JOURNAL OF FORENSIC SCIENCES

J Forensic Sci, July 2012, Vol. 57, No. 4
doi: 10.1111/j.1556-4029.2012.02077.x
Available online at: onlinelibrary.wiley.com

PAPER

PATHOLOGY/BIOLOGY

Ewelina Pośpiech,[1] M.S.; Jolanta Draus-Barini,[1] M.S.; Tomasz Kupiec,[1] Ph.D.; Anna Wojas-Pelc,[2] Ph.D., M.D.; and Wojciech Branicki,[1,3] Ph.D.

Prediction of Eye Color from Genetic Data Using Bayesian Approach*

ABSTRACT: Prediction of visible traits from genetic data in certain forensic cases may provide important information that can speed up the process of investigation. Research that has been conducted on the genetics of pigmentation has revealed polymorphisms that explain a significant proportion of the variation observed in human iris color. Here, on the basis of genetic data for the six most relevant eye color predictors, two alternative Bayesian network model variants were developed and evaluated for their accuracy in prediction of eye color. The first model assumed eye color to be categorized into blue, brown, green, and hazel, while the second variant assumed a simplified classification with two states: light and dark. It was found that particularly high accuracy was obtained for the second model, and this proved that reliable differentiation between light and dark irises is possible based on analysis of six single nucleotide polymorphisms and a Bayesian procedure of evidence interpretation.

KEYWORDS: Bayesian network, eye color prediction, forensic science, genetics, likelihood ratio, phenotyping

Prediction of phenotypic features from genetic data is considered to show promise in medicine as it delivers personal information that can improve the effectiveness of applied medical treatment (1). It may also be valuable in forensic examinations as it provides information about externally visible traits of an evidence sample donor. This may direct an investigation by defining the phenotype of the perpetrator in criminal cases or the victim in cases concerning identification of human remains (2,3). Human pigmentation is a visible trait that has been studied thoroughly, and the research has led to the discovery of many relevant genes and polymorphisms, which explain a significant proportion of the variation in eye, hair, and skin coloration. Several pigment-related genes have been discovered by their links with extreme phenotype forms like albinism (4–7). Many other genes have been found through large-scale analyses involving hundreds of so-called tagging single nucleotide polymorphisms (SNPs) (8–12). The most successful investigation has been linked with the genetics of iris color. Eiberg and Mohr (13) using linkage analysis were able to show that two loci on chromosome 15q were associated with blue/brown eye color variation. The presence of the *OCA2,* a known albinism-related gene in this region of the human genome, naturally attracted the attention of researchers working on the genetics of eye color (to this particular locus). The *OCA2* gene was soon reported as a major contributor to the variation observed in eye coloration in several independent studies (8,14,15). Another breakthrough was related to the discovery that the *HERC2* gene may play an important role in the determination of iris color in humans (9,10,16). Two independent research groups (9,17) showed that rs12913832 polymorphism in *HERC2,* located in a conserved region, may have a functional effect and by regulating the *OCA2* gene expression may be responsible for the determination of blue eye color in humans. Several SNPs from other pigmentary genes, *TYR, TYRP1, SLC24A4, SLC45A2, IRF4,* and *ASIP,* have been suggested to contribute to human eye color variation, but compared to rs12913832 in *HERC2*, the effect is significantly lower (8). These discoveries opened up possibilities for reliable prediction of human iris color based on DNA analysis, with a central significance for the rs12913832 position, being the best known predictor of eye color in humans. Liu et al. (18) performed a study of 37 polymorphisms located in eight known eye color–related genes on a large cohort of 6168 Dutch subjects and on this basis selected 15 SNP positions enabling blue and brown eye color prediction with high accuracies. The same group soon reported results of optimization and forensic validation of a first test for eye color prediction named IrisPlex, which assumes simultaneous analysis of the six best SNP predictors and prediction of eye color from the obtained data using a logistic regression model (19,20).

The likelihood ratio (LR) approach represents a method that is highly respected by forensic science as a tool used for the evaluation of the weight of evidence (21,22). Variables and their probabilistic dependencies considered by LR models may be represented graphically by Bayesian networks (BN), which make the LR approach more comprehensible. A BN gives a graphical representation of relationships between the observed data and may allow inference of the probability of an individual phenotype (such as eye color) on the basis of known genotypes assigned to an individual in the range of analyzed multiple SNP loci (variables) (23).

Therefore, we further investigated this issue and analyzed the same set of six eye color predictors in a population sample from

[1]Section of Forensic Genetics, Institute of Forensic Research, Westerplatte 9, 31-033 Kraków, Poland.
[2]Department of Dermatology, Collegium Medicum of the Jagiellonian University, Skawińska 8, 31-066 Kraków, Poland.
[3]Department of Genetics and Evolution, Institute of Zoology, Jagiellonian University, Ingardena 6, 30-060 Kraków, Poland.
*Supported by a grant from the Ministry of Science and Higher Education in Poland, No. ON301115136 (science fund for years 2009–2012).

