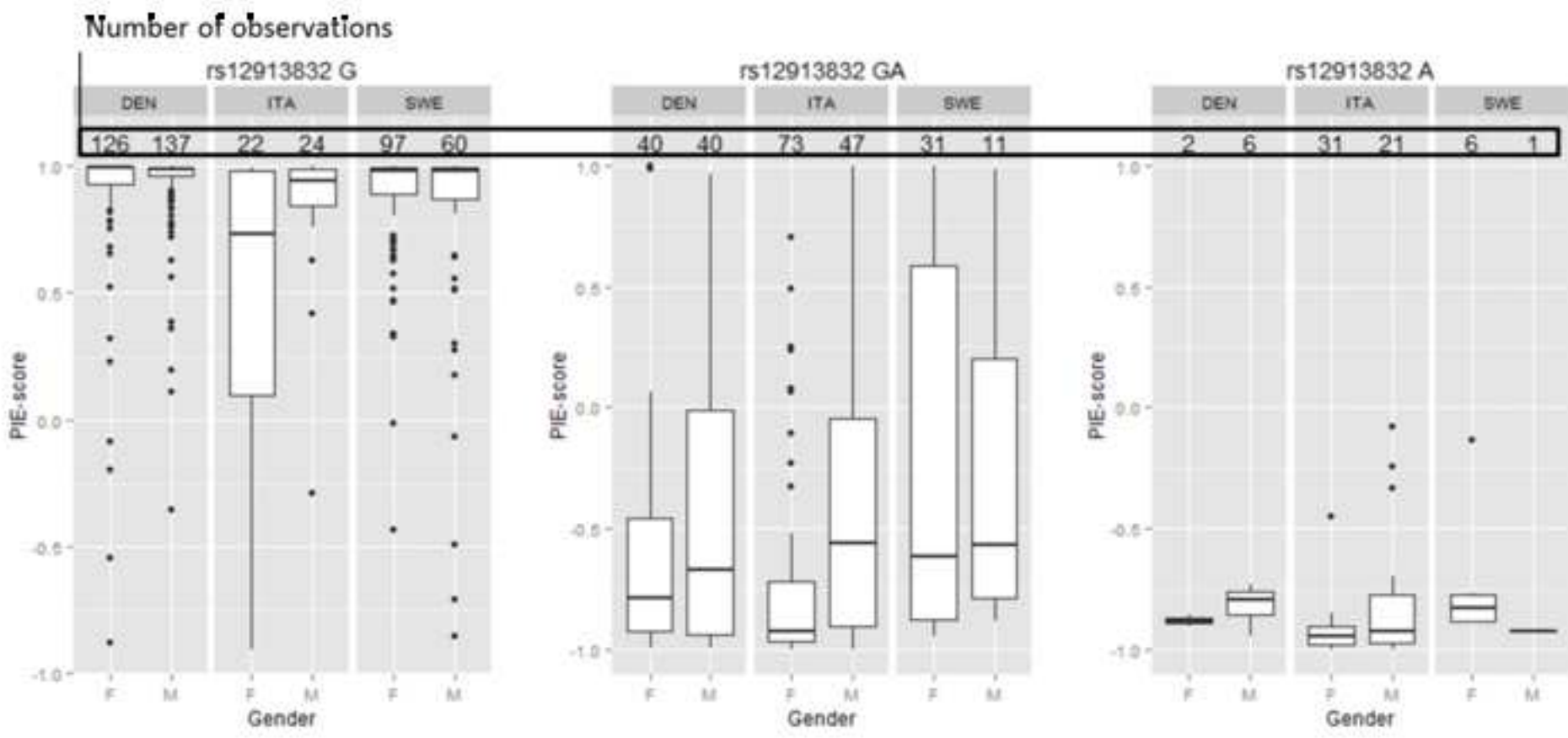
Figure 1

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**Figure 1.** Boxplots of PIE-scores for males (M) and females (F) in the Danish, Italian and the Swedish population samples based on the SNP-types of *HERC2* rs12913832. The numbers above each boxplot indicate the number of observations.

**Figure 2:** Comparison of the PIE-scores of individuals typed as rs12913832 GA with the eye colour classifications of the IrisPlex prediction methods. IrisPlex profiles with two or more observations were considered. The number of observations is shown above each plot. Genotypes are listed as *HERC2* rs12913832, *OCA2* rs1800407, *SLC24A4* rs12896399, *TYR* rs1393350, *SLC45A2* rs16891982 and *IRF4* rs12203592, respectively. Silent alleles were not considered by the prediction model. Homozygous genotypes are written as e.g. GG and not G, even though the presence of silent alleles cannot be excluded. Predictions from the IrisPlex are listed below the genotypes, brown (Br) and inconclusive (IC).

**Table 1.** Associations of gender and the six IrisPlex SNPs with the PIE-score.

**Table 2.** Association of gender with the PIE-score based on rs12913832.

**Table 3.** Predictors for quantitative eye colours in the Italian population sample.

**Table 4.** Accuracy of prediction models in the Italian population sample.

**Table 5.** Prediction outcomes of the IrisPlex prediction model.