Received 27 Jan. 2011; and in revised form 13 April 2011; accepted 4 June 2011.

Poland comprising individuals of European descent. Based on the gathered genetic data for 638 individuals with defined eye color, a BN model was created, which enables calculation of probabilities of having a particular eye color from genetic data based on a calculation of posterior probabilities. A BN was developed on the basis of the distribution of the six best SNPs (18) within groups of individuals representing four different eye colors with the parent node "eye color" categorized into four states (blue, green, hazel, and brown) or reduced to two states (light and dark). Finally, both model variations were tested for their prediction accuracy.

Materials and Methods

Samples

The study was approved by the Ethics Committee of the Jagiellonian University in Krakow (number KBET/17/B/2005) and the Commission on Bioethics of the Regional Board of Medical Doctors in Krakow (number 48 KBL/OIL/2008). Buccal swabs were collected from 638 unrelated males and females aged between 18 and 85 years old living in Southern Poland. The swabs were obtained by a dermatology specialist with their written consent. The dermatology specialist was responsible for the determination of eye color classifying all the participants into one of four eye color categories, that is, blue/gray (further referred to as blue), green, hazel, and brown/black (further referred to as brown).

DNA Extraction

DNA was extracted with a NucleoSpin® Tissue extraction kit (MACHEREY-NAGEL GmbH & Co., KG, Germany) using the protocol recommended for buccal swabs. Briefly, swabs were cut up, put into a 2-mL tube, and incubated in a water bath for 20 min at 70°C with 400 μL of TE buffer plus 400 μL of lysis buffer B3 and 25 μL of proteinase K. After incubation, 400 μL of 96% ethanol was added, and the 600 μL of content was transferred onto the NucleoSpin® Tissue Columns (Macherey-Nagel GmbH & Co, KG, Germany), centrifuged (1 min 11,000 × g), and washed with BW buffer and B5 buffer. Finally, columns were transferred into new 1.5-mL tubes, and DNA was retrieved with the warmed elution buffer BE.

Multiplex PCR Amplification

Details concerning PCR primers for six SNP positions, which has been selected as the best eye color predictors by Liu et al. (18), are given in Table S1. The multiplex PCR was optimized to enable their simultaneous amplification using the Qiagen Multiplex PCR kit (Qiagen, Hilden, Germany). The PCR consisted of 2.5 μL Qiagen multiplex PCR mixture, 0.5 μL Q solution, 0.5 μL primer premix (final concentration of 0.125 μM was used, except for rs12896399, where the final concentration was 0.25 μM) and 1.5 μL of template DNA (approx 1–10 ng). The temperature profile was as follows: 95°C/15 min, [94°C/30 sec, 58°C/90 sec, 72°C/90 sec] × 32, 72°C/10 min. The PCR products were purified by means of exonuclease I (Exo I) and shrimp alkaline phosphatase (SAP) treatment (Fermentas, Vilnius, Lithuania).

SNaPshot Reaction

The PCR products were subjected to minisequencing reaction using a developed multiplex SNaPshot procedure (Applied Biosystems, Foster City, CA). A single reaction consisted of 0.5 μL SNaPshot mix, 0.5 μL extension primer premix, 1 μL of purified PCR product, and DNase-free water up to 5 μL. The extension primer sequences and their final concentrations are shown in Table S2. The temperature profile was as follows: [96°C/10 sec, 50°C/5 sec, 60°C/30 sec] × 26. Products of minisequencing reactions were purified with SAP enzyme (Fermentas) and analyzed on an ABI 3100 Avant Genetic Analyser (Applied Biosystems) with the appropriate protocol for SNP analysis. All amplification and minisequencing reactions were carried out on a GeneAmp 9700 thermocycler (Applied Biosystems).

Statistical Analyses

The obtained genetic data for 638 samples were tested for agreement with Hardy–Weinberg expectations and degree of linkage disequilibrium using Arlequin v. 3.1 software (http://cmpg.unibe.ch/software/arlequin3). The data set was used to evaluate the frequency of SNPs in each eye color category, which was further used to establish conditional probability values for the conditional probability tables (CPTs). An additional set of 80 samples representing four different eye color categories (20 samples for each category) was selected for evaluation of the accuracy of the developed eye color prediction models. These external samples were randomly selected but with the assumption that each eye color category should include 20 samples. Area under the curve (AUC), which is the integral of area under the receiver operating characteristic (ROC) curves, was measured to assess the overall performance of the two developed model variants and blue, green, hazel, and brown eye color prediction. Values of AUC range from 0.5, which means a complete lack of prediction, to 1.0, which means perfect prediction. All association testing as well as ROC calculations were performed with PASW statistics v. 17 (SPSS Inc., Chicago, IL). Sensitivity, understood as the percentage of correctly predicted color type among the observed color type, specificity, understood as the percentage of correctly predicted noncolor type among the observed noncolor type, positive prediction value (PPV), understood as the percentage of correctly predicted color type among the predicted positives, and negative prediction value (NPV), understood as the percentage of correctly predicted noncolor type among the predicted negatives, were also calculated. All the parameters describing the model accuracy were calculated based on *a posteriori* probabilities (prediction based on maximum *a posteriori* probability).

Bayesian Model for Eye Color Prediction

Hugin Researcher v. 7.00 computer program (Hugin Expert A/S, Aalborg, Denmark) was used to develop a model of eye color prediction. The Hugin Researcher creates an environment for the construction of BN, which graphically represents probabilistic variables (predictors). The program implements a Bayesian paradigm, which is proposed in forensic science to test the significance of various hypotheses $H_k = 1 \ldots n$ concerning evidence. Here, we tested $n = 4$ or 2 hypotheses corresponded with four (blue, green, hazel, and brown) or two (light and dark) eye colors. The multilocus genotype of a given individual j Mj is scored at each of the analyzed six SNP positions. The genotype of each individual j at each of these loci is G_{ji}. Based on generated data set, we assessed the conditional probability P_{ji} of the observed genotype given each eye color H_k. Then, we assigned probability of the multilocus genotype M_j given each eye color H_k, that is, $\Pr(M_j|H_k) = \prod P_{ji}$. At the next stage, the posterior probability that a person has^i specific eye color given a particular multilocus genotype M_j was assigned using Bayesian

Eq. (1). The main component of this equation is LR, which is a quotient of two conditional probabilities, that is, probability of the observed multilocus genotype M_j given that a person under investigation has specific eye color $Pr(M_j \mid H_{k=x})$ and the sum of probabilities of the observed multilocus genotype M_j given that a person under investigation has different eye color $\sum_{k=1}^{n} \Pr(M_j|H_k)$. The Bayesian equation includes also *a priori* component, that is, a ratio of probability of having specific eye color in a given population $\Pr(H_{k=x})$and the sum of probabilities of having different eye colors in a given population $\sum_{k=1}^{n} \Pr(H_k)$. Here, we assigned the prior probabilities $\Pr(H_k) = 1/n$ for all k eye colors. However, in cases when the population of origin of a sample under examination is known, the frequencies of specific eye colors in a given population as *a priori* probabilities can be applied and this can further improve the prediction accuracy.

$$\Pr(H_{k=x}|M_j) = \frac{\Pr(M_j|H_{k=x})\Pr(H_{k=x})}{\sum_{k=1}^{n} \Pr(M_j|H_k)\Pr(H_k)} \quad (1)$$

A BN consists of nodes and directed arrows. Each node represents an uncertain state of a variable, and arrows between nodes represent links among these variables. An arrow introduced between two nodes from A to B determines that A is a parent node to B and B is a child node of A (23,24), and for example, a node ''rs12913832'' is a child node of a node ''eye color'' in Fig. 1. Each child node is characterized by a CPT, which is described as the probability of being in one state or another without evidence (given the state of the parental variable). The parent node is characterized by a table with *a priori* probabilities. Information put into both kinds of tables is called *a priori* beliefs. If the true state of a particular variable (node) is known with 100% confidence, this information could be entered as 100% probability for this particular state. This also means that we are sure of this state, and thus, this information is called hard evidence.

A model with two alternative variations was developed to predict the iris color probabilities from genetic data. The first model variant, BN–I, assumed the data within the parent node ''eye color'' to be categorized into four colors (blue, green, hazel, and brown), and the second one (BN–II) assumed the eye colors in parent node categorized into two states, that is, light (blue and green) and dark (hazel and brown) (Fig. 1). Thus, the parent node in the BN–I version had four states, while the BN–II version had only two states. The *a priori* conditional probabilities of these colors/states were entered as having the same value (0.25 for BN–I and 0.5 for BN–II) what assumed unknown population of origin.

Six variables (SNP positions) selected by Liu et al. (18) as best eye color predictors were introduced into these two alternative eye color prediction model versions as child nodes. The prediction model was comprised of positions: rs12913832, rs1800407, rs12896399 rs16891982, rs1393350, and rs12203592 from six different pigmentation genes, that is, *HERC2, OCA2, SLC24A4, SLC45A2, TYR,* and *IRF4.* Each child node in our models represents one SNP position defined by three possible states (e.g., homozygote CC, heterozygote CT, and homozygote TT in the case of rs12913832 polymorphism) and was characterized by CPT (see Tables S3 and S4). The CPT created for each SNP was based on the number of individuals possessing a defined genotype and belonging to one of four iris color categories, that is, blue, green, hazel, or brown in the BN–I model and belonging to one of two iris color categories: light and dark in the BN–II model. In particular, the probability of each genotype state was calculated as the

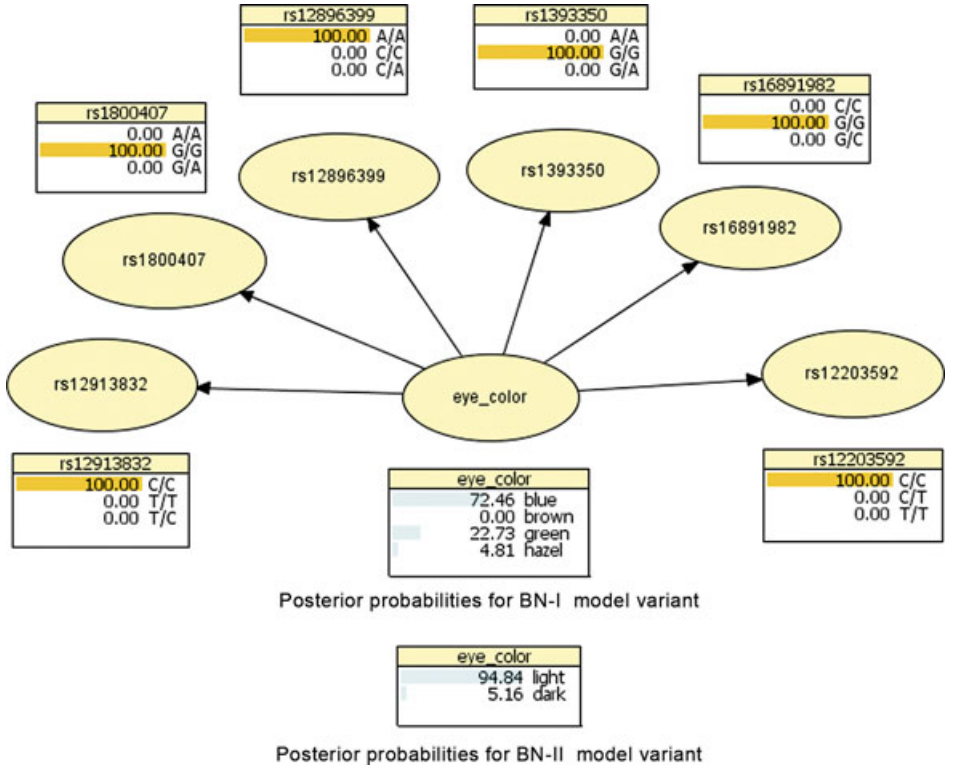

FIG. 1—*Example of calculation of a posteriori probabilities (parent node ''eye color'') after propagation of the ''hard evidences'' (the particular genotype of a person) on the SNP nodes using Bayesian network model variant BN–I and BN–II.*

frequency of this genotype in each eye color group (e.g., the frequency of homozygote CC for rs12913832). To assess the accuracy of both developed BN model variants for eye color prediction, 80 random individuals (external/test sample set consisting of 20 individuals for each iris color) were examined. Genotyping data were treated as hard evidences and put into a particular state of each child node state, that is, for an individual with CC genotype of rs12913832, the probability in the appropriate node for C/C state was entered as 100%, while the rest of the states were automatically set as 0%. The same was carried out for the other SNPs (Fig. 1). The flow of information via BN and changes of probability values of states of adequate nodes are results of entering of hard evidences. For each tested individual, a table of posterior probabilities for blue, green, hazel, and brown iris colors (BN–I) and light and dark (BN–II) was calculated. Maximum *a posteriori* probability was used for the prediction of iris color state. The LRs presented in the Supplementary Table S5 were calculated from *a posteriori* probabilities using Eq. (1). For example, the LR calculation from the BN–I testing results for the blue eye color was as follows: a ratio of *a posteriori* probability of having blue eyes and *a posteriori* probability of having the alternative eye color (green + brown + hazel) divided by a ratio of *a priori* probability of having blue eyes (0.25) and *a priori* probability of having alternative eye color (green + brown + hazel = 0.75). Giving an example, for an individual having the *a posteriori* probabilities presented in Fig. 1, the LR calculation for blue eye color (the highest probability obtained was for the blue = 72.5) was as follows: LR = (72.5/[22.7 + 4.8])/(0.25/0.75) = 2.64/0.333 = 7.9, alternatively LR for the light eye color was: LR = 94.8/5.2 = 18.23 as the ratio for prior probabilities was 1.

Results

Population Analyses

Among the studied 638 unrelated individuals, 346 (54.2%) had blue eye color, 76 (11.9%) green, 129 (20.2%) hazel, and 87 (13.6%) brown eye color. The collected population samples were subjected to analysis using an optimized genotyping assay based on simultaneous amplification and minisequencing analysis of six SNP polymorphisms within different pigment-related genes. After Bonferroni correction for multiple testing, no significant departures from Hardy–Weinberg equilibrium were detected for all the analyzed SNP positions ($p > 0.0083$) (Table 1). Linkage disequilibrium was noted for polymorphisms in *HERC2* and *OCA2*.

Evaluation of the Developed Bayesian Predictive Model Variants

To evaluate the predictive accuracy of the two developed Bayesian model variants, 80 random external (test) samples (20 for blue, green, hazel, and brown iris color) were genotyped and subjected to analysis using BN–I and BN–II and Hugin Researcher computer software (Hugin Expert A/S). Posterior probabilities and LR values obtained for all these samples are presented in Table S5. Results obtained for AUC as well as other analyzed parameters describing the predictive accuracy of the two developed model variants calculated from *a posteriori* probability values are presented in Table 2.

TABLE 1—*SNP positions included in prediction model.*

SNP-ID	Gene	Chr.	Position	Allele	MAF*	H-W†
rs16891982	*SLC45A2*	5	33987450	C/G	0.029	0.45941
rs12203592	*IRF4*	6	341321	C/T	0.077	0.79802
rs1393350	*TYR*	11	88650694	A/G	0.223	0.92733
rs12896399	*SLC24A4*	14	91843416	G/T	0.445	0.89832
rs1800407	*OCA2*	15	25903913	A/G	0.072	0.56832
rs12913832	*HERC2*	15	26039213	A/G	0.235	0.42604

*MAF, Minor allele frequency.
†H-W, Hardy–Weinberg equilibrium.

TABLE 2—*Parameters describing predictive accuracy of two developed eye color prediction model variants: BN–I and BN–II.*

	BN–I				BN–II	
	Blue	Green	Brown	Hazel	Light	Dark
AUC*	0.783	0.533	0.583	0.633	0.925	
Sensitivity [%]	80	0	35	55	85	100
Specificity [%]	76.7	93.3	81.7	71.7	100	85
PPV† [%]	53.3	0	38.9	39.3	100	87
NPV‡ [%]	92	73.7	79	82.7	87	100

*AUC, area under the receiver operating characteristic (ROC) curves.
†PPV, positive prediction value.
‡NPV, negative prediction value.

The BN–I Model Variant

The highest sensitivity was obtained for blue eye color and reached 80%, which means that 80% of blue-eyed individuals (16/20) were predicted correctly. The AUC for blue eye color was calculated to equal 0.783, where 1.0 means perfect prediction and 0.5 complete lack of prediction. The sensitivity values for hazel, brown, and green eye color were lower and amounted to 55, 35, and 0%, respectively. The corresponding AUC values for these eye colors equaled 0.633, 0.583, and 0.533, respectively. The highest specificity, 93.3%, was obtained for green eye color, which means that among all non-green-eyed individuals, 93.3% were recognized correctly as nongreen, which means that only 6.7% of non-green-eyed individuals were incorrectly classified into the group of green eye color. The specificity obtained for brown, blue, and hazel eye color was 81.7, 76.7, and 71.7%, respectively. The highest PPV was obtained for blue eye color at 53.3%. This value means that in all cases of assignments to the group of blue eyes, 53.3% of individuals in fact possessed blue eye color. The PPVs for hazel, brown, and green were, respectively, at the 39.3, 38.9, and 0% level. The highest NPV at the level of 92% was obtained for blue eye color, which means that out of all cases in the all-color group where individuals were classified into the non-blue-eyed group, in 92% of cases, they were classified correctly as non-blue-eyed individuals. The NPVs for hazel, brown, and green were 82.7, 79, and 73.7%, respectively.

The BN–II Model Variant

The AUC value for eye color defined as light versus dark in the BN–II model was calculated to equal 0.925. This model variant correctly classified 34 of 40 light-eyed individuals (blue + green) into the light category, so the sensitivity value for this eye color equaled 85%. The specificity for light eye color reached the highest value of 100%. This means that among all non-light-eyed individuals, all of them were classified correctly into the non-light eye color group. The PPV and NPV for light eyes were at the 100% and 87% level, respectively. The PPV value of 100% means that in all cases of assignments to the group of light eyes, all individuals in fact possessed light eye color. The NPV value of 87% means that out of all the cases in the all-color group where individuals were

classified into the non-light-eyed group, in 87% of cases, they were classified correctly as non-light-eyed individuals. For dark eye color, the sensitivity reached 100%, which means that all individuals with hazel and brown eye color were predicted correctly as dark eyed. The specificity decreased compared to light color and amounted to 85%. This means that among all non-dark-eyed individuals, 85% of them were classified correctly into the non dark eye color group. The PPV and NPV for dark eyes were at the 87% and 100% level, respectively.

Discussion

Iris color represents a human trait that can be reliably predicted from genetic data, and this information may be useful in certain forensic investigations. Although eye color is a typical multigenic trait, the predominant role in its determination is associated with two interplaying genes located on chromosome 15, that is, *OCA2* and *HERC2*. Protein encoded by the *OCA2* gene is an integral part of the melanosome membrane and is responsible for the regulation of intra-melanosome pH (25). It seems that the product encoded by *HERC2* is not directly involved in pigment production or distribution. Its biologic function, as was suggested by Bekker-Jensen et al. (26), is related to the process of ubiquitylation, that is, a post-translational modification with ubiquitin that directs proteins to the proteasome and thus promotes their degradation. It is assumed that *HERC2* may influence human pigmentation through the regulation of *OCA2* expression. This action is thought to be associated with position rs12913832, located in a highly conserved segment of the intron 86 in *HERC2* (9,17). The rs12913832 in *HERC2* and rs1800407 in *OCA2* are, respectively, in first and second position on the list of six best predictors according to the ranking presented by Liu et al. (18). A logistic model based on these six polymorphisms was reported by Walsh et al. (19) as highly accurate for blue and brown iris color prediction. Our method employs Bayesian procedure to predict iris color based on genetic data for the same six polymorphisms. Bayes' theorem formulates the interpretation of the probability often applied in forensic sciences to quantify the uncertainty about different hypotheses and to assess the weight of the evidence (22,23). Here, the Bayesian model for eye color prediction was created using the Hugin Researcher program, enabling high flexibility and usage. It is advantageous that the constructed model can be easily modified and updated in the light of new information, that is, new variables and thus gives perspectives for further development as new data appear (24). The BN was built based on the probabilities of genotypes for the CPTs, which were obtained from genotype frequencies calculated from a data set of 638 Europeans included in our database. Two Bayesian model variants were developed and tested for eye color prediction. The BN–I model variant assumed prediction of eye color divided into four categories, that is, blue, green, hazel, and brown. The BN–II model variant had lower resolution as eye color categories were reduced to only two states — light (blue and green) and dark (hazel and brown). The priority of accuracy over high resolution in forensic prediction of visible traits would seem to be obvious. Assuming this, it becomes clear that the more accurate BN–II model should be recommended as more appropriate for forensic purposes than BN–I, which, although it has higher eye color resolution, achieves significantly lower accuracy. The accuracy of BN–II as calculated from the AUC, which is the integral of the area under the ROC curves, reached 0.925 for light versus dark eye color. The sensitivity for dark eye color equaled 100%, which means that all dark-eyed individuals were classified into the dark eye color group. The specificity for dark eye color amounted to 85%, which means that among all non dark-eyed individuals, 85% of them were classified correctly into the nondark eye color group. The values of accuracy informative parameters calculated for BN–II for light eye color was as follows: sensitivity at the level of 85% and specificity amounted to 100%. It is therefore obvious that slightly diminished value of sensitivity in the case of the light eye color category is caused by the presence of green-eyed individuals in this group. More detailed analysis of this result showed that six of 20 green-eyed individuals were categorized incorrectly into the dark eye color group. The problem with the prediction of green eye color is clearly visible from testing results for the BN–I model variant (Table 2). The sensitivity for green iris color was 0 and AUC equaled 0.533 (where 0.5 means complete lack of prediction). A significantly lower prediction accuracy for intermediate eye colors compared to extreme eye colors was also reported by Liu et al. (18) who demonstrated that by employing multinomial regression–based algorithm, intermediate iris color can be predicted with a sensitivity of 1.1% and specificity of 99.6%. Spichenok et al. (27) tested seven SNPs for their predictive value in describing human pigmentation on samples from various populations, European descendants, African Americans, and Asians and also reported problems with more distinguishing predictions, in particular for green eye color. Detailed analysis of our BN–I and BN–II models indicates that besides problems with prediction of green eye color, we were also facing difficulties with differentiation between hazel and brown iris colors. Using BN–I, these two eye colors were often predicted reversely with very little difference between the obtained probability values (Table S5). This problem is reflected by the AUC and sensitivity values for brown and hazel eye colors (Table 2). We can speculate that the predictors included in the tested model variants are not powerful enough to distinguish between hazel and brown as well as green and other eye colors. However, especially for hazel and brown iris colors, it also seems to be possible that the problem lies in imprecise eye color classification. As we used the same SNP predictors as in the two above-mentioned reports (18,19), it was possible to test our 80 samples using the algorithm developed by Liu et al. (18) and implemented in the Excel macro provided by Walsh (19). High AUC and sensitivity values were observed for blue (AUC = 0.842, sensitivity = 100%) and brown (AUC = 0.800, sensitivity = 95%) eye color (data not present). However, we observed a complete lack of prediction for intermediate eye color (understood here as green and hazel). None of our 80 tested samples was classified as intermediate. Nineteen of the 20 green samples were categorized as blue with very high probabilities of around 0.9. The remaining one was categorized as brown. Five of the 20 hazel samples were classified as blue (although with a low probability of around 0.4–0.5), and the remaining 15 were predicted as brown. It is obvious that if our hazel samples were defined as brown (and the difference between these two colors is often very subtle), this would positively influence prediction accuracy. This suggests problems with phenotyping and proper eye color classification, which result from the continuous character of the trait. Various authors have proposed different eye color categorizations, but all of them have simplified the quantitative nature of this trait to a greater or lesser extent. Recently, a novel digital method of quantification of human eye color was proposed by Liu et al. (28), who measured continuous eye color variations (in terms of hue and saturation) using high-resolution digital full-eye photographs and ascribed values to them. In our study, we used a four eye color categorization, and phenotyping was based on a dermatology survey performed by a professional, which seems to be much more reliable than the frequently used method of self-reporting (29–31).

The mathematical model proposed by Liu et al. (18) and Walsh et al. (19) relies on the multinomial logistic regression method. However, the LR is often proposed as a good method of presentation of the weight of evidence in forensic science. This parameter can easily be calculated from the posterior probabilities produced by our BN, given knowledge of the *a priori* values. To compare our results with those obtained by Mengel-From et al. (31), who calculated LR values supporting particular eye colors based on analysis of SNPs in three genes, that is, *HERC2*, *OCA2,* and *SLC45A2,* we also calculated LRs for all the samples tested (results are presented in Table S5). The value of the LR representing the best discrimination between light and dark eye color calculated from our results equaled 32.3, which should be interpreted to mean that it is 32 times more probable to have dark eye color than to have light eye color, given this particular genotype. This value is in good concordance with the data obtained by Mengel-From et al. (31), who assessed the analogous LR value to equal 29.3 when predicting eye color using the light-dark eye color classification. We claim that prediction of eye color assuming these two categories (light–dark) may be sufficient in many forensic examinations, not only because of the high reliability of such a test but also because this kind of information may be sufficient to guide an investigation in a certain direction. Bayesian Eq. (1), besides an LR component that solely reflects conditional probabilities calculated on the basis of genotype distribution in particular categories of eye colors, includes an *a priori* component, which may reflect frequencies of eye colors in a particular population. In general, this *a priori* component was eliminated (by unification of the prior probabilities) in our calculations. This was done since we rarely have prior knowledge concerning ancestry of the identified subject, and thus, it is more reasonable to rely solely on genotypic data. However, the Hugin environment enables *a priori* values to be easily adjusted based on distribution of eye colors in the population of origin, and this may further improve accuracy of prediction in particular cases.

Further studies should reveal additional genes and polymorphisms contributing to variation in iris colors. Recently, Liu et al. (28) using a fine phenotyping approach have found that three new loci are associated with eye color and polymorphisms within them enable slightly more reliable prediction of intermediate eye colors. This shows that additional genetic data may allow us to go beyond reliable prediction of blue/brown eye colors. Furthermore, the prediction accuracy especially of intermediate eye colors may also be improved by including information concerning non additive effects between different genes, that is, gene–gene interactions.

Acknowledgments

We would like to thank all sample donors who participated in this research project. We also wish to thank Dr. Paulina Wolańska-Nowak and Dr. Grzegorz Zadora for helpful comments on the applied Bayesian procedure.

References

1. Brand A, Brand H, Schulte in den Bäumen T. The impact of genetics and genomics on public health. Eur J Hum Genet 2008;16:5–13.
2. Kayser M, Schneider PM. DNA-based prediction of human externally visible characteristics in forensics: motivations, scientific challenges, and ethical considerations. Forensic Sci Int Genet 2009;3:154–61.
3. Branicki W, Brudnik U, Wojas-Pelc A. Genetic prediction of pigmentary traits in forensic studies. Probl Forensic Sci 2005;64:343–57.
4. Tomita Y, Takeda A, Okinaga S, Tagami H, Shibahara S. Human oculocutaneous albinism caused by single base insertion in the tyrosinase gene. Biochem Biophys Res Commun 1989;164:990–6.
5. Richnik EM, Bultman SJ, Horsthemke B, Lee ST, Strunk KM, Spritz RA, et al. A gene for the mouse pink-eyed dilution locus and for human type II oculocutaneous albinism. Nature 1993;361:72–6.
6. Newton JM, Cohen-Barak O, Hagiwara N, Gardner JM, Davisson MT, King RA, et al. Mutations in the human orthologue of the mouse underwhite gene underlie a new form of oculocutaneous albinism, OCA4. Am J Hum Genet 2001;69:981–8.
7. Boissy RE, Zhao H, Oetting WS, Austin LM, Wildenberg SC, Boissy YL, et al. Mutation in and lack of expression of tyrosinase-related protein-1 (TRP-1) in melanocytes from an individual with brown oculocutaneous albinism: a new subtype of albinism classified as "OCA3." Am J Hum Genet 1996;58:1145–56.
8. Frudakis T, Thomas M, Gaskin Z, Venkateswarlu K, Chandra KS, Ginjupalli S, et al. Sequences associated with human iris pigmentation. Genetics 2003;165:2071–83.
9. Sturm RA, Duffy DL, Zhao ZZ. A single SNP in an evolutionary conserved region within intron 86 of the HERC2 gene determines human blue-brown eye color. Am J Hum Genet 2008;82:424–31.
10. Sulem P, Gudbjartsson DF, Stacey SN, Helgason A, Rafnar T, Magnusson KP, et al. Genetic determinants of hair, eye and skin pigmentation in Europeans. Nat Genet 2007;39:1443–52.
11. Sulem P, Gudbjartsson DF, Stacey SN, Helgason A, Rafnar T, Jakobsdottir M. Two newly identified genetic determinants of pigmentation in Europeans. Nat Genet 2008;40:835–7.
12. Han J, Kraft P, Nan H, Guo Q, Chen C, Qureshi A. A genome-wide association study identifies novel alleles associated with hair color and skin pigmentation. PLoS Genet 2008;4(5):e1000074.
13. Eiberg H, Mohr J. Assignment of genes coding for brown eye colour (BEY2) and brown hair colour (HCL3) on chromosome 15q. Eur J Hum Genet 1996;4:237–41.
14. Sturm RA, Frudakis TN. Eye colour: portals into pigmentation genes and ancestry. Trends Genet 2004;20:327–32.
15. Branicki W, Brudnik U, Kupiec T, Wolańska-Nowak P, Szczerbińska A, Wojas-Pelc A. Association of polymorphic sites in the OCA2 gene with eye colour using the tree scanning method. Ann Hum Genet 2008;72:184–92.
16. Kayser M, Liu F, Janssens AC, Rivadeneira F, Lao O, van Duijn K. Three genome-wide association studies and a linkage analysis identify HERC2 as a human iris color gene. Am J Hum Genet 2008;82:411–23.
17. Eiberg H, Troelsen J, Nielsen M, Mikkelsen A, Mengel-From J, Kjaer KW, et al. Blue eye color in humans may be caused by a perfectly associated founder mutation in a regulatory element located within the HERC2 gene inhibiting OCA2 expression. Hum Genet 2008;123:177–87.
18. Liu F, van Duijn K, Vingerling JR, Hofman A, Uitterlinden AG, Janssens AC, et al. Eye color and the prediction of complex phenotypes from genotypes. Curr Biol 2009;19:192–3.
19. Walsh S, Liu F, Ballantyne KN, van Oven M, Lao O, Kayser M. IrisPlex: a sensitive DNA tool for accurate prediction of blue and brown eye colour in the absence of ancestry information. Forensic Sci Int Genet 2011;5:170–80.
20. Walsh S, Lindenbergh A, Zuniga SB, Sijen T, de Knijff P, Kayser M. Developmental validation of the IrisPlex System: determination of blue and brown iris colour for forensic intelligence. Forensic Sci Int Genet 2011;5:464–71.
21. Evett IW, Weir BS. Interpreting DNA evidence. Statistical genetics for forensic scientists. Sunderland, MA: Sinauer Associates Inc, 1998.
22. Aitken CGG, Taroni F. Statistics and the evaluation of evidence for forensic scientists. Statistics in practice. Chichester, UK: John Wiley & Sons, 2004.
23. Taroni F, Aitken C, Garbolino P, Biedermann A. Bayesian network and probabilistic inference in forensic science. Chichester, UK: John Wiley & Sons, Inc., 2006.
24. Zadora G, Wolańska-Nowak P. Application of Bayesian network in forensic genetics and criminalistics. Probl Forensic Sci 2009;78:141–59.
25. Puri N, Gardner JM, Brilliant MH. Aberrant pH of melanosomes in pink-eyed dilution (p) mutant melanocytes. J Invest Dermatol 2000;115:607–13.
26. Bekker-Jensen S, Rendtlew Danielsen J, Fugger K, Gromova I, Nerstedt A, Lukas C, et al. HERC2 coordinates ubiquitin-dependent assembly of DNA repair factors on damaged chromosomes. Nat Cell Biol 2010;12:80–6.
27. Spichenok O, Budimlija ZM, Mitchell AA, Jenny A, Kovacevic L, Marjanovic D, et al. Prediction of eye color and skin color in diverse populations using seven SNPs. Forensic Sci Int Genet 2011;5:472–8.
28. Liu F, Wollstein A, Hysi PG, Ankra-Badu GA, Spector TD, Park D, et al. Digital quantification of human eye color highlights genetic association of three new loci. PLoS Genet 2010;6:e1000934.

29. Graf J, Hodgson R, van Daal A. Single nucleotide polymorphisms in the MATP gene are associated with normal human pigmentation variation. Hum Mutat 2005;25:278–84.
30. Duffy DL, Montgomery GW, Chen W, Zhao ZZ, Le L, James MR, et al. A three-single-nucleotide polymorphism haplotype in intron 1 of OCA2 explains most human eye-color variation. Am J Hum Genet 2007;80:241–52.
31. Mengel-From J, Børsting JJ, Sanchez C, Eiberg H, Morling N. Human eye colour and HERC2, OCA2 and MATP. Forensic Sci Int Genet 2010;4:323–8.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. PCR primer sequences.

Table S2. Extension primer sequences.

Table S3. Conditional probability table used in BN-I model variant calculated from the frequency of each pair of alleles for each SNP in four eye color groups: blue, green, hazel and brown.

Table S4. Conditional probability table used in BN-II model variant calculated from the frequency of each pair of alleles for each SNP in two eye-color groups: light and dark.

Table S5. Testing results for BN-I and BN-II model variants for 80 external samples.

Please note: Wiley-Blackwell is not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

Additional information and reprint requests:
Wojciech Branicki, Ph.D.
Institute of Forensic Research
Section of Forensic Genetics
Westerplatte 9
31-033 Kraków
Poland
E-mail: wbranicki@ies.krakow.pl